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RESEARCH ARTICLE



The rLVS $\Delta capB/iglABC$ vaccine provides potent protection in Fischer rats against inhalational tularemia caused by various virulent *Francisella tularensis* strains

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ABSTRACT

Francisella tularensis is one of the several biothreat agents for which a licensed vaccine is needed. To ensure vaccine protection is achieved across a range of virulent *F. tularensis* strains, we assembled and characterized a panel of *F. tularensis* isolates to be utilized as challenge strains. A promising tularemia vaccine candidate is rLVS $\Delta capB/iglABC$ (rLVS), in which the vector is the LVS strain with a deletion in the *capB* gene and which additionally expresses a fusion protein comprising immunodominant epitopes of proteins IgA, IgB, and IgC. Fischer rats were immunized subcutaneously 1–3 times at 3-week intervals with rLVS at various doses. The rats were exposed to a high dose of aerosolized Type A strain Schu S4 (FRAN244), a Type B strain (FRAN255), or a tick derived Type A strain (FRAN254) and monitored for survival. All rLVS vaccination regimens including a single dose of 10^7 CFU rLVS provided 100% protection against both Type A strains. Against the Type B strain, two doses of 10^7 CFU rLVS provided 100% protection, and a single dose of 10^7 CFU provided 87.5% protection. In contrast, all unvaccinated rats succumbed to aerosol challenge with all of the *F. tularensis* strains. A robust Th1-biased antibody response was induced in all vaccinated rats against all *F. tularensis* strains. These results demonstrate that rLVS $\Delta capB/iglABC$ provides potent protection against inhalational challenge with either Type A or Type B *F. tularensis* strains and should be considered for further analysis as a future tularemia vaccine.

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Introduction



Francisella tularensis is a gram-negative bacterium and the causative agent of tularemia. *F. tularensis* is one of the most infectious bacterial pathogens for humans as it has been reported that inoculation or inhalation of less than 10 organisms can cause disease.¹ Due to the threat posed by its low infectious dose, severity of the disease, and ability for aerosol dissemination, *F. tularensis* has been listed as a Tier 1 Select Agent by the United States (U.S.) Department of Health and Human Services.² The two *F. tularensis* subspecies that are a risk to humans are the highly virulent *F. tularensis* subsp. *tularensis* (Type A) and the less virulent *F. tularensis* subsp. *holarctica* (Type B). Typically, the Type A strains are limited to North America, whereas Type B strains are present across the Northern Hemisphere.^{3–5}

F. tularensis infects a wide range of animal species, including humans. Humans can be exposed through a number of routes, the most common being to the skin through the bite of an infected insect or other arthropod vector.^{5–8} *F. tularensis* can be transmitted to humans through the mucous membranes, gastrointestinal tract, and lungs. Thus, tularemia can range from an ulceroglandular to a much more severe pneumonic or

typhoidal disease. The greatest concern to the biodefense community is the intentional use of aerosolized *F. tularensis*.^{2,9–11} Despite this threat, a U.S. Food and Drug Administration (FDA) approved tularemia vaccine does not currently exist.

Live Vaccine Strain (LVS) was derived from an unknown Type B strain in the former Soviet Union. The vaccine is delivered by scarification, forms a small lesion, and provides some level of protection against challenge.^{1,12–14} However, LVS is available only as an Investigational New Drug vaccine in the U.S. In addition, LVS is not considered an ideal vaccine for several reasons, including significant cutaneous reactivity, and documented breakthrough in protection.^{1,15–18} Therefore, an improved vaccine to prevent tularemia is needed.

When evaluating a new vaccine, protection studies have typically been completed with the prototype Schu S4 strain that was obtained from a human ulceroglandular disease.¹⁷ However, the virulence of *F. tularensis* strains can vary between Type A and B isolates.^{19–23} Therefore, a successful pneumonic tularemia vaccine needs to protect against a wide variety of *F. tularensis* strains which may differ in geographic origin or virulence attributes. In order to facilitate the efficacy testing of new vaccines against pneumonic tularemia, we

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previously developed a well-characterized panel of *F. tularensis* strains which represents a variety of historical, clinical, and environmental isolates.¹⁹ In this study, we have utilized our *F. tularensis* panel and a rat aerosol model of pneumonic tularemia to test the efficacy of the rLVS $\Delta capB/iglABC$ vaccine, an improved version of the LVS vaccine. This vaccine vector, LVS $\Delta capB$, is a highly attenuated version of LVS that also serves as a platform to express recombinant immunoprotective antigens of target pathogens and thereby protect against several pathogens.^{24–26} We used a version of rLVS $\Delta capB$ that expresses a fusion protein comprising the immunodominant epitopes of *F. tularensis*, IglA, IglB, and IglC (IglABC).^{24,27} We show that this vaccine is able to protect Fischer rats from high challenge doses of aerosolized bacteria from multiple strains of *F. tularensis*.

Materials and methods

Bacterial strains and growth media

The fully virulent *F. tularensis* strains used in this study were previously described, prepared according to International Organization for Standardization/ISO standards and guidelines for biological reference materials, and generated as single-use vials.¹⁹ The down-selected strains were FRAN244, FRAN254, and FRAN255. FRAN244 refers to the Schu S4 strain obtained from BEI Resources (NR-10492).¹⁷ FRAN254 is a Type A1a strain obtained from a tick, and FRAN255 is a Type B isolate that was obtained from the pleura of a male in Kentucky, USA.¹⁹ The vaccine strains tested in this study were LVS and rLVS $\Delta capB/iglABC$ (rLVS).^{24,27} Frozen original stocks of these strains were thawed; diluted 1000-fold to $\sim 10^6$ CFU/ml; 0.1 ml ($\sim 10^5$ CFU) plated on chocolate agar plates containing 1% Isovitalax (Becton Dickinson) for 3 days at 37°C; colonies scraped into PBS, centrifuged, and the pellet suspended in PBS and 20% glycerol; the suspension dispensed into tubes (1 ml/tube); and the tubes stored at -80°C . Post-freeze titers were obtained on chocolate agar plates before the use of vaccines for immunization. Growth of all strains was performed on chocolate agar plates (Remel) or brain heart infusion broth supplemented with 1% Isovitalax at 37°C.

Vaccinations

Female Fischer 344 rats (6–8 weeks old) were obtained from Charles River Laboratories and allowed a week to acclimate prior to use. The frozen vaccine strains (LVS or rLVS) were thawed on ice, and then the intended CFU doses were administered to the rats subcutaneously in 100 μl injections. Naïve rats received only PBS via the same route. The vaccine dosing and intervals are described below with eight rats per group.

Aerosol challenges

F. tularensis challenge material was prepared from brain heart infusion cultures grown overnight in a 37°C shaker at 200 rpm. The cultures were adjusted for various challenge doses as determined by serial dilutions and plating. We administered aerosolized doses of the *F. tularensis* strains using a dynamic

30-l humidity-controlled Plexiglas whole-body exposure chamber, as previously described.²⁸ We obtained an estimate of the inhaled doses as previously described.^{29–31} For all challenge experiments, rats were monitored at least daily, and mortality rates (to include euthanasia when moribund in accordance with early endpoint criteria) were recorded for 21 days.

Pathology

Postmortem tissues (lung, spleen, and liver) were collected from the challenged rats and routinely processed. Briefly, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for hematoxylin and eosin staining. At least a single section of the above tissues were examined by a board certified veterinary pathologist and were subjectively graded on the severity of necrosis/inflammation: minimal (involving <10% of the tissue), mild (involving 11–25% of the tissue), moderate (involving 26–50% of the tissue), marked (involving 51–79% of the tissue), or severe (involving $\geq 80\%$ of the tissue).

Immunological analysis

Sera were obtained from anesthetized rats prior to vaccination (Pre-Vax), 16 days following the last dose of the vaccine (Post-Vax), and for the survivors at the end of the study at 21 days post challenge (Survivors) by either submandibular vein or cranial vena cava bleeds. During the bleeding process (Pre-/Post-Vax) to obtain sera, some of the rats did not recover from the procedure; the numbers of rats in each group in the protection studies are listed in the respective figures.

