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BRIEF REPORT

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# rLVS Δ*capB*/Yp F1-V single vector platform vaccine expressing *Yersinia pestis* F1 and LcrV antigens provides complete protection against lethal respiratory challenge with virulent plague bacilli

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**ABSTRACT**

*Yersinia pestis*, the causative agent of plague, is classified as a Tier I Select Agent of bioterrorism and is among a few pathogens of high concern as a potential cause of a future pandemic. Currently, there is no licensed vaccine against plague. Previously, we developed a live attenuated vaccine candidate, rLVS Δ*capB*/Yp F1-V, that utilizes a highly attenuated *capB* mutant of *Francisella tularensis* Live Vaccine Strain as a vector to express a fusion protein of *Y. pestis* F1 and LcrV antigens. We showed that homologous prime-boost vaccination with this vaccine provided potent protection in mice against lethal respiratory challenge with virulent *Y. pestis*. Here, we report on the immunogenicity and efficacy of rLVS Δ*capB*/Yp F1-V and additional LVS Δ*capB*-vectored vaccine candidates in mice. We demonstrate that three homologous prime-boost immunizations with an optimized dose of rLVS Δ*capB*/Yp F1-V provided complete protection against pneumonic plague in a stringent mouse model, outperforming other candidates and matching the survival efficacy of the toxic and unlicensed live attenuated *Y. pestis* EV76 strain vaccine; moreover, mice immunized with the rLVS Δ*capB*/Yp F1-V vaccine had minimal weight loss post-challenge that was significantly less than mice immunized with the EV76 vaccine. Protection induced by rLVS Δ*capB*/Yp F1-V correlates with F1 and LcrV-specific serum antibody levels. Our results highlight the potential of rLVS Δ*capB*/Yp F1-V to address the unmet need for a plague vaccine.

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## Introduction

*Yersinia pestis*, a Gram-negative bacterium, is the causative agent of plague including the 14th century Black Death pandemic. It is primarily transmitted to humans via flea bites but can also be spread through direct contact with infected animals or tissues, inhalation of infectious droplets, or ingestion of contaminated food or water. Among its four clinical forms (bubonic, pneumonic, pharyngeal, and septicemic), pneumonic plague is the most severe with mortality approaching 100% if not treated promptly.[1] Pneumonic plague can result from inhalation of infectious droplets or secondary spread of bubonic or septicemic plague.

As *Y. pestis* is highly infectious, causes significant morbidity and mortality, is relatively easy to culture and disperse, and has been weaponized in the past, it is classified as Tier I Select Agent of bioterrorism by the US Centers for Disease Control and Prevention (CDC). In addition to the need for a vaccine to protect against its use as a bioweapon, *Y. pestis* is among a small number of pathogens, and one of only two bacterial pathogens, prioritized for vaccine development to prevent a future pandemic by various interested organizations.[2] Post-exposure prophylaxis is impractical for public, military, or first-responder use, highlighting the need for a safe and effective pre-exposure vaccine to protect against intentional or nonintentional exposure to this pathogen.

Currently, there is no licensed vaccine to protect against plague. Early vaccines, such as the formalin-inactivated Greer USP whole cell vaccine (also known as Plague Vaccine USP, formerly produced by Cutter Biological, Ltd. In the USA) or the heat-activated CSL whole cell vaccine (produced by Commonwealth Serum Laboratories Ltd., Australia), offered partial protection against bubonic plague but were poorly effective against pneumonic plague and were highly reactogenic.[3,4] Another early vaccine, the live attenuated *Y. pestis* vaccine EV76 (produced in the former Soviet Union) was active against both bubonic and pneumonic plague but raised safety concerns due to its reactogenicity and residual virulence.[3,5] This has prompted the development of a new generation of vaccines that are more efficacious against pneumonic plague and safer.[6]

*Y. pestis* possesses three plasmids critical for virulence – pCD1, pMT1 (pFa), and pPCP1 (pPla). The 70-kb pCD1 plasmid encodes components of the Type III Secretion System (T3SS), including low calcium response V antigen (LcrV, 37.1 kDa, the T3SS structure needle tip protein), YscF (9.5 kD, the T3SS structure needle filament protein), and *Yersinia* outer membrane proteins. The 96-kb pMT1 encodes a highly immunogenic antiphagocytic capsular antigen Fraction 1 (Caf1 or F1, 17.6 kb) and other toxins. The 9.6-kb pPCP1 encodes plasminogen activator (Pla), a protease that promotes bacterial dissemination.

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Vaccines comprising F1 and LcrV antigens, including individual F1 and LcrV antigens, a mixture of F1 and LcrV, and F1-V fusion proteins expressed by bacterial and viral vectors, have shown significant protection against challenge with *Y. pestis* in rodents.[6–13] However, protection of non-human primates has been inconsistent and highly variable.[14] Naturally occurring F1-minus *Y. pestis* strains, which are as virulent as wild-type strains,[15] and the diversity in LcrV sequences may reduce the efficacy of F1/LcrV-based vaccines. Efforts to improve subunit vaccines have included using mutated F1 (F1mut) with increased solubility[12] and additional antigens like YscF,[16] which modestly enhances potency for F1-V and LcrV vaccines.[12,17,18]

We previously developed the *F. tularensis* subsp. *holarctica* LVS Δ*capB* vector,[19] a fully defined, further attenuated Live Vaccine Strain (LVS) with three major attenuating deletions (*capB*, *pilA*, and FTT 0918). LVS Δ*capB* is > 10,000-fold less virulent in mice than LVS, making it exceedingly safe.[19] Using LVS Δ*capB* as a single vector platform, we have developed vaccines expressing homologous antigens from *F. tularensis*[20–22] and heterologous antigens from *Bacillus anthracis*,[22] *Y. pestis*,[22] *Burkholderia pseudomallei*,[23] and SARS-CoV-2.[24,25] We have shown that these single vector platform vaccines abundantly express the homologous and heterologous antigenic proteins in broth and in infected murine macrophages. Moreover, we have shown that homologous priming-boosting with the LVS Δ*capB* single vector platform vaccines induces strong humoral and cell-mediated immunity, providing potent protection against lethal respiratory challenge with *F. tularensis*, *B. anthracis*, *Y. pestis*, and *B. pseudomallei* in mice,[20–23] lethal respiratory challenge with *F. tularensis* in Fischer rats,[26] and SARS-CoV-2 challenge in hamsters.[24,25] The protection in mice outperforms existing vaccines including LVS against virulent *F. tularensis* challenge, Anthrax Vaccine Adsorbed (AVA) against *B. anthracis* challenge, and BP82 against *B. pseudomallei* challenge, even with a single immunization.[22,23]

To optimize the efficacy of the LVS Δ*capB*-vectored vaccine against *Y. pestis*, we sought to broaden the antigenic repertoire, enhance the solubility and stability of the antigenic fusion proteins, and optimize the immunization dose. We constructed and characterized in broth culture multiple LVS Δ*capB*-vectored *Y pestis* vaccines expressing the fusion proteins of various forms of F1, LcrV and YscF, and we evaluated their immunogenicity and efficacy against respiratory challenge with the virulent *Y. pestis* CO92 strain in a mouse model. Here we show that three homologous prime-boost immunizations with an optimized dose of one of the vaccines, rLVS Δ*capB*/Yp F1-V, provide complete protection against *Y. pestis* respiratory challenge, and that the protection correlates with F1 and LcrV-specific serum antibody level.

