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Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines

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Abstract

Yersinia pestis causes plague, a disease with high mortality in humans that can be transmitted by fleabite or aerosol. A US Food and Drug Administration (FDA)-licensed plague vaccine is currently not available. Vaccine developers have focused on two subunits of *Y. pestis*: LcrV, a protein at the tip of type III secretion needles, and F1, the fraction 1 pilus antigen. F1-V, a hybrid generated via translational fusion of both antigens, is being developed for licensure as a plague vaccine. The rV10 vaccine is a non-toxigenic variant of LcrV lacking residues 271–300. Here we developed Current Good Manufacturing Practice (cGMP) protocols for rV10. Comparison of clinical grade rV10 with F1-V did not reveal significant differences in plague protection in mice, guinea pigs or cynomolgus macaques. We also developed cGMP protocols for rV10-2, a variant of rV10 with an altered affinity tag. Immunization with rV10-2 adsorbed to aluminum hydroxide elicited antibodies against LcrV and conferred pneumonic plague protection in mice, rats, guinea pigs, cynomolgus macaques and African Green monkeys. The data support further development of rV10-2 for FDA Investigational New Drug (IND) authorization review and clinical testing.

Introduction

Yersinia pestis is the causative agent of bubonic, septicemic and pneumonic plague [1, 2]. Due to its rapid spread and high mortality, plague has probably killed more people worldwide than any other infectious disease [3, 4]. *Y. pestis* has been used as a biological weapon with devastating effects [5]. To protect people from highly virulent *Y. pestis*, prophylactic measures against plague have been pursued for over a century [6, 7]. These efforts were initially based on the observation that clinical recovery occurs for some, but not all, bubonic plague cases and that recovery is associated with immunity to the disease [8, 9]. The first U.S. Food and Drug Administration (FDA) licensed vaccine was Plague Vaccine USP (Cutter), a killed whole-cell (KWC) vaccine derived by formalin treatment of the fully virulent strain *Y. pestis* 195/P [10]. Adverse reactions to immunization as well as the failure

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of Plague Vaccine USP to confer protection against pneumonic plague diminished interest in KWC vaccines [11–13].

Fully virulent *Y. pestis* strains harbor the high pathogenicity island (HPI) and pigmentation locus (*pgm*) on a 102 kb chromosomal DNA segment flanked by IS *100* elements [14, 15]. Spontaneous mutation or the loss of the entire HPI/*pgm* segment leads to non-pigmented plague strains with severe defects in virulence for animals and humans [14, 16]. Attenuated *pgm* isolates, for example *Y. pestis* EV76, have been used extensively as live whole-cell vaccines in humans [17]. However, *pgm* vaccines are associated with severe side effects, have been reported to revert to wild-type virulence and cannot elicit robust protection against pneumonic plague [18]. In the United States, *pgm* vaccines are not approved for use in humans.

The F1 (Caf1) protein is assembled into pili that accumulate on the surface of *Y. pestis* [19, 20]. Expression of F1 pili, which are encoded by the *caf* gene cluster on the pFra virulence plasmid, is associated with *Y. pestis* protection from macrophage phagocytosis as well as decreased uptake of the pathogen into epithelial cells [21, 22]. Nevertheless, F1 has only a contributory role for the pathogenesis of pneumonic plague in mice, rats, guinea pigs, non-human primates or humans [23–26]. The F1 protein therefore cannot be used as the sole antigen in plague subunit vaccines [25]. Burrows and Bacon discovered the protective antigen properties of LcrV [27, 28], which is absolutely essential for *Y. pestis* virulence [29]. The 35 kDa LcrV is secreted into extracellular media and deposited at the tip of type III needles [30]. LcrV enables *Y. pestis* type III machines to transport effector proteins (Yops) into host immune cells, thereby disabling phagocytic clearance of the pathogen in host tissues [31–34]. Several reports described the immuno-modulatory properties of LcrV, blocking inflammatory responses by stimulating the release of interleukin 10 (IL-10) [35] and preventing the release of pro-inflammatory cytokines [36]. LcrV appears to mediate its immuno-modulatory effects by interacting with TLR2/TLR6/CD14 signaling complexes on the surface of host cells [37].

LcrV and F1 are currently the only candidates for plague subunit vaccines. The U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) developed the F1-V fusion protein, which tethers the two antigens as a translational hybrid into a single molecule [38]. The fusion protein was designed to enable a one-protein-manufacturing process, which is considered an advantage over purifying two different polypeptides [38, 39]. F1-V maintains the antigenic properties of isolated F1 and LcrV subunits [40] and DynPort Vaccine Company LLC (DVC) is currently supporting the FDA licensure process for clinical grade F1-V vaccine [41]. F1-V immunization elicits protective immunity in mice, rats, guinea pigs and cynomolgus macaques [41, 42]. To improve expression, solubility, and recovery of F1-V for vaccine manufacturing purposes, recent efforts focused on the expression and purification of the hybrid from tobacco plants [43] as well as the construction of a reverse vaccine, V-F1, for purification from the periplasm of *Escherichia coli* [44]. Results from the manufacturing and vaccine efficacy trials of next generation F1-V variants are not yet available.

Because F1 is dispensable for the pathogenesis of pneumonic plague, our design of subunit vaccines focused on LcrV with the goal of producing variants that lack the immuno-modulatory attributes of this polypeptide [45]. rV10 harbors a deletion of amino acids 271–300 of LcrV (1–327)[45], fails to suppress the release of proinflammatory cytokines via the TLR2/TLR6/CD14 pathway [37], retains the ability to elicit plague protective immune responses in animals [46] and raises antibodies that block *Y. pestis* type III injection of effector proteins into immune cells [25]. Affinity tagged, recombinant rV10 protein was purified from batch cultures and adsorbed to aluminum hydroxide (Alhydrogel)[46]. When

used as a vaccine in mice, rats, guinea pigs and non-human primates, rV10 immunization offered significant protection against pneumonic plague challenge [24, 46–48]. Analysis of IgG responses to immunization revealed that, in contrast to wild-type LcrV, rV10 vaccine elicited almost exclusively antibodies that recognize conformational epitopes on the plague antigen [47, 48].

We report here the current Good Manufacturing Practice (cGMP) process for rV10. Clinical grade rV10 was compared with F1-V vaccine and no significant difference in protection against pneumonic plague challenge in mice, guinea pigs and cynomolgus macaques was observed. We also developed cGMP protocols for rV10-2, a variant of rV10 with an altered affinity tag, and analyzed preclinical vaccine efficacy for rV10-2 in mice, rats, guinea pigs, cynomolgus macaques and African Green monkeys.

Material and Methods

Cloning and expression of the rV10 vaccine

The design and features of the V10 expression system have been described previously [45]. The pET33b+ expression vector (Novagen) was prepared by restriction digest with *NdeI* and *BamHI*. The rV10 insert was excised with the same restriction enzymes and ligated into pET33b+ to generate pGMP-rV10, which was transformed into *E. coli* DH5 α . Plasmid pGMP-rV10 was isolated from a single positive clone, the DNA sequence determined, and transformed into *E. coli* BLR(DE3) using kanamycin (35 μ g/ml) selection. rV10 polypeptide harbors an N-terminal six-histidyl affinity tag and heart muscle kinase recognition sequence in addition to *Y. pestis* KIM D27 LcrV residues 2–271 and 300–327 with a glycyl-threonyl linker between the two LcrV segments.