Immunoglobulin (Ig) total IgG (IgG) and subclass IgG titers (IgG1, IgG2a, -2b, and -2c) from rats were determined by an ELISA performed in 96-well, Immulon 2 HB, round-bottom plates (Thermo Fisher). Suspensions of FRAN244 or FRAN255 were irradiated in a JL Shepherd irradiator Model 109–68 at approximately 21 kGy on dry ice and confirmed to be inactivated. These irradiated samples were individually used as antigens and diluted in 0.1 M carbonate buffer, pH 9.5, to a concentration of 10 $\mu\text{g}/\text{ml}$. Plates were covered and stored overnight at 4°C. The plates were washed five times with 1X wash buffer (1X PBS, 0.05% Tween 20) with a Biotek ELx405ts plate washer and incubated with a blocking buffer (1% Casein in PBS, Pierce/Fisher Scientific) for 30 min at 37°C. Blocking buffer was removed by washing as stated above, then twofold serial dilutions of rat sera, at a starting dilution of no less than 1:50, were made with antibody assay diluent (1X PBS, 0.25% Casein) in triplicate, and plates were incubated for 1 h at 37°C. After the plates were washed as previously mentioned, diluted goat anti-rat-IgG, -IgG1, -IgG2a, -IgG2b, or -IgG2c horseradish peroxidase conjugate (1:5,000; Southern Biotechnology Associates, Inc.) was added to the wells and the plates were incubated for 30 min at 37°C. After the plates were washed as previously stated, buffered hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine solution (Pierce, Thermo Fisher) were added to each well, and plates were incubated for 20 min at 37°C. The reaction was stopped with 2 N sulfuric acid, and the amount of bound antibody was determined colorimetrically by

reading at 450 nm with a reference filter (570 nm) using a Biotek ELx808 plate reader. The results are reported to be the reciprocal of the highest dilution giving a mean OD of at least 0.1 (which was at least twice the background) \pm 1 SD.

Statistics

The LD₅₀ values were estimated by Probit analysis, using log₁₀ dose as the predictor. The effect of strains on LD₅₀ is tested by a chi-square test based on the Probit model, under the assumption of a common effect of log₁₀ dose. The analysis is implemented in SAS PROC PROBIT, SAS PROC LIFETEST, and SAS PROC LIFEREG, and SAS version 9.4. The survival curves are compared by a Log-rank test for trends with an analysis completed with GraphPad Prism 9.2.0. Rat weight changes were compared between any two groups by one-way ANOVA with Tukey's post comparison test with analysis completed with GraphPad Prism 9.2.0. For the antibody titers, data were log₁₀ transformed prior to analysis. Pairwise treatment groups were compared using the Wilcoxon rank sum test. The analysis was implemented in SAS version 9.4 (Carey, NC).

Results

Determine rat LD₅₀ values with *F. tularensis* panel strains

In our previous study characterizing this panel of *F. tularensis* strains, we determined the LD₅₀ values in BALB/c mice to confirm virulence.¹⁹ In previous studies, we also demonstrated that the rLVS vaccine is highly protective in the BALB/c mouse model of lethal pneumonic tularemia.²⁴ However, mice show a different pattern of susceptibility to *F. tularensis* strains than humans, as unlike humans, mice are highly sensitive to LVS and *Francisella novicida*. In this respect, the Fischer rat, which is also resistant to LVS and *F. novicida*, more closely reflects human susceptibility.^{32,33} Therefore, we extended our studies to the Fischer rat model of lethal pneumonic tularemia.

As shown in Table 1 and Figure 1, we performed whole-body aerosols to determine the LD₅₀ value for Fischer rats with our down-selected strains FRAN244 (LD₅₀ = 433 CFU), FRAN254 (a Type A strain derived from a tick) (LD₅₀ = 23 CFU), and FRAN255 (a Type B strain) (LD₅₀ = 5,672 CFU). From these determinations, the estimated inhaled LD₅₀ for the three strains in rats had a wide distribution, and the FRAN254 and FRAN255 strains were significantly different from the FRAN244 strain. Furthermore, the range of LD₅₀ values obtained for this model using these strains is different as compared to what we previously reported for intranasal challenge in mice where the LD₅₀ for all virulent strains was <1 CFU.¹⁹

Table 1. Rat aerosol LD₅₀ analysis of *F. tularensis* strains.

Strain	Aerosol LD ₅₀ (CFU)	Significant Difference from FRAN244
FRAN244	433	–
FRAN254	23	<i>p</i> = 0.0004
FRAN255	5,672	<i>p</i> = 0.003

The rLVS vaccine protects rats against Schu S4 aerosolization challenge

Previously, it was demonstrated that the rLVS vaccine was able to protect mice against intranasal challenge with Schu S4.²⁴ Here, we demonstrate the efficacy of rLVS against challenge with aerosolized FRAN244 strain in Fischer rats. The rats were vaccinated with rLVS by subcutaneous inoculation with $\sim 10^6$ (2 doses), $\sim 10^7$ (1, 2, and 3 doses), or $\sim 10^8$ (2 doses) CFU as shown in Figure 2a and Table 2. As indicated, multiple-vaccine doses were given 3 weeks apart. The comparators for this study were rats receiving a single dose of parent LVS at $\sim 10^7$ CFU and naïve rats receiving a single dose of PBS. Prior to and during the vaccinations, the weights were collected weekly for all rats (vaccinated and naïve), and no weight loss or other adverse side effects were observed (Figure 2c), demonstrating that this vaccine is safe and well tolerated in rats.

Three weeks after the final vaccination, the rats were challenged by whole-body exposure to aerosolized FRAN244. Rats from each group were included in each of the multiple aerosol runs to ensure equal representation during the challenge sprays. The average inhaled dose for the FRAN244 challenged rats was 72 LD₅₀. As shown in Figure 2b, all rats administered a vaccine (either LVS or rLVS) survived the FRAN244 challenge, and no outwardly apparent clinical signs of infection were noted. rLVS provided 100% protection whether administered once, twice or three times at a dose of 10^7 CFU, and whether administered twice at a dose of 10^6 , 10^7 or 10^8 CFU. In contrast, all rats in the naïve group succumbed to infection or were euthanized in accordance with early endpoint euthanasia criteria by Day 8 (Figure 2b). At the end of the study, 21 days post-challenge, a portion of the lungs and spleens from the surviving rats were processed for pathology (described below), and the remainder of the organs were homogenized, diluted, and plated for CFU counts. None of the surviving animals had any detectable *F. tularensis* bacteria (limit of detection is 10 CFU).

The rLVS vaccine can protect rats against inhalational tularemia caused by diverse *F. tularensis* strains

Based upon the protection provided by rLVS against the FRAN244 strain, we proceeded to challenges of vaccinated rats with the down-selected *F. tularensis* panel strains (FRAN254 and FRAN255) (Figure 3). In addition, the vaccination strategy for rLVS was modified to consist of a single vaccination with $\sim 10^7$ CFU or two vaccinations at $\sim 10^8$ CFU. The controls for this study were again administered PBS (naïve) or the LVS parent with a single vaccination at $\sim 10^7$ CFU (Figure 3a and Table 3). Prior to and during vaccinations, the weights were collected weekly for all rats (vaccinated and naïve), and again no weight loss or other adverse side effects were observed for those rats receiving the vaccine compared to PBS alone (Figure 3).