## Material and methods

### Bacteria and vaccines

*F. tularensis* Live Vaccine Strain (LVS) with a deletion in *capB* (LVS Δ*capB*) was developed in our laboratory.[19] The *Y. pestis* attenuated EV76 strain was obtained from the Centers for Disease Control and Prevention, Fort Collins, CO. A virulent strain of *Y. pestis* (CO92), originally obtained from BEI Resources, was used in animal challenge experiments at Colorado State University (CSU). The *Y. pestis* bacteria were inoculated onto sheep blood agar; cultured at 35°C for 2 days; the colonies harvested into BHI broth supplemented with 10% glycerol; and the bacteria frozen and stored at − 80°C until use. The LVS Δ*capB* vector and recombinant LVS Δ*capB*-vectored *Y. pestis* candidate vaccine strains were prepared by scraping bacteria grown on chocolate agar into sterile PBS, collecting cells by centrifugation, washing them twice in PBS, resuspending them in 20% glycerol in PBS, and storing them at −80°C. Prior to each use for immunization of mice, vaccines were thawed at room temperature, diluted in sterile PBS, and used as described previously by us.[20,22] Similarly, the frozen stock of the *Y. pestis* challenge strain was thawed immediately prior to use, diluted in 0.025 ml sterile PBS, and used as described below.

### Mice

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratory (Wilmington, MA) and randomly assigned to experimental groups. Animals were maintained in a specific-pathogen-free animal facility prior to challenge with virulent *Y. pestis*. The animals were challenged intranasally (IN) with *Y. pestis* CO92 strain; relocated to and maintained in a BSL3 facility at CSU; and used according to protocols approved by the CSU Institutional Animal Care and Use Committee (IACUC).

### Proteins and antibodies

We obtained the following reagents through the NIH NIAID Biodefense and Emerging Infections Research Resources Repository: goat polyclonal antibody to LcrV, NR-31022; LcrV protein, NR-32875; F1 protein, NR-44223; and F1-V monomer protein, NR-2562.

### Construction of highly efficient *E. coli-Francisella* shuttle plasmids

Using LVS Δ*capB* as a vaccine vector and a highly efficient pFNL-derived *E. coli-Francisella* shuttle plasmid,[27] we constructed 25 recombinant LVS Δ*capB* (rLVS Δ*capB*) vaccine candidates expressing various forms of fusion proteins comprising the *Y. pestis* F1, LcrV and YscF antigens as we published previously.[22] Specifically, we constructed 25 pFNL-derived shuttle plasmids carrying an antigen expression cassette for various forms of *Y. pestis* fusion proteins, including F1-V [22], PepON43-F1-V, V-F1-YscFmut, V-F1mut-YscFmut, YscFmut-F1mut-V, and V-F1mut, respectively (Table S4). The F1-V and PepON43-F1-V antigen expression cassettes were cloned downstream of the *F. novicida* outer membrane protein promoter (*omp*, upstream of FTN_1451, amplified from *F. novicida* genomic DNA) in the shuttle plasmid and the other fusion protein expression cassettes were cloned downstream of the *F. tularensis bacterioferritin* promoter (*bfr*, upstream of FTT_1441, amplified from the genomic DNA of a clinical isolate of the Schu S4 strain).[22] The coding sequences

for the F1-V and PepON43-F1-V fusion protein were PCR amplified from the genomic DNAs of *Y. pestis* (BEI DD-494) as we described previously.[22]

To construct expression plasmids for fusion proteins of *Y. pestis* F1mut, LcrV and YscFmut, we purchased DNAs codon optimized for expression in LVS from ATUM (Newark, CA) and cloned the coding sequences for the fusion proteins of F1mut, LcrV and YscFmut into pFNL-derived shuttle vector using the Electra cloning strategy described previously by us.[23] Briefly, the DNAs encoding the fusion proteins of F1mut, LcrV, and YscF joined by a flexible peptide linker in between the three open reading frames were codon optimized and synthesized by ATUM. Subsequently, we amplified the coding sequence for LcrV, F1mut, and YscFmut by PCR, using a forward primer with a SapI site, the pM264 MOTHER vector with different overhangs (3'gca, 5'gca, 3'tct, and 5'tct), and sequence specific for codon optimized DNAs for F1, LcrV and YscFmut; and a reverse primer specific for the codon optimized DNA for F1mut, LcrV, and YscFmut, the sequence for the peptide linker in the primer tail, and the pM264 MOTHER vector with different overhangs, and a SapI site (Table S5). The sequence encoding the 3' end of the F1mut (aa 1–21) was included in the reverse primer to amplify F1mut from the codon optimized sequence for F1. The PCR product was subsequently cloned in the SapI cloning sites of the pM264 MOTHER vector with different overhangs (3'gca, 5'gca, 3'tct, and 5'tct) (Table S6). Fusions of two or three ORFs were obtained by combining pM264 MOTHER plasmids with compatible overhangs together with a pFNL DAUGHTER [pFNLdA-bfr-D1 (sacB)] plasmid using Electra cloning to generate the desired *E. coli-Francisella* shuttle plasmid for fusion protein expression in rLVS Δ*capB* strains, as we described previously.[23] A total of 25 shuttle plasmids were generated (Table S4) and the inserted sequences encoding the Yp fusion proteins were confirmed by nucleotide sequencing.

## Construction and characterization of rLVS Δ*capB*/Yp strains

The 25 shuttle plasmids were individually introduced by electroporation into LVS Δ*capB* electrocompetent cells to generate the corresponding rLVS Δ*capB*/Yp vaccine candidates (Table S2), which were then plated on chocolate agar containing 7.5 μg/mL kanamycin as described by us previously.[22,23] After 3–5 days incubation at 37°C, individual clones were picked for analysis by Western blotting for corresponding *Y. pestis* fusion protein expression. The shuttle plasmids were isolated from the vaccine candidates and further sequenced to confirm the inserted DNAs encoding the *Y. pestis* fusion proteins.