Fermentation and purification of bulk rV10 protein

Initial culture and expression conditions were scaled-up to a 400 L fermentor (New Brunswick Scientific) under cGMP. Cells were grown in Select APS Super broth supplemented with 0.5% glycerol and 35 μ g/ml kanamycin to an OD₆₀₀ 5.2. Cells were then induced for rV10 expression with 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) for 2.5 hours and sedimented by centrifugation. The cell paste was aliquoted and stored at –80°C. Laboratory purification process was scaled-up under cGMP, 500 g of cell paste were thawed and suspended in loading buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, pH 7.5) at a buffer:paste ratio of 5:1 (w/v) using an IKA Turrax homogenizer. Following cell disruption (M-110Y Microfluidizer, Microfluidics Corp), lysate was centrifuged and the rV10 protein was purified from the soluble fraction using a one step nickel affinity chromatography procedure. Supernatant was loaded onto Ni-NTA Superflow column (Qiagen) initially equilibrated with loading buffer. The column was washed with 20 column volumes of loading buffer supplemented with 1% Triton-X-100 followed by 10 column volumes of loading buffer supplemented with 20 mM imidazole. The rV10 protein was then eluted with loading buffer supplemented with 250 mM imidazole. Purified protein was dialyzed in phosphate buffer saline (PBS) (1.04 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 7H₂O, 154 mM NaCl, pH 7.4) using a 5,000 NMWC, size 5, hollow fiber filter, Model #UFP-5-C-5a (GE Healthcare) at a final concentration of 1.5 mg/ml, determined by the bicinchoninic acid method (BCA, Pierce), and sterile filtered.

Formulation, vialling and lot release tests for rV10

rV10 filtered bulk protein (BPR 879-00, Lot 1487) was diluted in PBS to a final concentration of 1 $\pm$ 0.1 mg/ml. Aliquots (700 $\pm$ 10% μ l) were aseptically transferred into sterile 3 ml vials (Wheaton, Cat # 223684), which were sealed with 13 mm Teflon coated stoppers (West, Cat # 10124671) and 13 mm plastic flip off crimps (Wheaton, Cat #

54131207). The rV10 vaccine vials (BPR 889-00, Lot 1503) were visually inspected for color, turbidity and homogeneity. Quantitative determination of endotoxin content in the rV10 filtered bulk protein (2.4 EU/ml) was performed using the LAL assay (QCL-1000; Cambrex, New Jersey).

Cloning and expression of rV10-2 vaccine

Plasmid pGMP-rV10-2 was generated by amplifying rV10 coding sequence from the original pV10 laboratory construct [45], using a forward primer 5'-TTCCATGGGCAGCAGCCATCATCATCATCACATTAGAGCCTACGAACAAAA CCC-3' and a reverse primer 5'-TAGGATCCTCATTACCAGACGTGTCATCTAGC-3'. The PCR product was cloned into pET33b+ (Novagen) following restriction with *NcoI* and *BamHI*. pGMP-rV10-2 was transformed into *E. coli* DH5 α . rV10-2 encompasses an N-terminal six histidyl tag as well as the glycyl-threonyl substitution for amino acids 271–300 of LcrV but lacks the heart muscle kinase recognition sequence near the N-terminus of rV10. Plasmid was isolated from a single positive clone, DNA sequence determined and transformed into *E. coli* BLR(DE3) using kanamycin (35 μ g/ml) selection. Following small scale expression and purification studies, a clone expressing rV10-2 was selected for cGMP development process. Glycerol stocks, namely Master Cell Bank (BPR 929-00, Lot 1573) and Production Cell Bank (BPR 930-00, Lot 1574), were generated at Walter Reed Army Institute of Research Pilot Bioproduction Facility and stored at -80°C . Plasmid was isolated from these clones and subjected to DNA sequencing to validate the rV10-2 coding sequence of prV10-2.

Fermentation and purification of bulk rV10-2 protein

Initial culture and expression conditions were scaled-up to a 400 L fermentor (New Brunswick Scientific) under cGMP. Cells were grown in Select APS Super broth supplemented with 0.5% glycerol and 35 μ g/ml kanamycin to OD₆₀₀ 13. Cells were induced for rV10-2 expression with 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) for 2.5 hours and sedimented by centrifugation. The cell paste was aliquoted and stored at -80°C . Under cGMP, 500 g of cell paste were thawed and suspended in loading buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, pH 7.5) at a buffer:paste ratio of 5:1 (w/v) using a IKA Turrax homogenizer. Following cell disruption (M-110Y Microfluidizer, Microfluidics Corp), lysate was centrifuged and the rV10-2 protein was purified from the soluble fraction using a one-step nickel affinity chromatography procedure. Supernatant was loaded onto Ni-NTA Superflow column (Qiagen) initially equilibrated with loading buffer. After being loaded with cell lysate, the Ni-NTA Superflow column (Qiagen) was washed extensively with 60 column volumes of loading buffer supplemented with 1% Triton-X-100 followed by 40 column volumes of loading buffer supplemented with 20 mM imidazole. rV10-2 protein was eluted with loading buffer supplemented with 250 mM imidazole. Purified protein was dialyzed in phosphate buffer saline (PBS) (1.04 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 7H₂O, 154 mM NaCl, pH 7.4) using a 5,000 NMWC, size 5, hollow fiber filter, Model #UFP-5-C-5a (GE Healthcare) at a final concentration of 1.5 mg/ml, determined by the bicinchoninic acid method (BCA, Pierce), and sterile filtered.

Formulation, vialling and lot release tests for rV10-2

rV10-2 filtered bulk protein (BPR 943-00, Lot 1595) was diluted in PBS to a final concentration of 1 ± 0.1 mg/ml. Aliquots ($700\pm10\%$ μ l) were aseptically transferred into sterile 3 ml vials (Wheaton, Cat # 223684), which were sealed with 13 mm Teflon coated stoppers (West, Cat # 10124671) and 13 mm plastic flip off crimps (Wheaton, Cat # 54131207). The rV10-2 vaccine vials were designated Vial A (BPR 948-00, Lot 1600). Adjuvant vials (Vial B, BPR 890-00, Lot 1505) for the rV10-2 Plague Vaccine were filled with $1.5\pm5\%$ ml of Alhydrogel 2% (Accurate Chemical Cat # A1090BS) diluted 1:5 (v/v) in

(PBS) (1.04 mM KH₂PO₄, 5.6 mM Na₂HPO₄, 7H₂O, 154 mM NaCl, pH 7.4). Vials A and B were visually inspected for color, turbidity and homogeneity.