Three weeks after the final vaccination, the rats were challenged by whole-body aerosolization with FRAN254 or FRAN255. The average inhaled dose for the FRAN254 challenged rats was 233 LD₅₀ and 73 LD₅₀ for FRAN255. As shown in Figure 3b, all rats immunized with either LVS or rLVS survived the high challenge dose with FRAN254 (Type A),

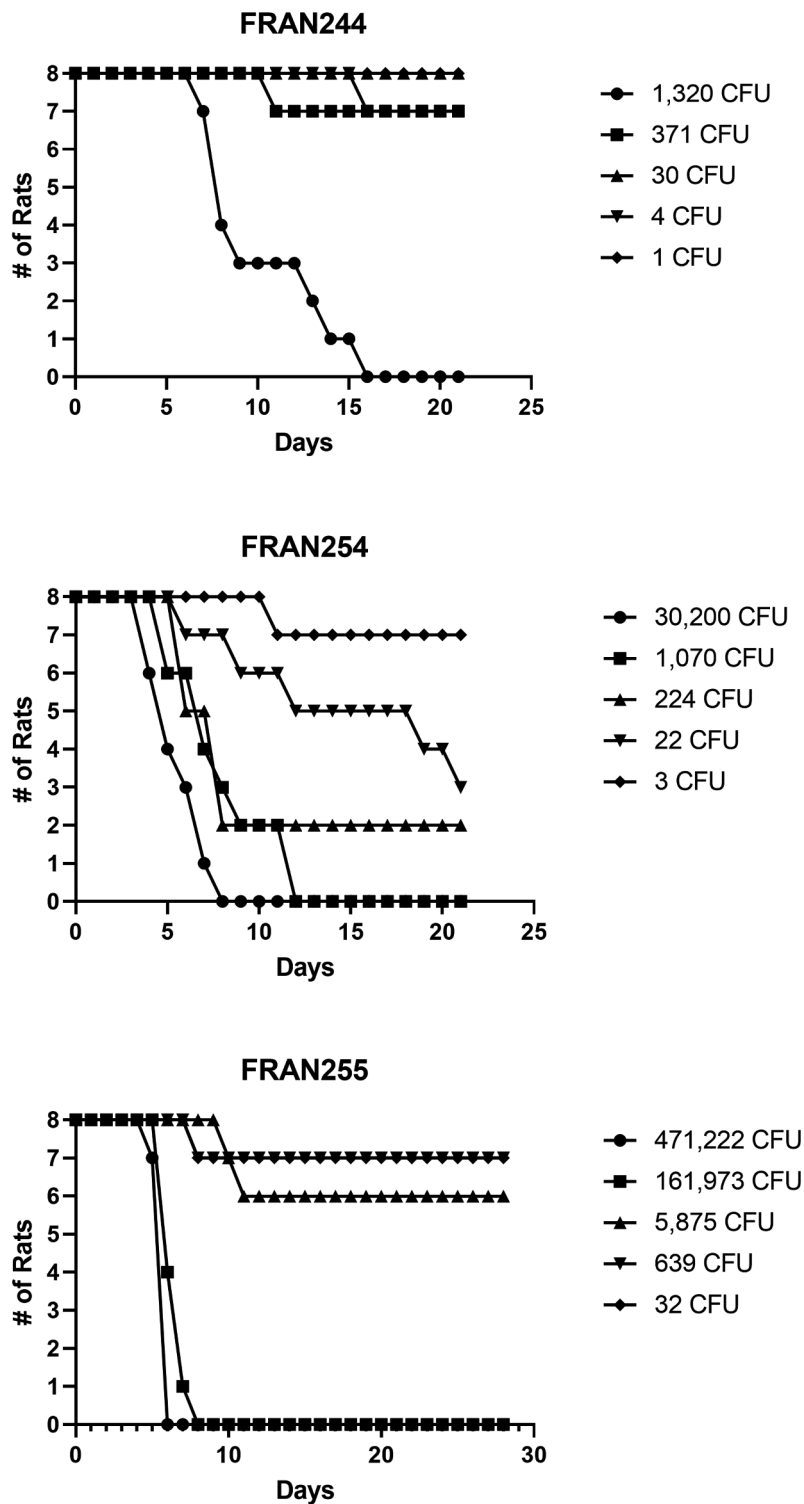


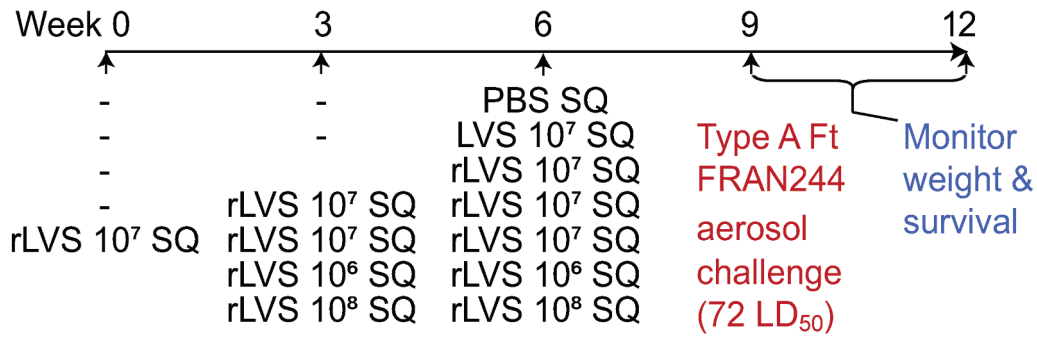
Figure 1. Survival data of rats challenged with aerosolized *F. tularensis* strains for determination of LD₅₀. Groups of Fischer rats ($n = 8/\text{group}$) were challenged by whole body aerosolization with strains FRAN244, FRAN254, or FRAN255 as indicated, and survival monitored. The calculated LD₅₀ values from these experiments are shown in Table 1.

and no clinical signs were observed post-challenge for all of the vaccinated groups. In contrast, all naïve rats challenged with FRAN254 succumbed to infection or were euthanized by Day 6 (Figure 3b, lower left graph). At the end of the study, 21 days post-challenge, a portion of the lungs and spleens from the surviving rats were processed for pathology (described below) and the remainder was homogenized, diluted, and plated for

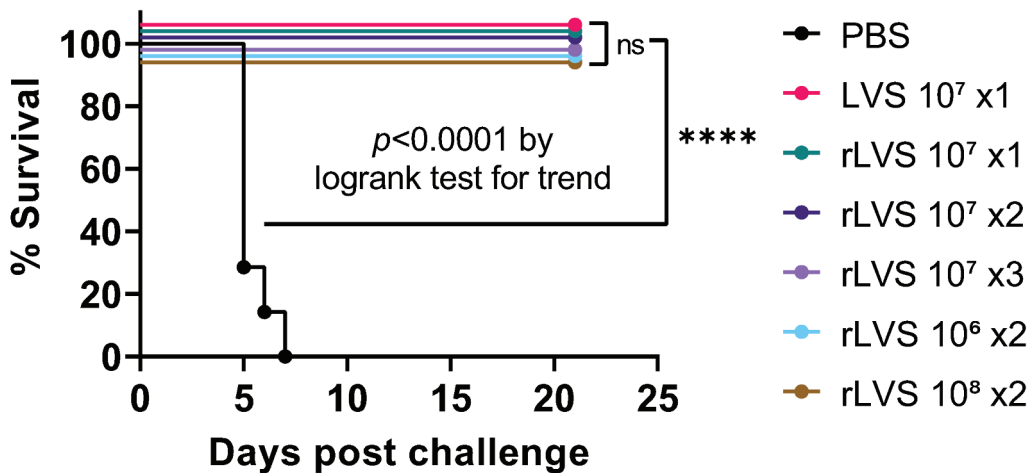
CFU counts. Again, there was no evidence of any *F. tularensis* bacteria in the examined organs in any of the surviving rats.

For those rats exposed to aerosols of the Type B strain FRAN255, all rats immunized with LVS or twice with rLVS (10^8 CFU) survived to the end of the study at Day 21 post-challenge (Figure 3b, lower right graph). For rats immunized just once with a low dose (10^7 CFU) of rLVS, all but one of the