To assess protein expression by rLVS Δ*capB*-vectored *Y. pestis* vaccines in broth culture, we inoculated each of the vaccine stocks into Medium T; grew them overnight; collected bacterial cells; lysed the cells in SDS buffer; applied equivalent amounts of lysates to SDS-PAGE; and analyzed protein expression via Western blotting. Secreted proteins in the supernatant of Medium T broth culture of rLVS Δ*capB*/Yp vaccines were concentrated by using protein Concentrators EPS [10 MWCO, Thermo Scientific 88513] and analyzed by Western blotting. Goat polyclonal antiserum specific to *Y. pestis* LcrV (BEI, NR-31022) was used as primary antibody in Western blotting. Growth kinetics of rLVS Δ*capB* vaccines in Medium T broth were assayed as we described previously.[21]

### Immunization and challenge

Efficacy of rLVS Δ*capB*/Yp vaccines was studied at CSU similarly to what was previously described by us.[19–22] Mice were sham-immunized intradermally (ID) once, immunized subcutaneously (SQ) three times with $10^6$ CFU EV76, or three times ID with $2 \times 10^6$ LVS Δ*capB* vector or rLVS Δ*capB*/Yp vaccines, 4 weeks apart. One week prior to challenge (Week 11), mice were bled via facial vein puncture and serum isolated and stored at −80° C until use. At week 12, four weeks after the last immunization, mice were challenged intranasally (IN) with 2100 CFU of virulent *Y. pestis* CO92 diluted in 0.025 ml sterile PBS (~10 LD50), weighed, and monitored for illness and death for 3 weeks. Mice that met predetermined humane endpoints for euthanasia were euthanized and counted as a death.

### Serum antibody

Sera were tested for IgG antibody response by enzyme-linked immunosorbent assay (ELISA) using standard procedures.[22] Briefly, ninety-six-well microtiter plates were coated with recombinant protein F1 (NR-44223) or LcrV (NR-32875) at 1 μg/mL each, diluted in carbonate buffer overnight at 4°C and afterward processed at ambient temperature. The plates were washed three times with 0.05% Tween 20-PBS, blocked in Blocker Casein in PBS (Thermo Scientific) for 1 h, incubated with each serum sample serially diluted 2-fold twelve times at a starting dilution of 1:20 in 1% BSA-PBS for 90 min, and washed again. Bound antibody was detected by using alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO), subtype IgG1 (Sigma), or subtype IgG2a (Abcam) diluted in 1% BSA-PBS, and incubating for 90 min. Plates were developed with 100 μL of p-nitrophenylphosphate substrate diluted in diethanolamine buffer (BioRad), and the A415 was read using a multiscan microplate reader (iMark, BioRad). The antibody endpoint titer is calculated as the mean $\log_{10}$ of the reciprocal of the highest immune serum dilution yielding an optical density (OD) exceeding the mean OD of Sham sera plus three standard deviations at the same dilution.

### Statistical analyses

Values of pre-challenge serum antibody endpoint titers and post-challenge weight loss were presented as Means ± standard deviations (SD). Statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test or ordinary one-way ANOVA with Tukey's test to correct for comparisons. Survival analysis was conducted using the Kaplan-Meier method with log-rank (Mantel-Cox) test to evaluate significant differences in survival curves between vaccinated and control mouse groups. Linear regression was performed to determine the slope, intercept, and correlation coefficient ($R^2$) between pre-challenge serum antibody endpoint titers and post-challenge median or mean survival times at 14 days post-

challenge. All statistical analyses were performed using GraphPad Prism 10.4.0 software (San Diego, CA).

## Results

### Construction and characterization of LVS Δ*capB*-vectored *Y. pestis* vaccines

To broaden the antigenic repertoire and enhance the solubility and stability of the antigenic fusion protein of our prototype LVS Δ*capB*-vectored plague vaccine (rLVS Δ*capB*/Yp F1-V, also referred to herein as rLVS Δ*capB*/F1-V or rLVS/F1-V), we modified rLVS Δ*capB*/F1-V, which expresses the fusion protein of F1 with its signal peptide sequence (F1ss-F1) and LcrV from a highly efficient shuttle plasmid,[22] by: 1) replacing the F1ss with the highly secreted *Francisella novicida* PepO (FTN_1186) protein N-terminal 43 residues, including the signal peptide and part of the mature peptide sequence (PepON43), so as to potentially improve secretion[28]; 2) utilizing the mutated F1 (F1mut) and mutated YscF (N35S/I67T) (YscFmut), proven to be immunoprotective as immunogenic antigens[12]; and 3) using LcrV and YscFmut as leaders in fusion protein constructs. In so doing, we constructed 25 rLVS Δ*capB*-vectored *Y. pestis* vaccine candidates expressing a fusion protein comprising various combinations of F1, F1mut, LcrV and YscFmut (Table S1, S2). Based upon their recombinant protein expression, we selected six vaccine candidates for further study as follows: 1) rLVS Δ*capB*/F1-V, our prototype vaccine candidate tested in mice previously,[22] expressing a fusion protein of F1ss-F1-V downstream of the potent promoter of *F. novicida* omp26 (FTN_1451, *omp*)[29]; 2) rLVS Δ*capB*/PepON-F1-V, replacing the F1ss with PepON43 to potentially enhance *Y. pestis* protein secretion in *Francisella*; 3) rLVS Δ*capB*/V-F1-YscFmut, expressing a fusion protein of LcrV, the F1 mature peptide, and YscFmut[12] downstream of the potent promoter of *Francisella tularensis bfr* [FTT_1441][30,31]; 4) rLVS Δ*capB*/V-F1mut-YscFmut, expressing a fusion protein of LcrV, F1mut,[12] and YscFmut downstream of the *bfr* promoter; 5) rLVS Δ*capB*/YscFmut-F1mut-V, expressing a fusion protein of YscFmut, F1mut and LcrV downstream of the *bfr* promoter; and 6) rLVS Δ*capB*/V-F1mut, expressing a fusion protein of LcrV and F1mut downstream of the *bfr* promoter (Figure 1a). In each of the fusion proteins, a flexible linker (GGSG or $[GGGS]_3$) was inserted between each antigen (Table S3). These vaccine candidates were then characterized with respect to protein expression and growth kinetics in broth and immunogenicity and efficacy in mice.

To assess fusion protein expression by the above-described vaccine candidates, we cultured the vaccines in Medium T broth overnight at 37°C with shaking. Whole bacterial cells were collected, and the culture supernates were concentrated. Protein expression was analyzed by Western blotting using a polyclonal antibody against LcrV (BEI Resources, NR-31022). As shown in Figure 1b, the six fusion proteins 1) F1-V (lane 4), 2) PepON-F1-V (lane 5), 3) V-F1-YscFmut (lane 6), 4) V-F1mut-YscFmut (lane 7), 5) YscFmut-F1mut -V (lane 8), and 6) V-F1mut (lane 9) were predominantly expressed as protein bands of 55-, 57-, 63- (and breakdowns), 64- (and breakdowns), 64-, and 54-kDa, respectively, as expected. No protein expression was detected by the LcrV-specific antibody in the whole cell lysates of the LVS Δ*capB* vector (Figure 1b, lane 3). The F1-V monomer protein (positive control) was detected as expected (Figure 1b, lane 10). Of note, the PepON-F1-V (Figure 1c, lane 5; Figure S1, panel c, lane 5) was abundantly present in the culture supernatant, indicating that the fusion protein is secreted via the PepO signal sequence.