Quantitative determination of endotoxin content in the rV10-2 filtered bulk protein (<2.4 EU/ml) was performed using the LAL assay (QCL-1000; Cambrex, New Jersey). The rV10-2 filtered bulk protein preparation was also tested for the presence of residual host cell protein using a quantitative ELISA to detect *E. coli* protein. Presence of *E. coli* DNA was tested via qPCR. The vaccine was subjected to additional quality control tests (data not shown).

rV10-2 vaccine purity, identity and stability testing

Protein purification samples and final product samples were separated by 15% SDS-PAGE with Tris-glycine buffer and stained with R250 Coomassie brilliant blue or electrotransferred to PVDF membranes. Following electrotransfer, proteins were immunoblotted with monoclonal antibody BA5, raised against LcrV [49]. Purity of the vaccine was tested by reversed-phase high performance liquid chromatography (RP-HPLC) using a PLRP-S column (300 Å, 5 µm)(Varian, Inc). The mobile phase consisted of solution A (0.1 % formic acid in water) and solution B (0.1 % formic acid in acetonitrile). The rV10-2 protein was eluted with a solvent B gradient from 1% to 60%, run from 5 to 50 min. The solvent flow rate was 0.5 ml/min. rV10-2 was detected by absorbance at 280 nm and peak eluate subjected to MALDI-TOF, which revealed an ion signal with *m/z* 34,993.89. Assuming a systematic error of 0.05% for the MALDI-TOF measurement, the observed ion signal was in agreement with the expected mass of full length rV10-2 (calculated average mass 34,995.34 Da).

Stability of the final product (Vial A) was determined by placing sample vials in ESI Environmental Chambers with an Envirotrac Monitoring System at temperature and humidity controlled at 25 °C ± 2 °C / 60 % RH ± 5 % RH. After 4 weeks, sample vials were compared to vaccine vials stored at -80°C (manufacturing storage condition). Purity and stability of the vaccine was assayed by examining appearance, SDS-PAGE mobility and fluorescence spectrum.

F1V and rV10 immunization protocols

Mice and guinea pigs were immunized with cGMP manufactured rV10 vaccine (clinical grade purified pilot protein at 1 mg/ml) or the cGMP F1-V fusion vaccine (BEI resources NR-4526, vials containing 1 ml of recombinant protein, in PBS, at a concentration of 2 mg/ml) at day 0 and day 21. Five-hundred µl of the rV10 vaccine were mixed with vial B (Alhydrogel adjuvant vial) prior to injections. Two hundred-and-fifty µl of F1-V vaccine and 250 µl of PBS were mixed with vial B prior to injections. Mice received intramuscular injections of 100 µl, i.e. 25 µg rV10 or F1-V mixed with 250 µg Alhydrogel. Guinea pigs received intramuscular injections of 200 µl, i.e. 50 µg rV10 or F1-V mixed with 500 µg Alhydrogel. For control groups, animals received injection of an equal volume of PBS (500 µl) mixed with vial B. Animals were subjected to blood collection for serum analysis at day 0 (prior to first immunization) and day 42. Forty-two days post immunization animals were challenged with 1,000 MLD of fully virulent *Y. pestis* CO92 or CAC1 via intranasal inoculation.

Cynomolgus macaques (CM) were immunized with adjuvant only (500 µg Alhydrogel), cGMP manufactured rV10 or cGMP manufactured F1-V prepared as described previously [48]. Animals received 3 intramuscular injections of 200 µl, i.e. 50 µg rV10 or F1-V mixed with 500 µg Alhydrogel, at 21 day intervals. Blood was collected for serum analysis on day 63. After 77 days, animals were challenged via aerosol exposure using head-only inhalation

with 300 MLD of fully virulent *Y. pestis* CO92 as previously described [48]. NHP experiments with rV10 and F1V were conducted at Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico.

rV10-2 immunization protocols

Small animals (mice, rats or guinea pigs) were immunized with cGMP manufactured rV10-2 vaccine at day 0 and day 21. Five-hundred µl of vial A: rV10-2 vaccine, were mixed with vial B (Alhydrogel adjuvant vial) prior to injections. Mice and rats received intramuscular injections of 100 µl, i.e. 25 µg rV10-2 mixed with 250 µg Alhydrogel. Guinea pigs received intramuscular injections of 200 µl, i.e. 50 µg rV10-2 mixed with 500 µg Alhydrogel. For control groups, animals received injection of equal volume of PBS (500 µl) mixed to vial B (Alhydrogel adjuvant vial). Animals were subjected to blood collection for sera analysis on day 0 (prior to first immunization) and day 42. Forty-two days post immunization, animals were challenged with 1,000 mean lethal doses (MLD) of the fully virulent *Y. pestis* isolates CO92 or CAC1 (*caf1A::IS1541*) via intranasal inoculation [25].

CM and African Green Monkeys (AGM) were immunized with adjuvant only (Alhydrogel) or clinical grade rV10-2 following. Animals received 3 intramuscular injections of 200 µl, i.e. 50 µg rV10-2 with 500 µg Alhydrogel in 21 day intervals. After 70 days, animals were challenged via aerosol exposure using head-only inhalation with approximately 50 LD₅₀ doses of fully virulent *Y. pestis* CO92. NHP experiments with rV10-2 were conducted at LRRI under direction from the National Institute of Allergy and Infectious Diseases (NIAID).

Serum analysis

For antibody detection, the levels of serum immunoglobulin G (IgG) reactive with specific antigens were determined by a custom enzyme-linked immunosorbent assay (ELISA) designed and performed by the Great Lakes Regional Center of Excellence for Biodefense and Infectious Diseases Research (GLRCE) Animal Research and Immunology Core (ARIC) at the Howard Taylor Ricketts Laboratory. Briefly, serum samples representative of the immunization groups were aliquoted on microtiter plates pre-coated with either histidine tagged rF1 [23], rLcrV [23], cGMP rV10, cGMP rV10-2 or cGMP F1-V antigens (1 µg/ml). Binding of serum antibody was detected with secondary antibodies against specific immunoglobulin (anti-mouse, anti-rat, anti-guinea pig or anti-monkey), Peroxidase AffiniPure IgG (H+L) from Jackson ImmunoResearch Laboratories, Inc. Additional experiments were performed to examine the contribution of anti-histidine antibodies to mean IgG titers. Findings revealed that the level of specific anti-histidine antibodies was negligible in the sera of animals immunized with cGMP rV10 and cGMP rV10-2 (data not shown). Statistical analysis of antibody levels was performed in pairwise comparison using the unpaired two-tailed Student's t-test.

For passive transfer experiments, sera were collected from mice, rats, guinea pigs or CM following the immunization process. Polyclonal rabbit serum was raised against cGMP rV10-2 (3 intramuscular injections at 21 day intervals of 700 µg cGMP rV10-2 emulsified 1:1 with Complete Freund's Adjuvant or Incomplete Freund's Adjuvant (2 boosts). Antisera were injected into the peritoneal cavity of naïve 6–8 weeks old BALB/c mice, one hour prior to subcutaneous infection with 20 MLD *Y. pestis* CO92 or CAC1.