a. Schedule for rLVS vaccine - immunization and challenge



b. Percent survival post immunization and respiratory challenge



c. Weight change post immunization and respiratory challenge

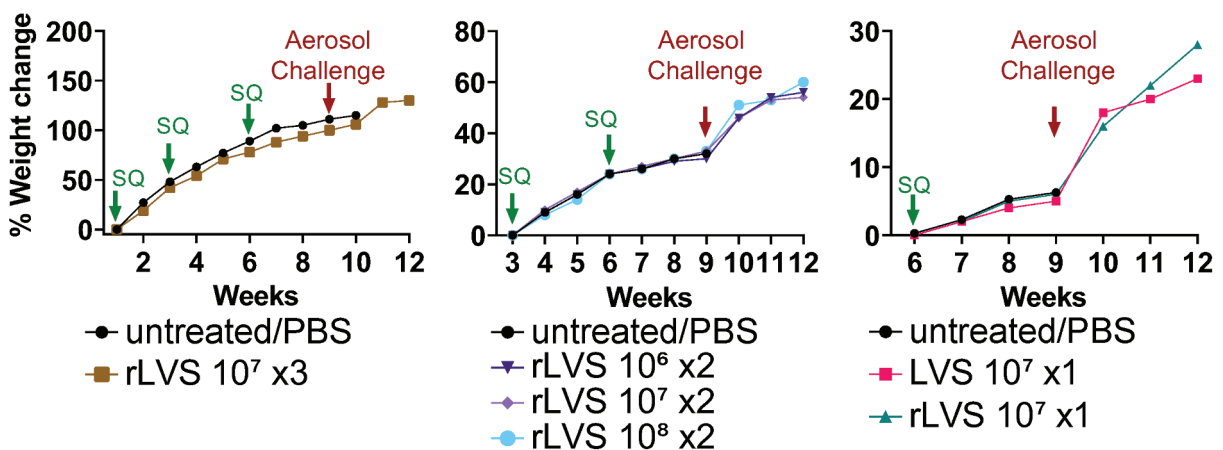


Figure 2. Efficacy of rLVS against aerosol challenge with *F. tularensis* (FRAN244) in Fischer rats. (a). Experimental schedule. Fischer rats (*n* = 7 or 8/group) were immunized subcutaneously (SQ) once with PBS (*n* = 7/group), 10⁷ CFU LVS (*n* = 8/group), or 10⁷ CFU rLVS (*n* = 7/group); twice with 10⁶ CFU rLVS (*n* = 8/group), 10⁷ CFU rLVS (*n* = 7/group), or 10⁸ CFU rLVS (*n* = 7/group) rLVS; or three times with 10⁷ CFU rLVS (*n* = 8/group), 3 weeks apart; challenged with aerosolized *F. tularensis* FRAN244 (72 LD₅₀) at Week 9; and monitored for signs of illness, weight change, and death for 3 weeks post challenge, as indicated. (b). Survival post immunization and respiratory challenge. The survival curves are compared by log-rank test for trend, ****, *p* < 0.0001. (c). All rats were weighed weekly. Weight change post immunization and post respiratory challenge.

rats survived to the end of the study; the one rat that succumbed died on Day 7. Among the surviving rats, low-level clinical signs of infection, such as chromodacryorrhea, were observed for some of the rats in all vaccinated groups. These first appeared on Day 5 post-challenge and resolved by Day 8 (data not

shown). In addition, weight loss (Figure 3b, upper right graph) was also detected for the vaccinated rats post-challenge. However, their weights did rebound by the second week post-challenge. The results for the naïve rats were drastically different, as all animals succumbed to infection by Day 7.

Table 2. Vaccine doses for testing protection against FRAN244 in rats.

Group	Vaccine Strain	Week 0 (CFU)	Week 3 (CFU)	Week 6 (CFU)
1	PBS- naïve	–	–	N/A
2	LVS	–	–	1.24×10^7
3	rLVS	–	–	1.21×10^7
4	rLVS	–	2.71×10^6	1.04×10^6
5	rLVS	–	1.15×10^7	1.21×10^7
6	rLVS	–	1.46×10^8	1.42×10^8
7	rLVS	1.92×10^7	1.15×10^7	1.21×10^7

Histopathological analysis of vaccinated rats

In vaccinated animals challenged with the FRAN244/SchuS4 strain, as described in Figure 2a, no significant pathological differences were observed among the challenged animals within any of the groups that received rLVS at different concentrations or among animals that received the traditional LVS vaccine (Figure 4, top row, middle, and rightmost images). All animals from every vaccinated group that were reviewed microscopically had similar histopathologic changes within the lungs, consisting of multifocal areas of alveolar inflammation comprising macrophages, lymphocytes, and plasma cells, with a severity ranging from mild to moderate. The lesions observed in all vaccinated animals were considered to be a consequence of aerosolized *F. tularensis* infection and resolving. In marked contrast, unvaccinated rats infected with FRAN244, all of which had succumbed to infection, showed alveolar necrosis and damage, more severe and widespread inflammation (moderate to marked) and hyperplasia of bronchus associated lymphoid tissue (Figure 4, top row, leftmost image).

Findings within the liver of vaccinated animals challenged with FRAN244 were minor and classified as incidental; these included minimal inflammatory infiltrates with occasional single-cell necrosis (supplementary Figure S1, left panel, top row). In contrast, unvaccinated rats challenged with FRAN244 displayed multifocal areas of necrosis within hepatic lobules as well as hepatocyte vacuolation and inflammation. In vaccinated rats that survived challenged with FRAN244, the spleen appeared normal in almost all animals; only a few animals displayed decreased cellularity in the marginal zone of the white pulp (supplementary Figure S1, right panel, top row). Again, in marked contrast, the spleens of unvaccinated rats, all of which succumbed to the challenge with FRAN244, showed necrosis in both the red and white pulp as well as lymphoid depletion, inflammation, and accumulation of fibrin and/or fibrin thrombi.

In rats challenged with FRAN254, those vaccinated with LVS or rLVS, all of whom survived the challenge, were again protected against lesions in the lung, liver, and spleen. Specifically, in the lung, the vaccinated animals were spared the necrosis/alveolar damage and severe inflammation throughout the lung observed in the naïve controls (Figure 4, middle row). In the liver, LVS or rLVS vaccination provided protection against the necrosis as well as more severe inflammation and hepatocyte vacuolation seen in naïve controls (supplementary Figure S1, left panel, middle row). In the spleen, vaccinated animals were protected against necrosis, more severe inflammation, fibrin accumulation, and lymphoid depletion observed in naïve controls

(supplementary Figure S1, right panel, middle row). Additionally, when comparing FRAN254 infected animals that had been administered two different rLVS regimens (Table 3), the lesion type, severity, and frequency were very similar between these groups, and the lesion severity was either minimal or mild in the majority of cases in the lung, liver, and spleen.

In rats challenged with FRAN255, the histopathologic analysis again showed those vaccinated with LVS or rLVS were protected against lesions in the lung, liver, and spleen compared with unvaccinated animals. Specifically, in the lung, this included protection against necrosis/alveolar damage (Figure 4, bottom row). Inflammation (alveolar, perivascular) in the lung remained elevated in vaccinated animals, similar to the severity seen in naïve controls although with a different character (e.g., many foamy macrophages and absence of neutrophils). In the liver, vaccinated rats were protected against necrosis, as well as more severe inflammation and hepatocyte vacuolation seen in naïve animals (supplementary Figure S1, left panel, bottom row). In the spleen, vaccinated rats were protected against necrosis, more severe inflammation, fibrin accumulation and/or thrombi and lymphoid depletion seen in naïve animals (supplementary Figure S1, right panel, bottom row).

When comparing the pathology among rats immunized by different vaccination regimens, the lesion type, severity, and frequency were very similar among the groups and the lesion severity was minimal in the liver and spleen. Interestingly, some differences were noted among the groups in the lungs of the survivors (Figure 4). Inflammation and BALT hyperplasia were elevated in the lung in all three vaccinated groups and ranged in severity from mild to marked. In a subset of animals, those immunized once with rLVS had lower severity scores for inflammation and BALT hyperplasia than the animals immunized with rLVS twice or with LVS. There was a single FRAN255 infected animal vaccinated once with a low dose (10^7) of rLVS that was found dead on day 7; this rat had lesions more consistent with those seen in control animals, suggesting that this single animal was not protected by the vaccine protocol. Supplementary Table S1 provides additional details on the histopathological analysis of these challenge experiments.