To evaluate the growth kinetics of each vaccine candidate in broth, we sub-cultured the vaccine candidates and the LVS Δ*capB* vector in Medium T broth. Cultures were adjusted to an initial absorbance at 540 nm ($A_{540}$) of 0.1 and grown for 6 hours, or an initial $A_{540}$ of 0.005 and grown for 24 hours. As shown in Figure S2, no significant differences were observed in $A_{540}$ (Figures S2a, 2b) or doubling time (Figures S2c, 2d) between the vaccine candidates and the vaccine vector at the end of the 6-hour (Figures S2a, 2c) or 24-hour (Figures S2b, 2d) growth periods. These results indicated that *Y. pestis* fusion protein expression did not impact the growth of the vaccine candidates.

### Efficacy of optimized dose of rLVS Δ*capB*/Yp vaccines in a mouse model of pneumonic plague

Previously, we showed that three homologous ID immunizations with a dose of $1 \times 10^6$ CFU of the rLVS Δ*capB*/Yp F1-V vaccine expressing the fusion protein of F1 and LcrV (F1-V) provided significant but incomplete protection to mice against lethal respiratory challenge with the virulent *Y. pestis* CO92 strain.[22] Subsequently, a dose-response study of an LVS Δ*capB*-vectored melioidosis vaccine indicated that the dose of $1 \times 10^6$ CFU was suboptimal, and less than half as effective as a higher dose of 2, 4, or $8 \times 10^6$ CFU, which were all comparably effective.[23] We therefore evaluated the efficacy of three homologous immunizations of rLVS Δ*capB*/Yp vaccines at a dose of $2 \times 10^6$ CFU in the mouse model. We immunized six- to eight-week-old BALB/c mice, 8 per group, ID three times, four weeks apart, with PBS (Sham), rLVS Δ*capB*/F1-V, rLVS Δ*capB*/PepON-F1-V, rLVS Δ*capB*/V-F1mut, rLVS Δ*capB*/V-F1-YscFmut, rLVS Δ*capB*/V-F1mut-YscFmut, rLVS Δ*capB*/YscFmut-F1mut-V, or, or subcutaneously (SQ) three times with EV76, a toxic and unlicensed vaccine that served as a positive control. Animals immunized with the rLVS Δ*capB*/Yp vaccines did not show any adverse reactions to the vaccine after either the prime or two booster immunizations. Animals immunized with the EV76 vaccine showed no adverse reactions after the prime immunization, but when boosted 4 weeks later, 5 of 8 mice developed dime-sized lesions of hair loss and wrinkled skin within 5 days of immunization. These lesions did not appear necrotic or infected and resolved within 2 weeks. The animals showed no adverse reactions to the EV76 vaccine after the third immunization. One week prior to challenge (Week 11), mice were bled and sera isolated and assayed for the level of LcrV and F1 specific antibodies. One week later (Week 12 - four weeks after the last immunization), mice were challenged intranasally (IN) with 2100 CFU (~10 $LD_{50}$) of the highly virulent *Y. pestis* CO92 strain and monitored for signs of illness and weight loss for three weeks