Plague challenge experiments

Y. pestis CO92 [50, 51] and its variant CAC1 [25] have been previously described. For the pneumonic plague model, animals were anesthetized with a cocktail of ketamine (Ketavet:Vedco) and xylazine (Sigma) (administered intraperitoneally, doses were adjusted

for each species) and challenged by intranasal inoculation with 20 μ l of *Y. pestis* CO92 or CAC1. For these experiments, *Y. pestis* was grown in heart infusion broth (HIB) supplemented with 2.5 mM calcium at 37°C overnight, conditions that mimic the ambient temperature during man-to-man transmission of pneumonic plague [52]. For the bubonic plague model, *Y. pestis* was grown in HIB at 26°C overnight. Growth of *Y. pestis* at 26°C is thought to mimic the ambient temperature in infected fleas prior to transmission of bubonic plague [52]. Plague bacilli were washed and diluted in sterile PBS to the required concentration. All infected animals were observed for morbidity, mortality or recovery over a course of 14 days. Analysis of the statistical significance of mortality studies was performed using the Fisher's exact test.

Compliance

All NHP protocols and any amendment(s) or procedures involving the care and use of animals in these studies were reviewed and approved by LRRRI's Institutional Animal Care and Use Committee before the studies were conducted. During the studies, the care and use of animals was in accordance with the guidelines of the U.S. National Research Council. All small animal and plague experiments were performed at the Howard Taylor Ricketts Laboratory in accordance with institutional guidelines following experimental protocol review and approval by the Institutional Biosafety Committee, Select Agent Committee and the Institutional Animal Care and Use Committee at The University of Chicago.

Results

Comparison of rV10 and F1-V plague vaccines in mice and guinea pigs

To test whether or not rV10, the single subunit antigen derived from LcrV [45], elicits vaccine protection similar to the F1-V vaccine [38], we manufactured clinical grade rV10 vaccine for comparison with clinical grade F1-V vaccine [41]. BALB/c mice ($n=30$) were immunized in a prime-booster schedule with 25 μ g rV10 or F1-V adsorbed to 250 μ g Alhydrogel injected on day 0 and day 21. On day 42, blood was collected and sera were subjected to ELISA for antibody titer determination. Each vaccine raised antigen-specific antibodies (rV10 and F1-V) (Figure 1A). For mice vaccinated with F1-V, ELISA tests with isolated protective antigens revealed higher antibody titers for rLcrV than for rF1 ($P<1 \times 10^{-6}$, Figure 1A). On day 42, mice were challenged by intranasal instillation of 1,000 MLD of *Y. pestis* CO92 or *Y. pestis* CAC1 (*caf1A::IS1541*), an rF1-vaccine escape variant of *Y. pestis* CO92 with an IS1541 insertion in *caf1A*, encoding the outer membrane usher for F1 pilus assembly [25]. Mock (PBS/Alhydrogel) immunized animals ($n=10$) succumbed to infection within three days of challenge with *Y. pestis* CO92 or *Y. pestis* CAC1 (Figure 1BC). Mice ($n=30$) that had been immunized with rV10 or F1-V survived infection with 1,000 MLD *Y. pestis* CO92 ($P<0.0001$) (Figure 1B). When infected with 1,000 MLD *Y. pestis* CAC1, 29 rV10-immunized animals and 27 F1-V vaccinated mice survived the challenge (Figure 1C). Animals that died during this experiment succumbed to plague infection, as necropsy experiments revealed pathological features of plague disease (data not shown). Comparison of rV10 and F1-V vaccine efficacy in mice with the Fisher's exact test did not reveal significant differences ($P=0.24$).

Hartley guinea pigs ($n=14$) were immunized by intramuscular injection with 50 μ g of rV10 or F1-V adsorbed to 500 μ g Alhydrogel on day 0 and 21 of a prime-booster protocol. Animals were bled on day 42 and serum samples analyzed for humoral immune responses. Each vaccine raised antigen-specific antibodies (rV10 and F1-V) (Figure 2A). For guinea pigs vaccinated with F1-V, ELISA tests with isolated protective antigens revealed similar antibody titers for rLcrV than for rF1 ($P=0.5$, Figure 2A). On day 42, animals were challenged by intranasal instillation with 1,000 MLD of *Y. pestis* CO92 (1 MLD represents

1,000 CFU)[47]. In contrast to PBS/Alhydrogel immunized animals ($n=8$), guinea pigs vaccinated with rV10 or F1-V survived the challenge ($P<0.0001$) (Figure 2B). Intranasal challenge with 1,000 MLD *Y. pestis* CAC1 (*caf1A::IS1541*) generated similar results [47]. Mock immunized animals succumbed to the challenge, whereas guinea pigs vaccinated with rV10 were protected from pneumonic plague (Figure 2C). For F1-V vaccinated guinea pigs, 13 animals were protected from the challenge, however one animal died (Figure 2C). Necropsy revealed that this guinea pig suffered *Y. pestis* CAC1 induced pneumonic plague (data not shown). Comparison of rV10 and F1-V vaccine efficacy in guinea pigs with the Fisher's exact test did not reveal significant differences ($P=0.5$).

Comparison of rV10 and F1-V plague vaccines in Cynomolgus macaques

Cynomolgus macaques (CM, $n=4$) were immunized with PBS/Alhydrogel, F1-V and or rV10 using a prime-two booster protocol with intramuscular injection of 50 μ g vaccine adsorbed to 500 μ g Alhydrogel in 21 day intervals. On day 63, blood samples were collected and serum antibody titers determined by ELISA (Table 1). Mock immunized animals did not harbor antibodies against rV10, F1-V, rLcrV or rF1. rV10 immunized animals developed antigen-specific antibodies that cross-reacted with rLcrV and F1-V, but not with rF1. F1-V vaccinated animals developed antigen-specific antibodies that cross-reacted with rV10, rLcrV and rF1 (Table 1). To assess the value of vaccine-specific immune responses, both non-immune and immune CM sera (500 μ l) were injected into naïve BALB/c mice one hour prior to subcutaneous challenge with 20 CFU *Y. pestis* CO92. Non-immune sera failed to protect BALB/c mice from lethal plague challenge (Table 1). Immune sera of rV10-immunized CM conferred protection (60–100%) from lethal plague challenge (Table 1). Four of the five immune sera from F1-V vaccinated macaques conferred protection (80–100%) against lethal plague challenge in mice. The immune serum from one F1-V immunized CM (NHP ID06750) conferred partial protection, i.e. two of the five passively immunized mice survived the challenge (Table 1).

On day 77, all CM were challenged via aerosol exposure with 250 MLD *Y. pestis* CO92 (Table 1). Three of the four mock immunized NHPs suffered pneumonic plague disease and died. The one animal that survived the challenge did not develop pneumonic plague (Table 1). This observation is in agreement with other reports that CM display individual variations in innate lung defenses, modulating their susceptibility to pneumonic plague [53] (*vide infra*). All rV10 vaccinated CM survived the lethal challenge, indicating that the prime-two booster regimen afforded protection from pneumonic plague (Table 1). Three of the four CM that were immunized with F1-V were protected from the lethal challenge. One macaque succumbed to pneumonic plague disease (NHP ID 06750), the same animal whose immune serum failed to protect BALB/c mice in passive transfer experiments (*vide supra*) (Table 1). Taken together these data suggest that immunization of CM with rV10 elicits protection against pneumonic plague similar to the F1-V vaccine.