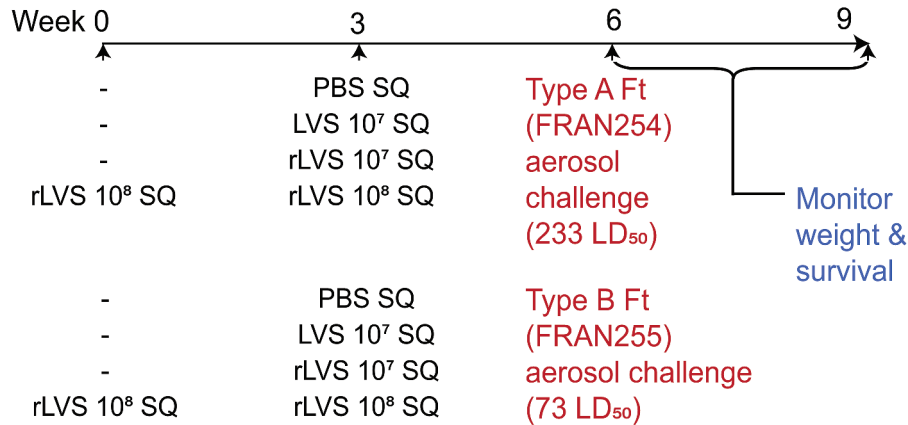
Antibody analysis

Total serum IgG response was assessed at three discrete time-points in rats: 1) prior to vaccination (Pre-vax), 2) 16 days following the last dose of the vaccine (Post-vax), and 3) from the survivors at the end of the study at 21 days post challenge (Survivors). Antibody titers were evaluated in all animals against irradiated FRAN244 (Type A), FRAN255 (Type B), or both antigens as listed. In Fischer rats, IgG2b/2c indicates a Th1 type response and IgG1/2a indicates a Th2 type response.^{34,35}

Experiment 1: challenge with FRAN244

Following vaccination but prior to challenge with Schu S4, the highest total IgG responses directed against irradiated

a. Schedule for rLVS vaccine - immunization and challenge



b. Weight and survival post immunization and respiratory challenge

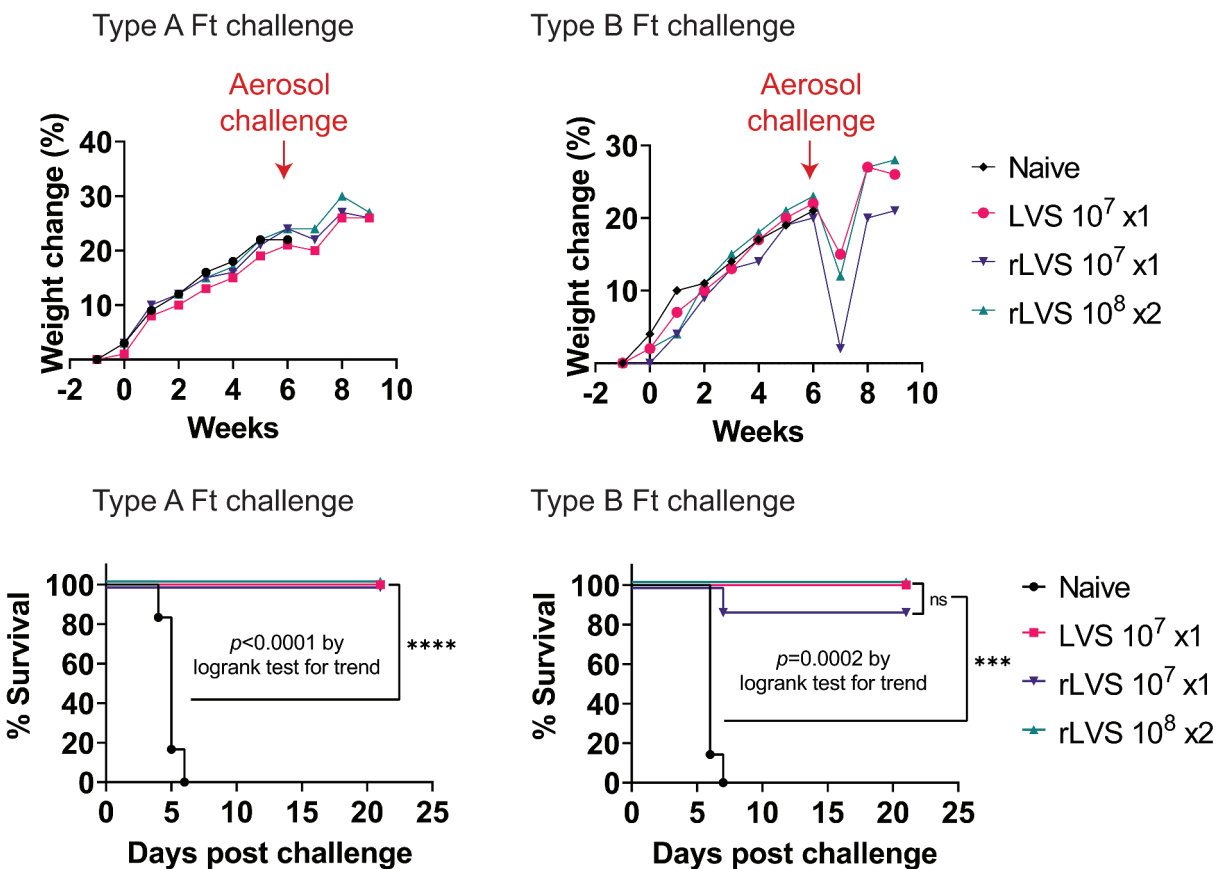


Figure 3. Efficacy of rLVS against aerosol challenge with *F. tularensis* FRAN254 and FRAN255 in Fischer rats. **(a).** Experimental schedule. Fischer rats were immunized subcutaneously (SQ) once at Week 0 with PBS (naïve, $n = 6$ and 7 /group for Type A and Type B challenge, respectively), 10^7 CFU LVS ($n = 7$ and 8 /group for Type A and Type B challenge, respectively), 10^7 CFU rLVS ($n = 8$ /group for both Type A and Type B challenge) or twice at Week 0 and Week 3 with 10^8 CFU rLVS ($n = 8$ /group for both Type A and Type B challenge); challenged with aerosolized *F. tularensis* Type A (FRAN254, 233 LD₅₀) or Type B (FRAN255, 73 LD₅₀) at Week 6; and monitored for signs of illness, weight change, and death for 3 weeks, as indicated. **(b).** Weight change and survival after vaccination and challenge. Weight changes were compared between any two groups by one-way ANOVA with Tukey's post comparison's test. No significant differences were found. The survival curves are compared by log-rank test for trend (Prism 9.2.0). ***, $p < 0.001$; ****, $p < 0.0001$.

F. tularensis (FRAN244) were observed in rats vaccinated with rLVS x2 (10^8 CFU) followed by rats vaccinated with rLVS x3 (10^7 CFU) (Figure 5a). Of the four IgG subclass antibodies (IgG1, IgG2a, IgG2b, and IgG2c), the LVS-based vaccines appear to mediate an IgG2b dominant response

following vaccination (Figure 5a). Rats vaccinated with rLVS x2 (10^8 CFU) and rLVS x3 (10^7 CFU) had significantly greater IgG2b titers than other vaccine groups ($p < 0.05$). In addition, with respect to the IgG2a response, rats vaccinated with rLVS x3 (10^7 CFU) had a significantly greater endpoint

Table 3. Vaccine doses for testing protection against FRAN254 and FRAN255 in rats.

Group	Vaccine Strain	Week 0 (CFU)	Week 3 (CFU)
1	PBS- naïve	-	N/A
2	LVS	-	5.2×10^7
3	rLVS	-	5.8×10^7
4	rLVS	9.7×10^7	8.8×10^7