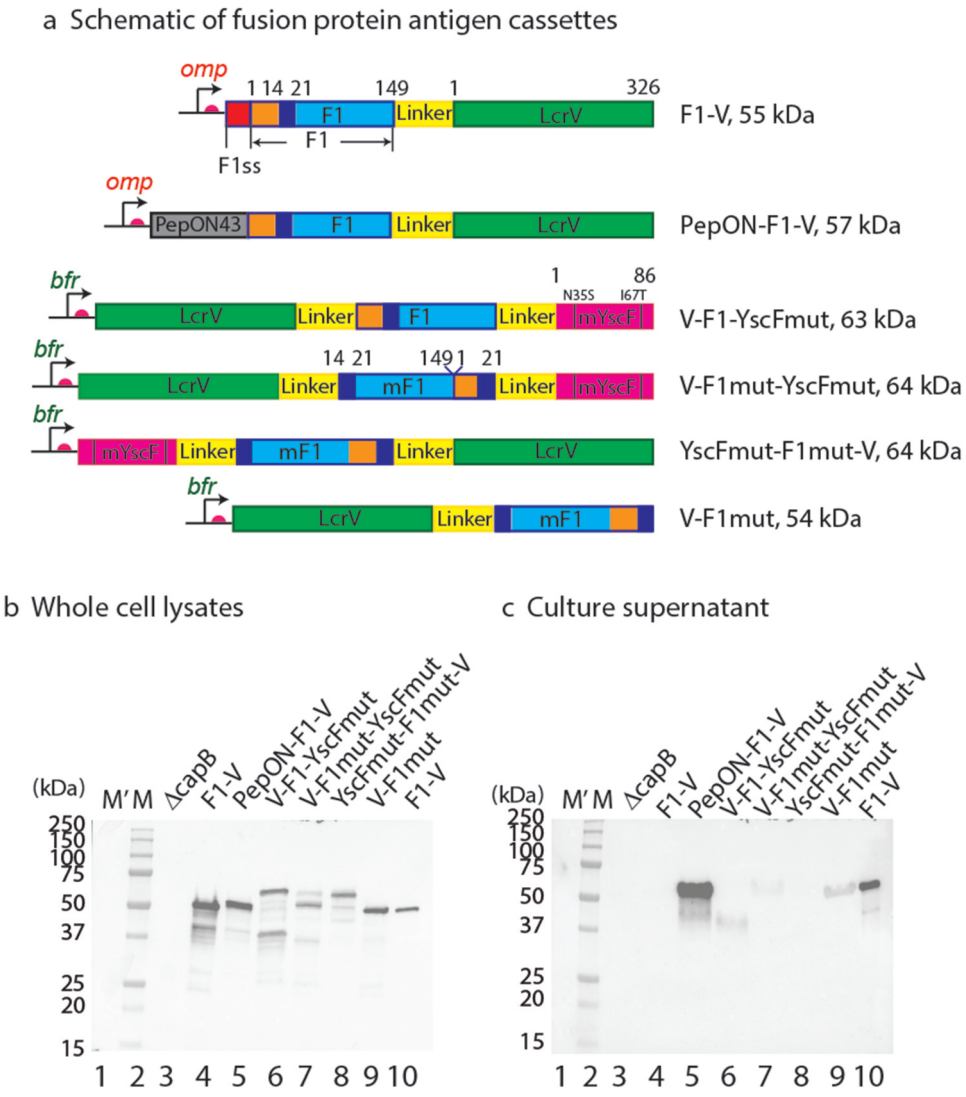


**Figure 1.** Fusion proteins of *Y. pestis* F1, LcrV, and YscF expressed by the LVS Δ*capB*-vectored vaccine strains. a. Schematic of fusion protein antigen cassettes. the coding sequence for F1 includes a signal peptide sequence (F1ss, 21 aa, shown in red) and the mature F1 peptide (F1, 149 aa), comprising a donor β-strand (aa 1–14, orange), a T-cell epitope (aa 15–21, dark blue), and the remaining F1 sequence (aa 22–149, light blue).[12] Linker sequences [GGSG or $(GGGGS)_3$] between F1, LcrV, and YscF are depicted in yellow. The full-length LcrV coding sequence (aa 1–326) is shown in green. The N-terminal 43 aa (PepON43) of the *Francisella tularensis* subsp. *novicida* major secreted protein PepO (GenBank accession DQ230367), secreted via the type IV secretion system, are shown in gray.[28] the coding sequence for the mutant YscF (YscFmut, N35S I67T)[12] is shown in magenta. Numbers above the boxes representing F1, LcrV and YscF correspond to aa residues of F1, LcrV, and YscFmut, respectively. F1mut (mF1) and YscFmut (mYscF) denote the mutant form of F1 and YscF.[12] potent *francisella* promoters *omp* and *bfr* are indicated by arrows and the Shine-Dalgarno sequence indicated by a magenta half circle. b & c. Expression of recombinant *Y. pestis* proteins by LVS Δ*capB*-vectored vaccine candidates. lysates of bacterial whole cells grown in medium T (equivalent to 0.1 mL of broth culture) (b) and culture filtrate proteins (equivalent to 0.075 mL of broth culture) (c) were analyzed by SDS-PAGE and Western blot analysis using goat polyclonal anti-LcrV antibody (BEI NR-31022) followed by rabbit anti-goat IgG-HRP. F1-V monomeric proteins served as a positive control (lane 10 of each blot). Unstained standards (M′) are visible only on the stain-free gel (Supplemental Figure 1, panels b, c, top panels), while pre-stained standards (M) are invisible on the stain-free gel but visible in merged colorimetric and chemiluminescence images of the Western blots.

(Figure 2a). Our results showed that BALB/c mice immunized ID three times with rLVS Δ*capB*/F1-V had 100% survival after respiratory challenge with *Y. pestis* CO92, equivalent to mice immunized with the EV76 positive control strain, and mice immunized with the rLVS Δ*capB*/PepON-F1-V had 75% survival; survival of mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V was significantly greater than that of sham-immunized mice ($p = .0001$ and $p = .0006$, respectively), mice immunized with the LVS Δ*capB* vector ($p = .0001$), and mice immunized with the other rLVS Δ*capB*-vectored Yp vaccine candidates (Figure 2b). Mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V had transient modest weight loss but rapidly recovered to the pre-challenge weight level at Day 5 post challenge; in contrast, mice immunized with PBS, LVS Δ*capB* and the other rLVS Δ*capB*/Yp vaccine candidates lost 20% of their total body weight by Day 3 post challenge (Figure 2c). Post-challenge weight losses of mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V were significantly less than mice immunized with PBS (sham) ($p < .0001$) or LVS Δ*capB* vector ($p < .0001$), and notably significantly less than mice immunized with EV76 ($p < .0001$ and $p = .0002$, respectively). The post-challenge weight loss of mice immunized with the LVS Δ*capB* vector was equivalent to mice immunized with PBS (Sham) and