Clinical grade rV10-2 vaccine

rV10-2 encompasses an N-terminal six histidyl tag and a glycyl-threonyl substitution for amino acids 271–300 of LcrV (Figure 3A). Figure 3B displays a flow chart for the overall cGMP process of rV10-2. Vaccine sample analysis revealed a single protein species on Coomassie stained SDS-PAGE, that, by immunoblotting, was recognized by LcrV specific monoclonal antibody BA5 [49](Figure 3C). One-hundred μ g rV10-2 vaccine was subjected to RP-HPLC (Figure 3D). Identity of full length rV10-2 polypeptide was confirmed by Edman degradation and mass spectrometry. Results from the analysis of the Bulk Protein Lot PR943 of the rV10-2 vaccine are displayed in Figure 3E. Accelerated stability testing for rV10-2 stored at 25°C indicated that the vaccine maintained its protective efficacy (Figure S1).

rV10-2 immunization prevents pneumonic plague in mice, rats and guinea pigs

rV10-2 vaccine preclinical studies for efficacy were conducted in several animal species. Immunizations used a prime-single booster schedule 21 days apart with 25 µg rV10-2 doses for mice ($n=30$) as well as rats ($n=10$) and 50 µg rV10-2 doses for guinea pigs ($n=10$); rV10-2 was pre-adsorbed to 1:10 Alhydrogel. Blood was drawn 42 days following the initial immunization and IgG antibody titers to rV10-2 vaccine antigen were examined by custom ELISA [46]. In comparison to PBS/Alhydrogel immunized animals, rV10-2 immunization of BALB/c mice ($P=0.003$, Figure 4A), Brown Norway rats ($P=0.001$, Figure 4C), as well as Hartley guinea pigs ($P=0.04$, Figure 4E) elicited significant antibody titers against the vaccine antigen. On day 42, immunized animals were anesthetized, challenged by intranasal instillation with 1,000 MLD of *Y. pestis* CO92 and monitored for disease progression. In contrast to PBS/Alhydrogel immunized cohorts, which succumbed to pneumonic plague within 3–5 days, rV10-2 vaccinated BALB/c mice ($P<0.0001$, Figure 4B), Brown Norway rats ($P<0.0001$, Figure 4D) and Hartley guinea pigs ($P<0.0001$, Figure 4F) were protected against lethal plague challenge. As a test whether or not vaccine protection extends to F1-mutant plague strains, rV10-2 immunized mice as well as guinea pigs were challenged with 1,000 MLD of *Y. pestis* CAC1. In contrast to PBS/Alhydrogel immunized animals, rV10-2 vaccinated BALB/c mice ($P<0.0001$, Figure 4B) or Hartley guinea pigs ($P<0.0001$, Figure 4F) were protected against pneumonic plague challenge with the F1-mutant strain.

rV10-2 immunization prevents pneumonic plague in non-human primates

Vaccine efficacy of rV10-2 was examined in NHP models of plague disease, including CM and African Green monkeys (AGM). Control animals ($n=6$) of CM and AGM cohorts received 3 intramuscular injections (prime-two booster schedule) of PBS/Alhydrogel (500 µg) in 21 day intervals. Vaccine groups ($n=12$) of CM and AGM cohorts each received 50 µg of clinical grade rV10-2 adsorbed to 500 µg Alhydrogel, following the same prime-two booster schedule. On day 70 following the immunization primer, NHPs were infected via aerosol exposure with approximately 50 MLD of *Y. pestis* CO92 and monitored for signs of the disease. As before, only some (3 out of 6) of the PBS/Alhydrogel immunized CM succumbed to pneumonic plague aerosol challenge with *Y. pestis* CO92 (Figure 5A). All CM ($n=12$) that had been immunized with rV10-2 survived the lethal plague challenge, indicating that the rV10-2 prime-two booster immunization schedule is able to protect CM against pneumonic plague challenge ($P=0.0079$, Figure 5A).

In contrast to CM, inhalation of *Y. pestis* CO92 aerosols by AGM leads to rapidly progressive, uniformly lethal pneumonic plague disease with an MLD of 350 CFU [54]. For this reason, AGM may be a preferred NHP model for the licensure of plague vaccines and post-exposure prophylaxes [54, 55]. Following inhalation of 50 MLD of *Y. pestis* CO92, all of the PBS/Alhydrogel immunized AGM control animals ($n=6$) succumbed to pneumonic plague with a calculated mean time-to-death (MTD) of 4.1 ± 0.37 days (Figure 5B). Four of the twelve AGM that had been immunized with rV10-2 vaccine, were protected from lethal plague challenge ($P=0.0002$, Figure 5B). Vaccinated animals that succumbed to the infection reached a MTD at 6.25 ± 1.7 day post challenge (Figure 5B). Thus, the rV10-2 immunization protocol described here (prime-two booster with 50 µg rV10-2 adsorbed to 500 µg Alhydrogel) achieved only partial protection against pneumonic plague in AGM.

In several plague models, antigen-specific IgG titers appear to correlate with disease protection, suggesting that protective immunity is based on B-cell mediated antibody responses to the plague protective antigens (LcrV and F1)[56]. We wondered whether antigen-specific IgG titers of rV10-2 immunized non-human primates could be correlated with protection against pneumonic plague. For rV10-2 immunized CM, the maximum average titer for antigen-specific IgG was measured two weeks following the second

injection, i.e. day 56 of the prime-two booster schedule. On day 70, the time of challenge, the average titer of antigen-specific IgG had dropped by about one third (Figure 5C). For AGM immunized with rV10-2, the maximum average titer of antigen-specific IgG was detected 14 days after the second booster (day 56). Two weeks later (day 70), the average level of rV10-2 specific IgG had dropped by about 50% (Figure 5D). rV10-2 immunized AGM were categorized into animals with ($n=4$) and without ($n=8$) protection against pneumonic plague. At the time of challenge (day 70), average antigen-specific IgG titers were higher in animals with protection ($1: 24,000 \pm 5,900$) than in the AGM cohort that lacked protection ($1: 12,000 \pm 6,100$, $P<0.01$). A significant difference in average antigen-specific IgG responses to immunization could not be detected 14 days following injection of the rV10-2 vaccine primer ($P=0.23$, day 35). Higher antibody titers were, however, observed two-weeks following the second booster immunization on day 56 ($P=0.028$, Figure 5D). Thus, antigen-specific IgG concentrations in rV10-2 immunized AGM appear correlated with protection against pneumonic plague.

To analyze antigen-specific antibody responses to vaccination in NHP (CM and AGM), sera of animals obtained on day 70 were analyzed by ELISA using the previously described LcrV peptide arrays [48]. Serum antibodies from NHPs, irrespective of whether these animals were protected against plague challenge or not, recognized the folded antigen (rV10-2 or LcrV) but displayed very little immune-reactivity towards LcrV oligopeptides (Figure S2).