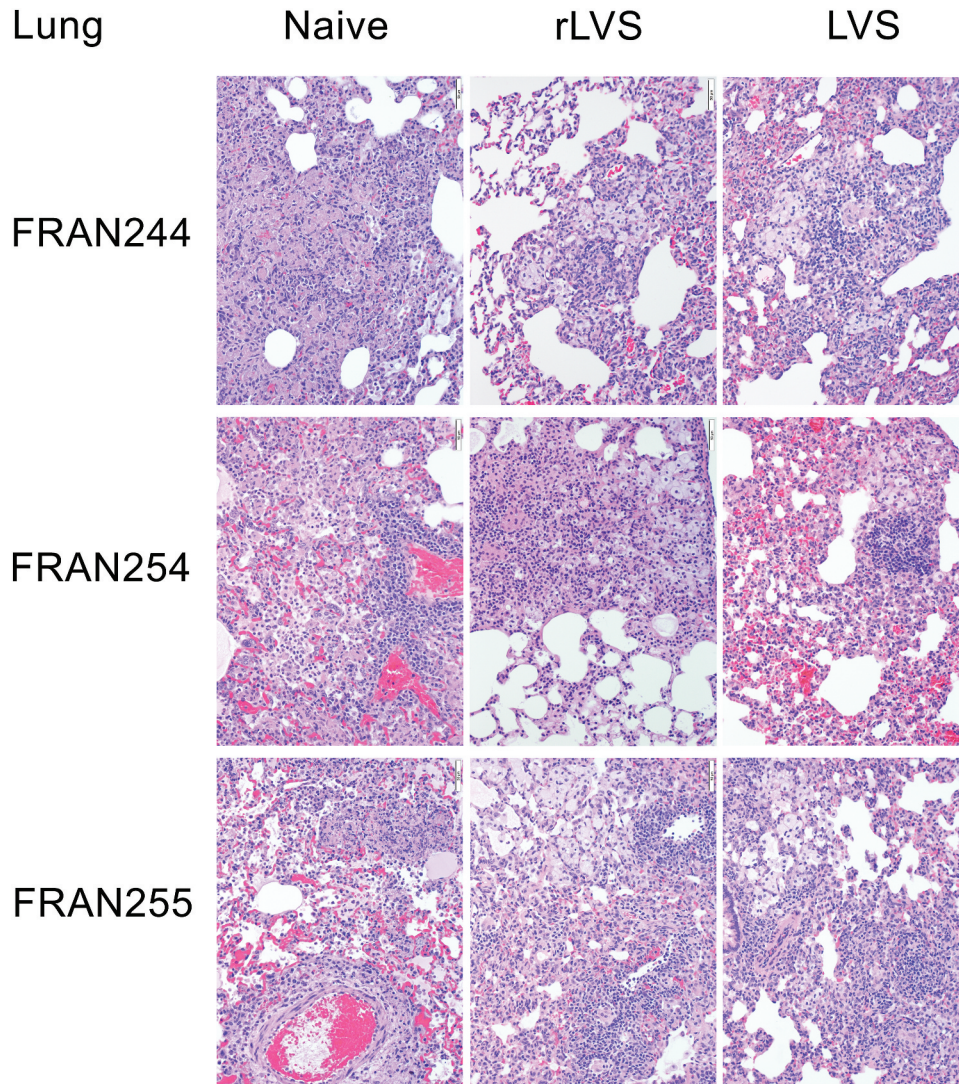


Figure 4. Histopathology of lungs from *F. tularensis* challenged rats. Rats were unvaccinated (naïve) or vaccinated with rLVS, or LVS, as indicated; challenged with FRAN244 (top), FRAN254 (middle), or FRAN255 (bottom); and monitored for survival for 21 days. All naïve rats were necropsied at death or following euthanasia when moribund. Vaccinated rats survived and were euthanized and necropsied at the end of the experiment; however one rLVS (single dose) rat challenged with FRAN255 did succumb). Lungs were examined for histopathology. A representative animal from each group is shown. For the rLVS group, all images shown are from the single dose 10^7 CFU vaccinated group.

titer (50,625) relative to the other vaccine groups ($p < 0.05$). The IgG1 titers in all vaccinated groups were near background levels and similar to those of the control group. As expected, a higher concentration and a greater number of doses induced higher antibody responses.

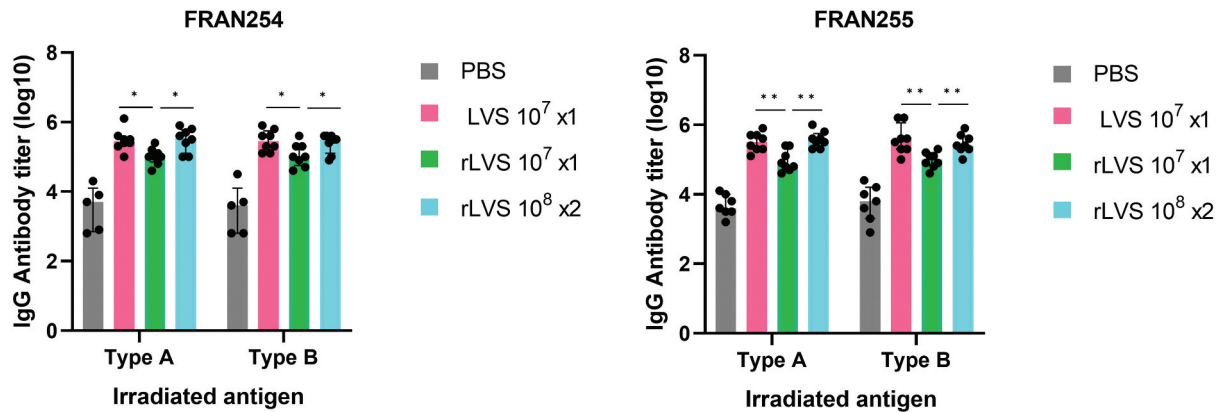
With respect to survivors of FRAN244 challenge, the total IgG antibody response of most vaccine groups was substantially increased relative to the pre-challenge antibody titers (Figure 5b). Furthermore, the IgG2b responses were still the most pronounced in all vaccine groups followed by the IgG2a and then the IgG2c responses, while the IgG1 response did not appear to be affected by the

challenge. Surprisingly, survivors of the rLVS x2 (10^8 CFU) vaccinated group did not generate as notable of an increase in antibody titers relative to other vaccine groups (Figure 5b). Unlike the antibody responses following vaccination, the antibody responses following the challenge did not appear to be as dependent upon the concentration or number of vaccine doses.

Experiment 2: challenge with FRAN254 and FRAN255

Prevaccination baseline antibody titers for Type A and Type B antigens were obtained in all groups; there were

a. Total serum IgG response prior to challenge with Type A FRAN254 and Type B FRAN255



b. Total serum IgG response in rat survivors after aerosol challenge with FRAN254 and FRAN255

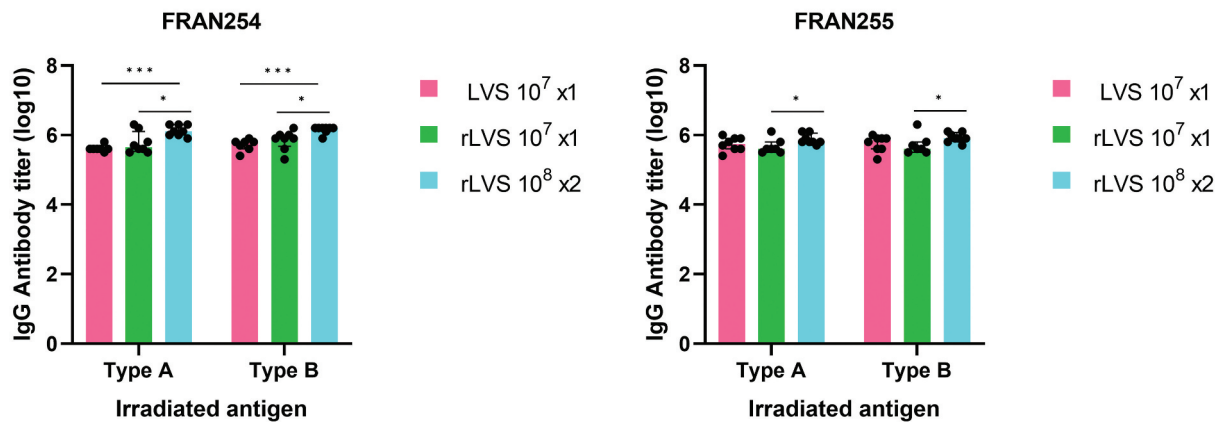
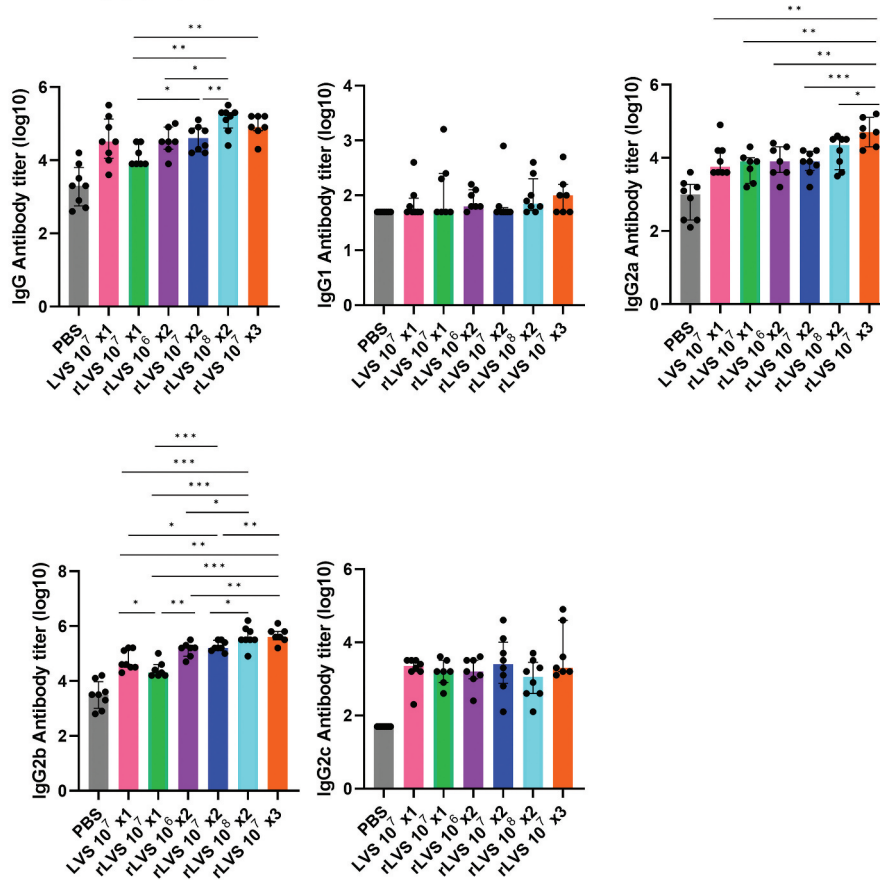