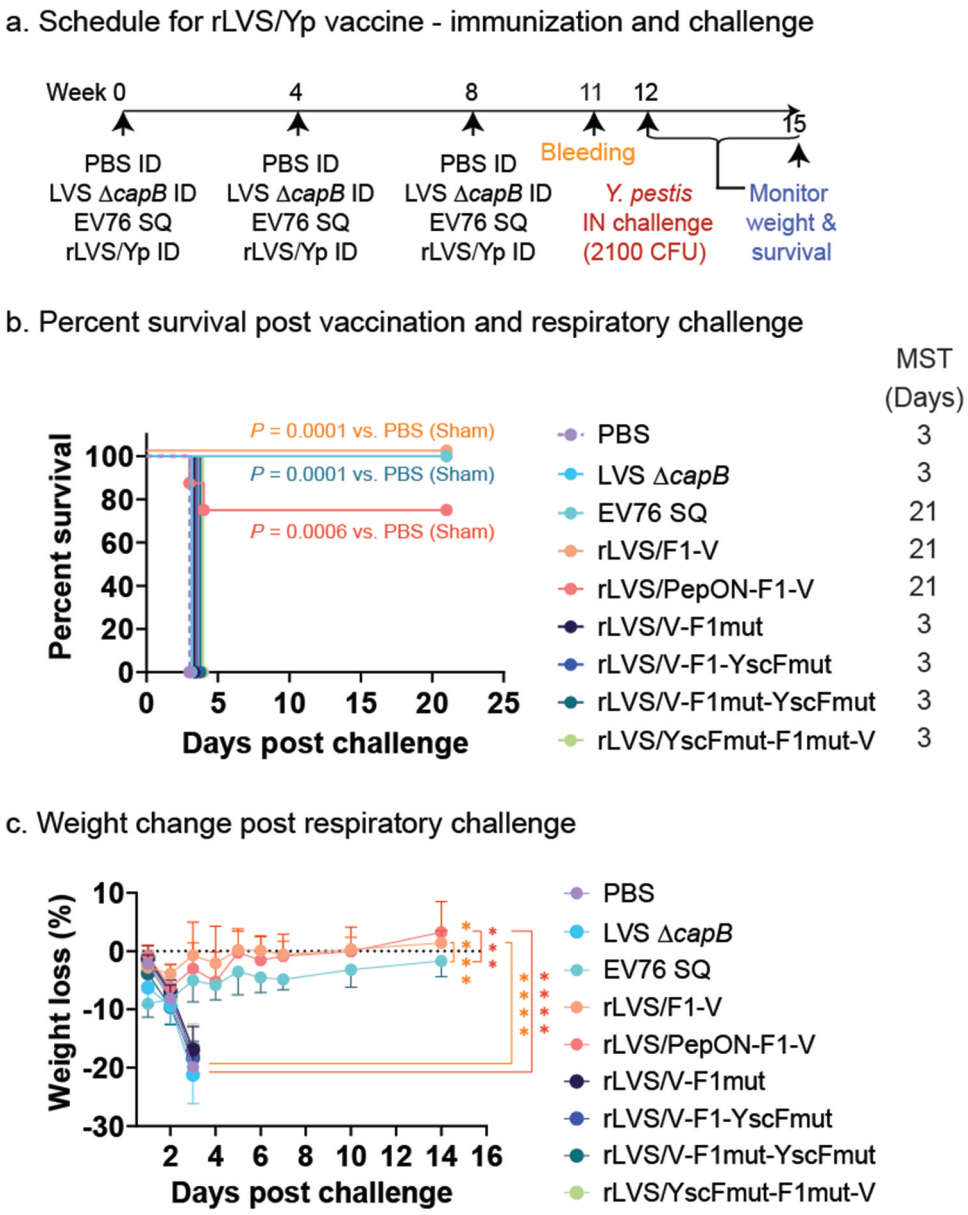


**Figure 2.** Three homologous prime-boost immunizations with the rLVS Δ*capB*/F1-V vaccine confers complete protective immunity against a lethal respiratory challenge with virulent *Y. pestis*. a. Experimental schedule. BALB/c mice (n = 8 per group) were immunized as follows: intradermally (ID) once with PBS (sham) at week 0; subcutaneously (SQ) three times with $10^6$ CFU of EV76 (an attenuated *Y. pestis* strain pathogenic in primates); or ID three times with $2 \times 10^6$ CFU of either LVS Δ*capB* (vector control) or rLVS Δ*capB*/Yp (abbreviated as rLVS/Yp, expressing various fusion proteins of *Y. pestis* antigens F1, LcrV, and YscF, as detailed in the legends for panels b and c at weeks 0, 4, and 8. One week before challenge (week 11), all mice were bled by facial vein puncture, and sera were tested for F1- and LcrV-specific antibodies (see Figure 3). At week 12, all mice were challenged intranasally (IN) with 2100 CFU of *Y. pestis* CO92 (~10× $LD_{50}$) and monitored for weight change and survival over 3 weeks. b. Survival post-challenge. survival rate (%) and median survival time are shown up to 3 weeks when the experiment was terminated. Survival curves were compared between each vaccine group and the PBS (sham) group using simple survival analysis (Kaplan-Meier) with log-rank (mantel-cox) and Gehan-Breslow-Wilcoxon tests (Prism 10.4.0). Significant *p* values comparing test groups to the sham group are indicated with colored values next to the respective groups. MST, median survival time shown to the right of the panel. c. Weight change post-challenge. Significant differences in weight change over the first 2 weeks post-challenge between test groups and the Sham group are marked with colored asterisks to the right of brackets connecting compared groups, analyzed by two-way ANOVA with Tukey's correction. ***, $p < .001$, ****$p \leq .0001$.

significantly greater than that of mice immunized with EV76 ($p < .0001$).

### *Vaccine immunogenicity and identification of correlates of protection*

To evaluate the humoral immune response induced by the rLVS Δ*capB*/Yp vaccines, we assayed the pre-challenge immune serum for F1 and LcrV-specific antibodies by enzyme-linked immunosorbent assay (ELISA) using standard procedures.[22] As shown in Figure 3, mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V produced significantly elevated total serum IgG antibody specific to F1 (Figure 3a), not significantly different from mice immunized with the EV76 strain. Levels of subtype IgG2a antibody were also highly elevated and equivalent for these

three vaccines; however, the rLVS Δ*capB*/PepON-F1-V vaccine induced significantly lower levels of subtype IgG1 antibody than either EV76 or rLVS Δ*capB*/F1-V (Figure 3c). Mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V also produced significantly elevated total serum IgG antibody specific to LcrV (Figure 3b), and these levels were significantly greater than that produced by mice immunized with EV76 ($p < .0001$). Mice immunized with rLVS Δ*capB*/F1-V and rLVS Δ*capB*/PepON-F1-V also produced similarly significantly elevated levels of subtype IgG1 and IgG2a antibody specific to LcrV (Figure 3d). Analysis of the ratios between IgG subclasses specific to F1 (Figure 3c) and LcrV (Figure 3d) showed a balance between IgG1 (Th2) and IgG2a (Th1), with a ratio of IgG1/IgG2a of ~ 1 (Figure 3e,f).

Importantly, the pre-challenge serum IgG levels to both F1 ($p < .0001$) and LcrV ($p < .01$) were highly correlated with median survival time for the six vaccines (Figure 4). Among the antibody subtypes, pre-challenge IgG1 and especially pre-challenge IgG2a antibody to F1 were also highly correlated with median survival time ($p = .03$ and $p = .0003$, respectively).

## Discussion

We have shown that three optimized doses of rLVS Δ*capB*/Yp F1-V provides 100% protection against pneumonic plague in