Passive transfer experiments with rV10-2 immune sera

As an experimental demonstration that rV10-2 protection is based on antibody responses, passive transfer experiments were performed with immune and non-immune sera [57]. Sera from immune (rV10-2 immunized) and non-immune (mock immunized) mice, rats or guinea pigs were injected into the peritoneal cavity of naïve BALB/c mice one hour prior to subcutaneous challenge with 20 CFU of *Y. pestis* CO92 or the *Y. pestis* CAC1 variant. Non immune sera did not confer protection (Table 2). In contrast, rV10-2 immune sera from mice, rats or guinea pigs provided protection against lethal plague challenge with *Y. pestis* CO92 or *Y. pestis* CAC1 in passively immunized BALB/c mice (Table 2). These results indicate that antigen-specific IgG in the serum of rV10-2 immunized animals is sufficient to confer protection against plague. This hypothesis was also corroborated with passive transfer experiments involving rV10-2 immunized rabbits, which generated high antigen-specific IgG titers ($1:780,000$). Immune and non-immune rabbit sera were diluted in PBS and subjected to mouse passive protection experiments. Undiluted as well as 1:2, 1:4, 1:8 and 1:10 diluted sera were injected into naïve mice. Animals receiving immune sera (undiluted or diluted) were protected from lethal plague challenge with either *Y. pestis* CO92 or *Y. pestis* CAC1 (Table 3).

Discussion

Several milestones in the developmental process for biodefense vaccines must be reached before human trials can be considered. These include a large scale manufacturing process to generate inexpensive vaccines with sufficient purity and stability, preclinical and clinical safety as well as efficacy determinations. US Food and Drug Administration (FDA) regulations concerning the approval of new biological products when human efficacy studies are neither ethical nor feasible are known as "the Animal Rule" (21 CFR 314.600 for drugs; 21 CFR 601.90 for biological products). Under the Animal Rule, FDA may grant marketing approval based on adequate and well-controlled animal studies when the results of those studies establish that the drug or biological product is reasonably likely to produce clinical benefit in humans.

Here we summarize a developmental process aimed at licensure of the University of Chicago clinical grade rV10-2 plague vaccine. rV10-2 was derived from the original laboratory construct rV10 [45] for suitable use in humans. Master Cell Bank (MCB) and Production Cell Bank (PCB) were prepared following BPR 929-00 and BPR 930-00, respectively. A 300 L fermentation yielded 6.8 Kg cell paste (Lot 1586) and 500 g of cell paste yielded a total of 9.6 g of filtered purified rV10-2 bulk protein (Lot 1595), demonstrating that large scale manufacturing of the rV10-2 Plague Vaccine was achievable. Lot release specifications for the vaccine at clinical grade were established during the manufacturing process. An accelerated stability protocol was used to demonstrate that the clinical grade rV10-2 vaccine was stable when stored at room temperature and that overall efficacy of the vaccine was not impaired.

A set of preclinical data was generated to demonstrate efficacy of rV10-2 in relevant animal models. Experiments with mice, rats, guinea pigs as well as cynomolgus macaques demonstrated that rV10-2 vaccine induced robust protection against lethal pneumonic plague challenge with *Y. pestis* CO92 or the F1⁻ variant CAC1. Side-by-side comparison of F1-V and rV10 plague vaccines in mice, guinea pigs and cynomolgus macaques did not detect significant differences. These data suggest that the rV10 and the rV10-2 vaccines, which generate plague immunity by raising antibodies only against the LcrV antigen, are not inferior to a plague vaccine that raises antibodies against both protective antigens, F1 and LcrV (F1-V).

The FDA Animal Rule may grant approval based on experiments with a single animal species, assuming the animal model is sufficiently well-characterized. Nevertheless, the biodefense vaccine field generally appreciates that efficacy must be demonstrated in more than one animal species. The essential data elements for the development and evaluation of animal models sustaining the FDA animal rule licensing usually include the characterization of the challenge agent, its pathogenic determinants, the route and quantification of exposure. Host susceptibility and response to the etiologic agent should also be documented, as well as disease progression and manifestations. In this report, rV10-2 efficacy as a protective vaccine against *Y. pestis* infection is supported by experiments with five animal models for pneumonic plague. Two of the three rodent models, rats and mice, presented in this study are well established in the plague vaccine field [24, 46, 58]. Recently, we developed a guinea pig model for pneumonic plague and vaccine evaluation [47]. Guinea pigs offer considerable advantages as a model for lung infections, as the respiratory tract of these animals is larger than that of mice or rats and resembles the respiratory tract of primates. In this report, guinea pigs were used to assay the efficacy of the clinical grade rV10-2 vaccine candidate as well as to compare the two clinical grade plague vaccines. In both studies, the guinea pigs demonstrated strong immune response to both plague vaccines and were protected against lethal challenge with *Y. pestis* when vaccines were administered. Importantly, and unlike the CM model, all mock immunized animals succumbed to the infection and displayed disease manifestations that resemble the symptoms of pneumonic plague in humans [47].

NHPs likely represent the most important model to test the efficacy of human plague vaccines. Several studies used CM to evaluate plague vaccines, however these animals are only partially susceptible to pneumonic plague challenge [53]. In contrast, AGM appear uniformly susceptible and may be the most suitable NHP model for the study of plague disease [54]. Similar results were observed here, as AGM displayed uniform mortality following lethal plague challenge via aerosol exposure with *Y. pestis* CO92. AGM generated robust immune responses using a prime-two booster schedule with 50 µg rV10-2 antigen and 500 µg Alhydrogel. Of note, antibody titers rose up to two weeks after the last immunization but then dropped quickly in the following fourteen days, i.e. the time of

challenge. Indeed, the rV10-2 immunization schedule described above generated only 33% protection against 50 MLD *Y. pestis* CO92 aerosol challenge. To date, the AGM results represent the only animal model where rV10-2 immunization generated partial protection. AGM protection from pneumonic plague appears to be correlated with the serum concentration of LcrV-specific immunoglobulin, as animals with the lowest antibody titers following the rV10-2 immunization schedule were not protected. Clearly, more work is needed to develop AGM as a NHP model for the licensure of plague vaccines and to establish correlates for protective immunity between this model and humans.

Further development of the rV10-2 vaccine awaits the completion of GLP toxicology/safety studies in rabbits as well as GLP long term (3 year) stability studies of the vaccine in order to support a future FDA IND application. rV10-2 specific IgG responses in human volunteers could then be analyzed for the attributes of protecting passively immunized mice against lethal plague challenge or of blocking *Y. pestis* type III injection of macrophages [25, 49, 59]. In conjunction with vaccine efficacy trials using both rodents and NHPs, for example guinea pigs and AGM, these assays may be useful as correlates for protective immunity against plague in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Yersin A. La peste bubonique à Hong-Kong. Ann Inst Pasteur. 1894; 2:428–430.
2. Simond PL. La propagation de la peste. Ann Inst Pasteur. 1898; 12:625–687.
3. Perry RD, Fetherston JD. *Yersinia pestis*- etiologic agent of plague. Clin Microbiol Rev. 1997; 10:35–66. [PubMed: 8993858]
4. Raoult D, Aboudharam G, Crubezy E, Larrouy G, Ludes B, Drancourt M. Molecular identification by “suicide PCR” of *Yersinia pestis* as the agent of medieval black death. Proc Natl Acad Sci USA. 2000; 97:12800–12803. [PubMed: 11058154]
5. Metcalfe N. A short history of biological warfare. Med Confl Surviv. 2002; 18:271–282. [PubMed: 12201085]
6. Haffkine WM. Remarks on the plague prophylactic fluid. Br Med J. 1897; 1:1461–1462.
7. Quenee LE, Schneewind O. Plague vaccines and the molecular basis of immunity against *Yersinia pestis*. Hum Vaccin. 2009; 5:817–823. [PubMed: 19786842]
8. Burrows TW. Virulence of *Pasteurella pestis* and immunity to plague. Ergebn Mikrobiol. 1963; 37:59–113.
9. Wake A, Morita H, Wake M. Mechanisms of long and short term immunity to plague. Immunology. 1978; 34:1045–1052. [PubMed: 680791]