Figure 5. Total serum IgG antibody in rats pre- and post-challenge (survivors) with Type A *F. tularensis* FRAN244/Schu S4 strain. Animals were immunized and challenged as described in the legend to Figure 2. Serum IgG antibody and subclasses specific to irradiated FRAN244 (Type A antigen) were assayed. a. Total serum antibody and subclasses in immunized rats prior to challenge with aerosolized *F. tularensis* Type A FRAN244/Schu S4 strain. For IgG, IgG2a, IgG2b, and IgG2c, all vaccinated groups had significantly greater titers than the PBS group ($p < 0.01$ – $p < 0.001$). For IgG1, all vaccinated groups except LVS, rLVS 10^7 x1 and rLVS 10^7 x2 groups had significantly greater titers than the PBS group ($p < 0.05$ – $p < 0.001$). Values represent median with interquartile range (Q1 and Q3 for the whiskers) of serum antibody for $n = 7$ or 8 per group. b. Total serum antibody and subclasses in immunized rats surviving challenge with aerosolized *F. tularensis* Type A strains. Values represent median with interquartile range (Q1 and Q3 for the whiskers) of serum antibody. Notes: in Fischer rats, IgG2b/2c indicates a Th1 type response and IgG1/2a indicates a Th2 type response. Values that are significantly different between two groups are marked with asterisk(s) over an open horizontal line crossing above the two groups. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Additional descriptive statistical values are included in Supplemental Tables S2–5.

no statistically significant differences among groups (supplementary Figure S2). Sixteen days following vaccination, the antibody response to both antigens [irradiated FRAN244 (Type A) and FRAN255 (Type B)] was significantly enhanced in all vaccine groups ($p \leq 0.0016$ vs. PBS) and the magnitudes of the antibody titers were comparable against both antigens. However, the antibody titer following a single low dose of the rLVS (10^7 CFU) was significantly lower relative to the other vaccine groups ($p < 0.05$ vs. LVS or rLVS $\times 2$) (Figure 6a).

Three weeks following the challenge, the antibody response in surviving animals was substantially increased in all vaccine groups relative to pre-challenge titers. Following the challenge with FRAN254, rats vaccinated with a double dose of rLVS had significantly higher endpoint titers against both FRAN244 (Type A) and FRAN255 (Type B) antigens ($p < 0.05$ vs. LVS and rLVS). Following FRAN255 challenge, the higher titer of the rats immunized with a double dose of rLVS only reached significance relative to rats administered a single dose of rLVS ($p < 0.05$ vs. rLVS). Surviving rats from the LVS and rLVS vaccinated groups

a. Serum IgG and subclasses prior to challenge with *F. tularensis* FRAN244/Schu S4



b. Serum IgG and subclasses of survivors after challenge with *F. tularensis* FRAN244/Schu S4

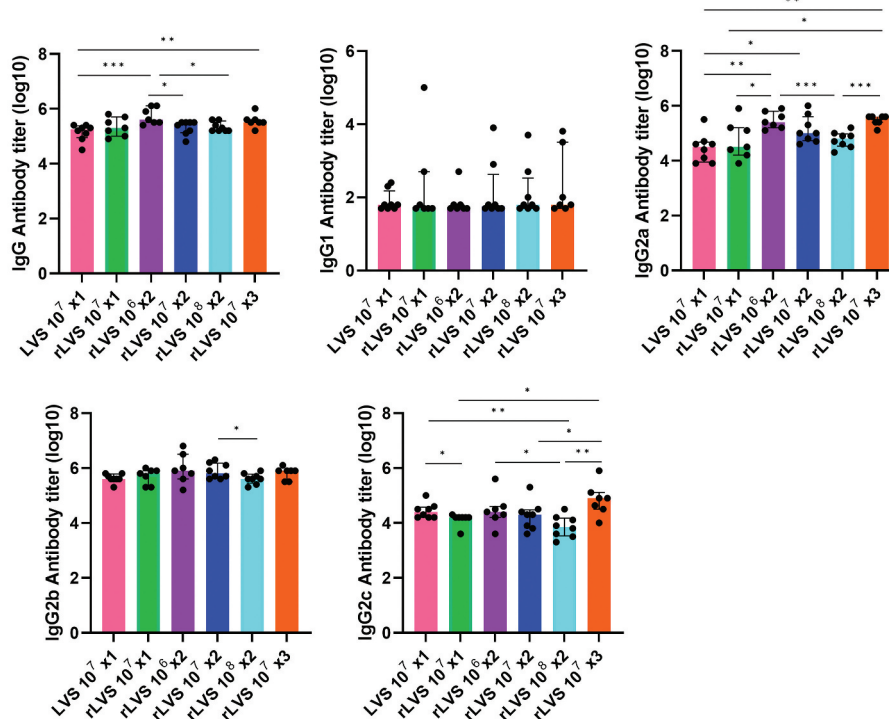


Figure 6. Total serum IgG antibody in rats immunized with rLVS vaccines prior to and post challenge with Type A *F. tularensis* FRAN254 or Type B FRAN255 strain. Animals were immunized and challenged as described in the legend to Figure 3. Sera were collected in immunized rats prior to and after challenge with Type A or Type B strains and assayed for total IgG antibody specific to irradiated FRAN244 (Type A antigen) and FRAN255 (Type B antigen). **(a).** Prior to challenge; All vaccinated groups in Panel a had significantly greater titers than the PBS group ($p < 0.01$ - $p < 0.0001$). **(b).** Post challenge (survivors only). Values represent median with interquartile range (Q1 and Q3 for the whiskers) of serum antibody titers for $n = 6-8$ /group. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Additional descriptive statistical values are included in Supplemental Tables S6,7.

had similar antibody titers relative to each other at the conclusion of the study (Figure 6b).

Discussion

Currently, the U.S. biodefense community is lacking an approved FDA vaccine to prevent tularemia. Attenuated strains of pathogens have the potential to be used as live-attenuated vaccines that establish mild infections in the host and mimic the natural infection which allows for the presentation of diverse antigens to the host immune system to promote the induction of both antibody and cell-mediated immune responses.^{36,37} Some of the concerns with the unlicensed LVS are in regard to its safety and reactogenicity, despite its having two major attenuating deletions (*FTT0918* and *pilA*) and several minor deletions.³⁸ However, a modified version of LVS (rLVS $\Delta capB$), with a third major attenuating deletion in *capB*, was demonstrated to be much less virulent than the parent LVS (intranasal LD₅₀ > 10,000 fold that of LVS in highly susceptible mice) and has the potential to be used as a vaccine platform to express immunogenic proteins against multiple pathogens in addition to *F. tularensis* (*Yersinia pestis*, *Bacillus anthracis*, and severe acute respiratory syndrome coronavirus 2).^{24–26,39} The rLVS $\Delta capB$ platform was engineered to express epitopes of the *F. tularensis* type VI secretion proteins IglA, IglB, and IglC. The vaccine rLVS $\Delta capB/iglABC$ (rLVS), administered intradermally or intranasally, was shown to be highly protective in mice against respiratory challenge with the Schu S4 strain of *F. tularensis*. Here, we further demonstrate the potential of rLVS as a vaccine in the rat model of tularemia by whole-body aerosol challenge with not only the prototype Schu S4 strain but additional strains of *F. tularensis* from a diversity panel.¹⁹