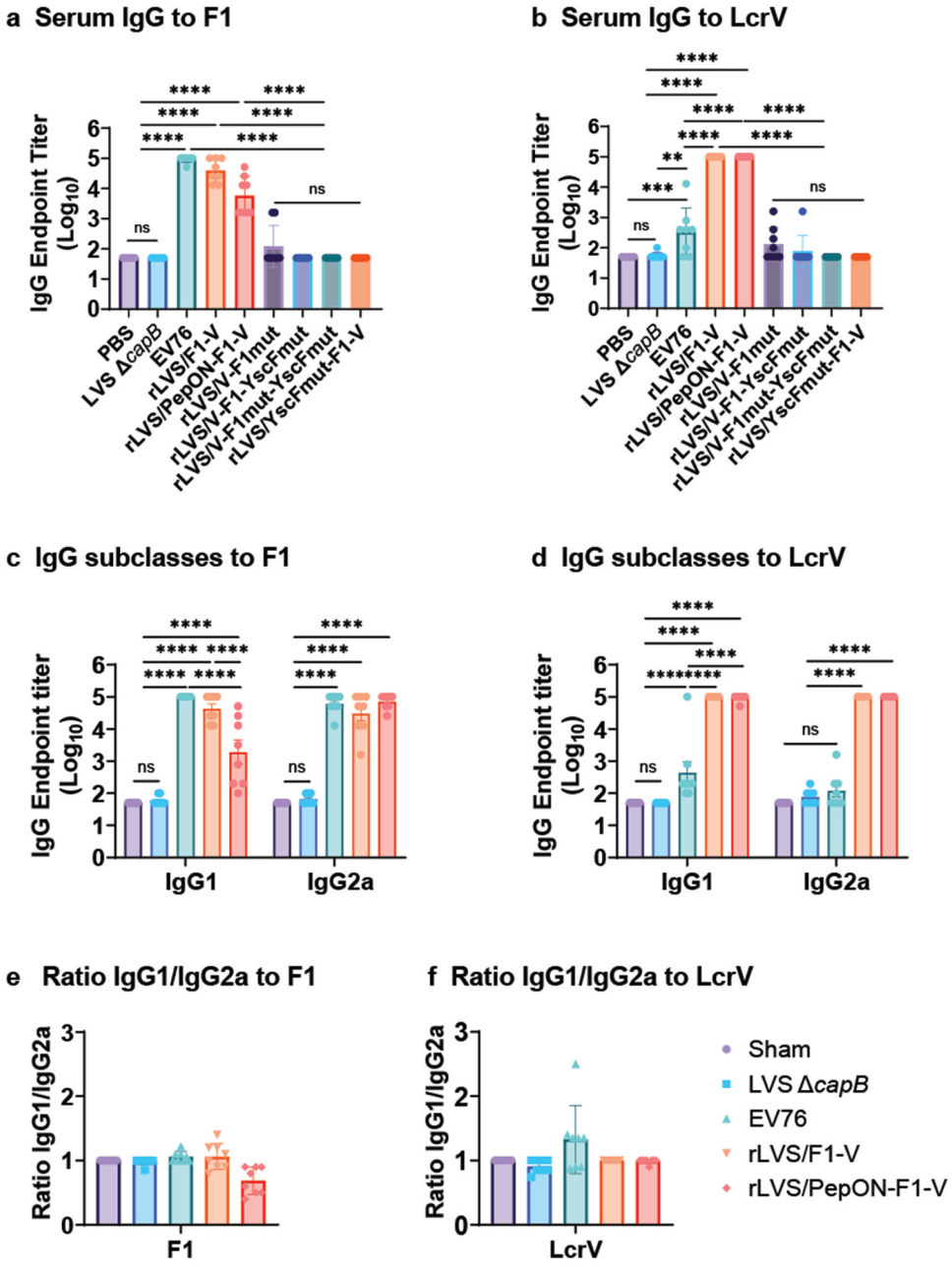


**Figure 3.** Serum IgG and subclass antibody levels pre-challenge specific to *Yersinia pestis* F1 and LcrV proteins. Mice were immunized and bled as described in Figure 2a. Sera were assayed for endpoint titers ($log_{10}$) of total IgG and IgG subclasses (IgG1 and IgG2a), as well as the IgG1/IgG2a ratio, specific to *Y. pestis* F1 (panels a, c, e) and LcrV (panels b, d, f) proteins. Data represent the mean ± SD of serum antibody endpoint titers for $n = 8$ mice per group. Significant differences between groups are indicated by asterisks above horizontal lines spanning the compared groups, determined by two-way ANOVA with Tukey's correction. ns, not significant; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$.

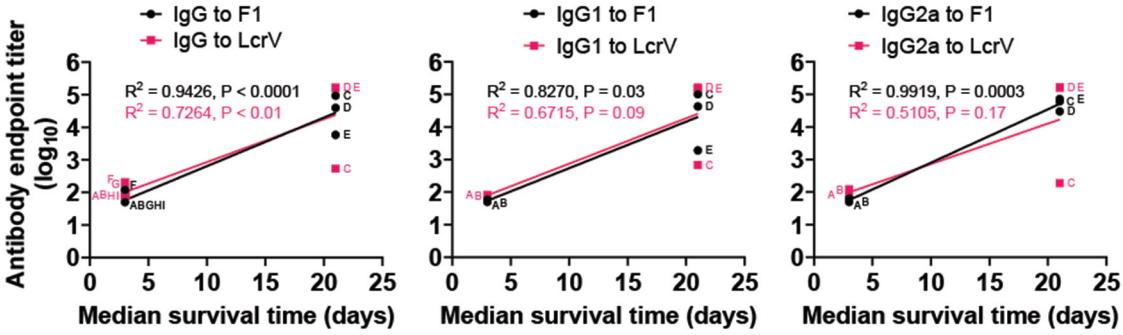


**Figure 4.** Correlation between serum antibody levels and median survival time. Linear regression was performed to determine the slope, intercept, and correlation coefficient ($R^2$) between pre-challenge group mean serum IgG (left panel), IgG1 (middle panel) and IgG2a (right panel) antibody levels specific to F1 and LcrV proteins and post-challenge group median survival time at 21 days post-challenge. Each point represents a single vaccine group. Two-tailed P-values for the correlation were calculated using Prism 10.4.0. The letters (black, antibody to F1 and magenta, antibody to LcrV) represent the experimental groups as follows: A, Sham; B, LVS Δ*capB*; C, EV76; D, rLVS/F1-V; E, rLVS/PepON-F1-V; F, rLVS/V-F1mut; G, rLVS/V-F1-YscFmut; H, rLVS/V-F1mut-YscFmut; and I, rLVS/YscFmut-F1mut-V.

a stringent mouse model, outperforming other candidates and matching the survival efficacy of the toxic and unlicensed EV76 strain vaccine. Furthermore, the rLVS Δ*capB*/Yp F1-V vaccine protected significantly better than the EV76 vaccine against weight loss post-challenge. Protection correlated with high F1- and LcrV-specific antibody levels. The balanced Th1/Th2 response indicates robust and versatile immune activation, potentially enhancing protection against diverse *Y. pestis* strains.

rLVS Δ*capB*-vectored vaccine candidates with inclusion of YscFmut or F1mut in fusion proteins yielded disappointing results, as these vaccines did not enhance protection despite previous reports that these antigens provide modest improvements of subunit vaccines. Their lack of efficacy suggests that these antigens may not be suitable in this vector system or that they require a different configuration (e.g., alternative promoters, linkers, or co-expression strategies).

rLVS Δ*capB*/PepON43-F1-V was highly efficacious, providing 75% survival of challenged mice; however, the PepON43 modification did not improve upon the efficacy of rLVS Δ*capB*/Yp F1-V despite enhancing secretion (Figure 1c). This indicates that secretion-enhancing strategies may need refinement, including alternative secretion signals and protein stabilization strategies. Future structural study of the fusion proteins could guide improvement of our vaccine design. Further studies also could explore if additional *Y. pestis* antigens can enhance protection, e.g. against F1-minus strains.

While mice immunized with the rLVS Δ*capB*/Yp vaccines showed no adverse reactions, mice immunized with the EV76 vaccine developed transient severe skin lesions after being boosted 4 weeks after the prime immunization. This pattern of reactogenicity of the EV76 vaccine contrasted with one of our previous studies in which mice immunized with EV76 became noticeably ill for several days after a single prime immunization. The reason for the discrepancy in the pattern of reactogenicity between the two studies is not known.

Protection by the vaccines, as assessed by median survival time post challenge, was correlated with pre-challenge serum IgG antibody levels to both F1 and LcrV and with pre-challenge serum antibody subtypes IgG1 and IgG2a to F1. If antibodies to these antigens also correlate with protection in a second animal model, especially non-human primates, then such antibodies may provide a convenient correlate of protection for the rLVS Δ*capB*/Yp F1-V vaccine in future clinical studies.