10. Meyer KF. Effectiveness of live or killed plague vaccines in man. Bull WHO. 1970; 42:653–666. [PubMed: 4988692]
11. Cavanaugh DC, Elisberg BL, Llewellyn CH, Marshall JD Jr, Rust JH Jr, Williams JE, et al. Plague immunization. V. Indirect evidence for the efficacy of plague vaccine. J Infect Dis. 1974 May; 129(Suppl):S37–S40. [PubMed: 4596518]
12. Meyer KF, Smith G, Foster LE, Marshall JD, Cavanaugh DC. Plague immunization. IV. Clinical reactions and serological responses to inoculations of Haffkine and freeze-dried plague vaccine. J Infect Dis. 1974; 129:S30–S36. [PubMed: 4825247]
13. Titball RW, Williamson ED. Vaccination against bubonic and pneumonic plague. Vaccine. 2001 Jul 20; 19(30):4175–4184. [PubMed: 11457543]
14. Fetherston JD, Schuetze P, Perry RD. Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol Microbiol. 1992; 6:2693–2704. [PubMed: 1447977]
15. Buchrieser C, Prentice M, Carniel E. The 102-kilobase unstable region of *Yersinia pestis* comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. J Bacteriol. 1998; 180:2321–2329. [PubMed: 9573181]
16. Brubaker RR. Mutation rate to non-pigmentation in *Pasteurella pestis*. J Bacteriol. 1969; 98:1404–1406. [PubMed: 5788712]
17. Girard G. Plague. Annu Rev Microbiol. 1955; 9:253–277. [PubMed: 13259466]
18. Meyer KF, Smith G, Foster L, Brookman M, Sung MH. Live, attenuated *Yersinia pestis* vaccine: virulent in non-human primates, harmless to guinea pigs. J Infect Dis. 1974; 129:S85–S112. [PubMed: 4207627]
19. Baker EE, Sommer H, Foster LE, Meyer E, Meyer KF. Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. J Immunol. 1952; 68:131–145. [PubMed: 14927919]
20. Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, et al. Structure and biogenesis of the capsular F1 antigen from *Yersinia pestis*: preserved folding energy drives fiber formation. Cell. 2003; 113:587–596. [PubMed: 12787500]
21. Du Y, Rosqvist R, Forsberg A. Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. Infect Immun. 2002; 70:1453–1460. [PubMed: 11854232]
22. Liu F, Chen H, Galvan EM, Lasaro MA, Schifferli DM. Effects of Psa and F1 on the adhesive and invasive interactions of *Yersinia pestis* with human respiratory tract epithelial cells. Infect Immun. 2006; 74:5636–5644. [PubMed: 16988239]
23. Quenee L, Cornelius CA, Ciletti NA, Elli D, Schneewind O. *Yersinia pestis* *cafI* (F1) variants and the limits of plague vaccine protection. Infect Immun. 2008; 76:2025–2036. [PubMed: 18347051]
24. Anderson DA, Ciletti NA, Lee H, Elli D, Segal J, DeBord KL, et al. Pneumonic plague pathogenesis and immunity in brown Norway rats. Am J Pathol. 2009; 174:910–921. [PubMed: 19164505]
25. Cornelius CA, Quenee LE, Elli D, Ciletti NA, Schneewind O. *Yersinia pestis* IS1541 transposition provides for escape from plague immunity. Infect Immun. 2009; 77:1807–1816. [PubMed: 19237527]
26. Friedlander AM, Welkos SL, Worsham PL, Andrews GP, Heath DG, Anderson GW Jr, et al. Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. Clin Infect Dis. 1995; 21:S178–S181. [PubMed: 8845449]
27. Burrows TW. An antigen determining virulence in *Pasteurella pestis*. Nature. 1956; 177:426–427. [PubMed: 13309325]
28. Burrows TW, Bacon GA. The effect of loss of different virulence determinants on the virulence and immunogenicity of strains of *Pasteurella pestis*. Br J Exp Pathol. 1958; 39:278–291. [PubMed: 13546547]
29. Perry RD, Harmon PA, Bowmer WS, Straley SC. A low-Ca²⁺ response operon encodes the V antigen of *Yersinia pestis*. Infect Immun. 1986; 54:428–434. [PubMed: 3021629]
30. Mueller CA, Broz P, Muller SA, Ringler P, Erne-Brand F, Sorg I, et al. The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles. Science. 2005; 310:674–676. [PubMed: 16254184]