Our results show that the rLVS vaccine was able to protect against the SchuS4 strain in addition to two down-selected strains, FRAN254, a highly virulent Type A strain derived from a tick, and FRAN255, a clinically derived Type B strain. We previously characterized these strains and determined the LD₅₀ in a mouse intranasal challenge model. The LD₅₀ for each of these three strains in BALB/c mice was only 1 CFU.¹⁹ However, when testing these strains in the Fischer rat model following whole-body aerosol challenge, the differences in LD₅₀ for these strains became apparent (Table 1). The differences in the LD₅₀ for FRAN254 (Type A & LD₅₀ = 23 CFU) and FRAN255 (Type B & LD₅₀ = 5,672 CFU) were each statistically significant when compared to the prototype Schu S4 (designated as FRAN244 for this study) having an LD₅₀ of 433 CFU (Table 1). The higher aerosol LD₅₀ value determined for FRAN255 as when compared to the Type A strains would not be unexpected as Type B strains are typically described as being less virulent than Type A strains.⁴⁰ In contrast, FRAN254, an additional Type A strain derived from a tick, was found to have a lower LD₅₀ as compared to the Schu S4 strain when tested in rats. These data further underscore the high level of susceptibility of mice to *F. tularensis* infection and support the choice of the rat model, which better reflects human susceptibility to *F. tularensis* than mice, as an appropriate rodent species for vaccine efficacy studies.^{32,33,41–44} More importantly, these results highlight the LD₅₀ differences between isolates of *F. tularensis*

and the critical need to test prospective tularemia vaccine candidates against more than just the prototype Schu S4 laboratory strain to assure protection against multiple strains.

As described here, rats receiving the rLVS vaccine in any of the doses/series provided were protected against high LD₅₀ challenges of the respective strains. Complete protection was observed following challenges with either of the Type A strains used, FRAN244 or a tick derived strain FRAN254. Interestingly, following a challenge with the high LD₅₀ Type B strain (FRAN255), one rat which only received one low dose of rLVS succumbed to infection after challenge. In addition, we noted that all rats vaccinated with either LVS or rLVS displayed some low-level clinical signs of infection, which were quickly resolved. During histopathologic analysis at the end of the study, lungs from survivors of FRAN255 challenge showed elevated levels of inflammation (alveolar, perivascular) that consisted of many foamy macrophages and no neutrophils. It is possible that these results following challenge with FRAN255 are due to the higher CFU numbers of bacteria needed to reach 70 LD₅₀ of the less virulent Type B strain versus the equivalent CFU numbers to reach 70 LD₅₀ of FRAN244. We note that some of these pathologic differences between the naïve control and vaccinated groups may not only be due to the effect of vaccination but also to the different time points of sampling in the course of disease (i.e., naïve animals that succumbed during the acute stage/day 5 versus vaccinated animals that survived to the end of study/subacute-chronic stage/day 21). Nevertheless, the rLVS vaccine offered protection and considerable melioration of disease caused by the Type B strain. Additional experimentation could include sampling the vaccinated versus naïve rats at set time points post-challenge to allow a better comparison of pathologic differences in protection and to determine what level of infection, as assessed by CFU counts from lungs and spleens, occurs for the vaccinated animals. In addition, considerations should be given to exploring the protection provided by rLVS against challenge with additional Type B *F. tularensis* strains.

Following vaccination with two doses of rLVS, a robust total IgG response was induced that was comparable to a single LVS vaccination (Figure 5). Due to the targeted deletion of the capsule synthesis gene (*capB*), the rLVS was strikingly more attenuated in BALB/c mice, with an intranasal LD₅₀ dose that was >10,000-fold relative to the parental LVS. Furthermore, rLVS replication in the lungs was significantly lower at the peak of infection and was cleared from the lungs, liver, and spleen faster relative to LVS.³⁹ Hence, when administered at the same relatively low dose (10⁷), a double dose of rLVS was necessary to achieve antibody titers similar to those observed with LVS. Furthermore, increasing the rLVS dose improved the endpoint titers that surpassed titers produced following LVS vaccination. The predominant IgG2b as well as IgG2c response, of which the latter was much less induced, was indicative of a Th1 skewed response, which increased following vaccination with increasing dose and number of injections.³⁴ Likewise, the Th2 associated IgG2a response was much less induced, while the IgG1 tiers remained close to baseline levels (Figure 5a).³⁵ A similar trend in total IgG titers was observed in survivors following the challenge with FRAN244, although the challenge served as a heterologous boost and drastically increased the overall titers in all vaccinated groups (Figure 5b). A strong

humoral response plays a critical role in protecting against *Francisella* infection. Previous studies have shown that prophylactic treatment of rats with purified immune IgG prior to Schu S4 challenge provided significant protection and diminished bacterial growth.⁴⁵ In addition, in both mouse and rat models, the protection afforded by antibodies was linked to strong T cell dependent responses for bacterial clearance of which the CD8 T cells appear to play a more critical protective role relative to CD4 T cells, although both cell populations are necessary.^{45,46}

The antibody response was also similar against both FRAN244 (Type A) and FRAN255 (Type B) strains following vaccination with either LVS or rLVS in sera collected pre-challenge and in the surviving rats (Figure 6). Of note, there was roughly a 10-fold difference in titers between the two vaccine studies (Figure 5a vs. Figure 6a). Rats vaccinated in the second experiment (Table 3) received roughly ~4- to 8-fold more bacteria per dose relative to the doses in the initial study (Table 2) and thus, this increased vaccine dose may have contributed to higher antibody titers. Furthermore, it is well established that the phenotypic diversity of *Francisella* colony morphology termed phase or antigenic variation is prominent and contributes to vaccine lot variability and dramatically affects virulence and immunogenicity based on a multitude of phenotypic attributes and their prominence within each lot, such as the ratios of the blue (induce protective immune response) vs. gray (do not induce immunity) variants.^{12,17,47–50} This confounding factor during the preparation of these vaccines may also lead to variability in vaccine efficacy and impede precise reproducibility of antibody titers across current studies. However, this limitation may be addressed with cultivation of these vaccines under-improved cGMP conditions, which have been shown to yield 100% blue phenotypes that are associated with conferring protection.⁴⁷ However, for the studies described here, although the vaccination studies were performed at different times, the LVS and rLVS used were from the same frozen preparations and therefore any variants present in these vaccines would have been the same. Furthermore, the frozen vaccine stocks were prepared by rapid growth on chocolate agar so as to minimize the likelihood of phase variation.⁵¹

Our current study has focused on the use of the rLVS vaccine as this is the first vaccine tested using our *F. tularensis* diversity panel to demonstrate protection against aerosol challenge in the Fischer rat model. Several other live and subunit vaccines continue to be explored with some showing promising results, though these studies have been usually limited to mouse models or challenges with only Schu S4.^{52–60} Additional live tularemia vaccine candidates, such as the *Francisella novicida* *iglD* and the *F. tularensis* Schu S4- Δ *clpB* mutant strains, have published studies demonstrating protection with these vaccines in rats or nonhuman primate models of pneumonic tularemia with the Schu S4 strain.^{61,62} It would be important to test these vaccine candidates against additional *F. tularensis* strains to assure protection against multiple virulent strains.

We have shown that immunization with the rLVS vaccine induces high levels of protection against exposure to three different *F. tularensis* strains in Fischer rats to the same level of protection as observed with the parent LVS. However, rLVS offers additional benefits that LVS does not, such as a decreased level of virulence (at least in a murine model of tularemia),

which could address one of the issues hindering LVS from receiving FDA approval.³⁹ In addition, rLVS has the ability to be engineered to express multiple protective antigens against multiple pathogens rather than just *F. tularensis* alone; this would simplify not only vaccine administration but also manufacturing and regulatory approval compared with that of multiple disparate vaccines.^{24–26} Finally, the rLVS vaccine can be administered by multiple routes including intranasally, intradermally, subcutaneously, intramuscularly, and even orally.^{24–27} Based upon the current lack of an approved tularemia vaccine in the U.S., consideration should be given to advancing the rLVS Δ *capB/iglABC* vaccine to further studies in a nonhuman primate model of tularemia challenge to confirm protection.^{63–65}

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Disclosure statement

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