While comparing antibody levels induced by vaccines between two separate experiments can be problematic, it is noteworthy that the levels of serum IgG and subtypes IgG1 and IgG2a antibody to F1 antigen induced by the rLVS Δ*capB*/Yp F1-V vaccine in this experiment, where the dose was twice as high as in the previous experiment ($2 \times 10^6$ vs. $1 \times 10^6$ CFU), were substantially higher than in the previous experiment (0.7, 1.4, and 0.8 logs higher for IgG, IgG1, and IgG2a, respectively), whereas the levels of IgG and subtypes IgG1 and IgG2a antibody induced by the EV76 vaccine, given at the same dose as in the previous experiment but three times instead of once, were relatively similar (0.2, 0, and 0.1 logs difference for IgG, IgG1, and IgG2a, respectively).[22] Levels of total serum antibody to LcrV induced by the two vaccines were similar between these experiments, although both vaccines and especially the rLVS Δ*capB*/Yp F1-V vaccine induced substantially more subtype IgG1 antibody (0.9 and 0.8 logs higher for rLVS Δ*capB*/Yp F1-V and EV76, respectively). This suggests the possibility that increased antibody levels to the F1 antigen and perhaps increased IgG1 antibody level to the LcrV antigen may have contributed to the enhanced efficacy of the higher dose of the rLVS Δ*capB*/Yp F1-V vaccine in this study (100% vs. 50% survival), although other immunoprotective mechanisms, including cellular immune mechanisms, that were not assayed in this study may also have played a role.

A large number of vaccines have been developed against plague.[3] Early vaccines included killed whole cell vaccines and the live attenuated EV76 vaccine, both of which had major deficits. The killed vaccines were highly reactogenic and efficacious against bubonic but not pneumonic plague in animal studies. The EV76 vaccine, employed as a positive control in this study, was active against both bubonic and pneumonic plague but also was highly reactogenic and virulent in the setting of iron overload. More recently developed vaccines include alternative live attenuated vaccines with novel attenuating deletions as well as subunit vaccines comprising antigens and adjuvants, DNA and mRNA-based vaccines, and bacterial and viral-vectored vaccines.[3] Many of these latter vaccines, as with our vaccine, have focused on the highly immunoprotective F1 and LcrV antigens and shown excellent efficacy in

animal models. Hence, the relative attractiveness of the more recently developed vaccines centers to a great extent on attributes beyond efficacy, especially safety, cost of production, storage and administration, and convenience.

With respect to safety, the LVS Δ*capB* vector has an especially promising profile. First, it is an attenuated derivative of a tularemia vaccine, LVS, that has been administered to >60 million people; the LVS vaccine already had two major attenuating deletions and several minor ones before being subjected to the additional *capB* major attenuating deletion. Second, in studies in highly sensitive mice, the LVS Δ*capB* vector administered IN is > 10,000 fold less virulent than its parent LVS.[19] Third, mice administered the LVS Δ*capB* vector ID at doses ranging from $10^6$ to $10^8$ CFU showed no weight loss (in fact they gained weight) or other signs of illness except local erythema at the injection site at doses of $10^7$ and $10^8$ CFU, doses much higher than the dose of $2 \times 10^6$ CFU ID used in this study.[19] Fourth, in a previous study of the rLVS Δ*capB*/Yp F1-V vaccine in which mice were administered the vaccine ID [or intramuscularly (IM)] at $1 \times 10^6$ CFU and weighed post-immunization, mice showed no weight loss or signs of illness. Finally, we have conducted extensive research on the safety of multiple LVS Δ*capB*-vectored vaccines in animal models including vaccines expressing homologous immunoprotective proteins of *Francisella tularensis* and heterologous immunoprotective proteins of *Bacillus anthracis, Burkholderia pseudomallei*, and SARS-CoV-2 in mouse, Fisher rat, and hamster models,[20–26] and we have not observed any significant toxicity including weight loss after vaccination with any of these vaccines at the doses used. Consistent with this profile, no adverse signs were noted in this study after ID administration of the LVS Δ*capB* – vectored plague vaccines.

With respect to cost, the self-replicating vaccine can be manufactured very inexpensively by bacterial fermentation such that hundreds of millions of doses can be grown overnight in simple broth medium free of animal products and prepared without the need for extensive purification, in contrast to viral-vectored vaccines and protein-adjuvant vaccines. Lyophilized, the vaccine can be stored and transported conveniently at refrigerator temperature, and potentially even at room temperature after vitrification. Moreover, if the plague vaccine, one of several utilizing the LVS Δ*capB* vector platform that has been demonstrated efficacious against Tier 1 Select Agents and SARS-CoV-2, ultimately is part of a suite of vaccines sharing the same vector platform, then manufacture, regulatory approval, and clinical evaluation of the vaccines would be simplified, further potentially dramatically lowering costs. Finally, no adjuvant is required, further reducing cost.

With respect to convenience, as a vaccine sharing the same vector platform as other Tier 1 select agent vaccines, our plague vaccine could be administered concurrently with these other vaccines, allowing for a vaccination regimen more acceptable to people than that likely required of multiple vaccines of disparate construction. Moreover, a single multi-pathogen vaccine against Tier 1 Select Agents that utilizes the LVS Δ*capB* vector is a future possibility. Furthermore, while the plague vaccine described here was delivered intradermally, other vaccines utilizing this vector have demonstrated excellent efficacy with intranasal and oral delivery, allowing needle-free vaccine administration and delivery not requiring highly trained personnel.[20–26]

The safety profile of the LVS Δ*capB* vector noted above and the safety and 100% efficacy demonstrated by the rLVS Δ*capB*/Yp F1-V vaccine in protecting mice against lethal respiratory challenge with the highly virulent wild-type *Y. pestis* CO92 strain in this study make it a promising vaccine candidate for further development. Future preclinical studies might assess the vaccine's efficacy in protecting against challenge with *Y. pestis* F1-minus strains and LcrV variants as well as its efficacy in a second animal model so as to satisfy the FDA's animal rule for vaccines for which human efficacy studies are not feasible or ethical.

In conclusion, our study demonstrates that rLVS Δ*capB*/Yp F1-V is a highly effective and safe vaccine against pneumonic plague in mice, providing 100% protection, and that protection is highly correlated with pre-challenge serum antibody to the F1 and LcrV antigens. rLVS Δ*capB*/Yp F1-V is thus a prime candidate for further preclinical and clinical development to address the unmet need for a safe and effective plague vaccine.

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## Author contributions

CRediT: **Qingmei Jia:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing; **Richard A. Bowen:** Data curation, Formal analysis, Investigation, Project administration, Supervision, Writing – review & editing; **Marcus A. Horwitz:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Notes on contributor

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## Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

## Ethics statement

All animal studies were conducted according to protocols approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC) (#5740).

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