31. Petterson J, Holmstrom A, Hill J, Frithz-Lindsten E, von Euler-Matell A, Carlsson E, et al. The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. *Mol Microbiol.* 1999; 32:961–976. [PubMed: 10361299]
32. Lee VT, Tam C, Schneewind O. LcrV, a substrate for *Yersinia enterocolitica* type III secretion, is required for toxin targeting into the cytosol of HeLa cells. *J Biol Chem.* 2000; 275:36869–36875. [PubMed: 10930402]
33. DeBord K, Lee VT, Schneewind O. On the Role of LcrG and LcrV during the type III targeting of effector Yops by *Yersinia enterocolitica*. *J Bacteriol.* 2001; 183:4588–4598. [PubMed: 11443094]
34. Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O. Plague bacteria target immune cells during infection. *Science.* 2005; 309:1739–1741. [PubMed: 16051750]
35. Brubaker RR. Interleukin-10 and the inhibition of innate immunity to yersiniae: roles of Yops and LcrV (V antigen). *Infect Immun.* 2003:3673–3681. [PubMed: 12819047]
36. Nakajima R, Motin VL, Brubaker RR. Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infect Immun.* 1995; 63:3021–3029. [PubMed: 7622225]
37. DePaolo RW, Tang F, Kim IY, Han M, Levine N, Ciletti NA, et al. TLR6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis. *Cell Host Microbe.* 2008; 4:350–361. [PubMed: 18854239]
38. Heath DG, Anderson GW Jr, Mauro JM, Welkos SL, Andrews GP, Adamovicz JJ, et al. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1- V antigen fusion protein vaccine. *Vaccine.* 1998; 16:1131–1137. [PubMed: 9682370]
39. Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, et al. A new improved subunit vaccine for plague: the basis of protection. *FEMS Immunol Med Microbiol.* 1995; 12:223–230. [PubMed: 8745007]
40. Williamson ED, Packer PJ, Waters EL, Simpson AJ, Dyer D, Hartings J, et al. Recombinant (F1+V) vaccine protects cynomolgus macaques against pneumonic plague. *Vaccine.* 2011; 29:4771–4777. [PubMed: 21570437]
41. Powell BS, Andrews GP, Enama JT, Jendrek S, Bolt C, Worsham P, et al. Design and testing for a non-tagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. *Biotechnol Prog.* 2005; 21:1490–1510. [PubMed: 16209555]
42. Warren R, Lockman H, Barnewall R, Krile R, Blanco OB, Vasconcelos D, et al. Cynomolgus macaque model for pneumonic plague. *Microb Pathog.* 2011; 50:12–22. [PubMed: 21040776]
43. Chichester JA, Musychuk K, Farrance CE, Mett V, Lyons J, Mett V, et al. A single component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic plague. *Vaccine.* 2009; 27:3471–3474. [PubMed: 19200825]
44. Goodin JL, Powell BS, Enama JY, Raab RW, McKnown RL, Coffman GL, et al. Purification and characterization of a recombinant *Yersinia pestis* V-F1 "reversed" fusion protein for use as a new subunit vaccine against plague. *Protein Expr Purif.* 2011; 76:136–144. [PubMed: 21055471]
45. Overheim KA, Depaolo RW, DeBord KL, Morrin EM, Anderson DM, Green NM, et al. LcrV plague vaccine with altered immunomodulatory properties. *Infect Immun.* 2005; 73:5152–5159. [PubMed: 16041032]
46. DeBord KL, Anderson DM, Marketon MM, Overheim KA, DePaolo RW, Ciletti NA, et al. Immunogenicity and protective immunity against bubonic and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infect Immun.* 2006; 74:4910–4914. [PubMed: 16861680]
47. Quenee LE, Ciletti NA, Berube BJ, Krausz T, Elli D, Hermanas T, et al. Pneumonic plague in guinea pigs and its prevention by subunit vaccines. *Am J Pathol.* 2011; 178:1689–1700. [PubMed: 21406168]
48. Cornelius CA, Quenee LE, Elli D, Ciletti NA, Schneewind O. V10 subunit vaccine protects cynomolgus macaques from lethal pneumonic plague. *Infect Immun.* 2008; 76:5588–5597. [PubMed: 18794281]
49. Quenee LE, Berube B, Segal J, Elli D, Ciletti NA, Anderson DM, et al. Amino acid residues 196–225 of LcrV represent a plague protective epitope. *Vaccine.* 2010; 28:1870–1876. [PubMed: 20005318]

50. Doll JM, Zeitz PS, Ettestad P, Bucholtz AL, Davis T, Gage KL. Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. *Am J Trop Med Hyg.* 1994; 51:109–114. [PubMed: 8059908]
51. Parkhill J, Wren BW, Thompson NR, Titball RW, Holden MT, Prentice MB, et al. Genome sequence of *Yersinia pestis* the causative agent of plague. *Nature.* 2001; 413:523–527. [PubMed: 11586360]
52. Samoilova SV, Samoilova LV, Yezhov IN, Drozdov IG, Anisimov AP. Virulence of pPst+ and pPst- strains of *Yersinia pestis* for guinea-pigs. *J Med Microbiol.* 1996; 45:440–444. [PubMed: 8958247]
53. Koster F, Perlin DS, Park S, Brasel T, Gigliotti A, Barr E, et al. Milestones in progression of primary pneumonic plague in cynomolgus macaques. *Infect Immun.* 2010; 78:2946–2955. [PubMed: 20385751]
54. Layton RC, Brasel T, Gigliotti A, Barr E, Storch S, Myers L, et al. Primary pneumonic plague in the African Green monkey as a model for treatment efficacy evaluation. *J Med Primatol.* 2011; 40:6–17. [PubMed: 20722770]
55. Layton RC, Mega W, McDonald JD, Brasel TL, Barr EB, Gigliotti AP, et al. Levofloxacin cures experimental pneumonic plague in African green monkeys. *PLoS Negl Trop Dis.* 2011; 5:e959. [PubMed: 21347450]
56. Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW. An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model. *Clin Exp Immunol.* 1999; 116:107–114. [PubMed: 10209513]
57. Motin VL, Nakajima R, Smirnov GB, Brubaker RR. Passive immunity to yersiniae mediated by anti-recombinant V antigen and Protein A-V antigen fusion peptide. *Infect Immun.* 1994; 62:4192–4201. [PubMed: 7927675]
58. Sebbane F, Gardner D, Long D, Gowen BB, Hinnebusch BJ. Kinetics of disease progression and host response in a rat model of bubonic plague. *Am J Pathol.* 2005; 166:1427–1439. [PubMed: 15855643]
59. Hill J, Leary S, Smither S, Best A, Petterson J, Forsberg A, et al. N255 is a key residue for recognition by a monoclonal antibody which protects against *Yersinia pestis* infection. *Vaccine.* 2009; 27:7073–7079. [PubMed: 19786138]

Figure 1. Comparison of clinical grade rV10 and F1-V plague vaccines in mice

Animals were immunized intramuscularly with 250 μ g of Alhydrogel ($n=10$ for each cohort) or with 25 μ g of clinical grade plague vaccine, rV10 or F1-V, adsorbed to 250 μ g Alhydrogel ($n=30$ for each cohort). Injections occurred at day 0 and 21. Specific antibody response to clinical grade proteins and laboratory grade recombinant subunits (rLcrV and rF1) was determined by ELISA on day 42 (A). Mock (PBS/Alhydrogel) immunized as well as rV10 or F1-V vaccinated animals were challenged 42 days after the first vaccine administration via intranasal instillation of 1,000 MLD of *Yersinia pestis* CO92 (B). The F1-mutant *Y. pestis* CAC1 strain (*cafIA::IS154I*) was used as challenge strain (1,000 MLD,

intranasal) to measure vaccine efficacy (C). Animals were monitored for disease progression and survival over 14 days.

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Figure 2. Comparison of clinical grade rV10 and F1-V plague vaccines in guinea pigs

Hartley guinea pigs were immunized intramuscularly with 500 μ g Alhydrogel ($n=8$ for each cohort) or with 50 μ g of clinical grade rV10 or F1-V, adsorbed to 500 μ g Alhydrogel ($n=14$ for each cohort). Injections occurred on day 0 and 21. Specific antibody response to clinical grade proteins and laboratory grade recombinant subunits (rLcrV and rF1) was determined by ELISA on day 42 (A). Mock (PBS/Alhydrogel) immunized as well as rV10 or F1-V vaccinated animals were challenged 42 days after the first vaccine administration via intranasal instillation of 1,000 MLD of *Y. pestis* CO92 (B). *Y. pestis* CAC1 (F1⁻) was used as challenge strain (1,000 MLD intranasal instillation) to measure vaccine efficacy (C). Animals were monitored for disease progression and survival over 14 days.

Figure 3. Manufacturing of clinical grade rV10-2

(A) Schematic representation of rV10-2, harboring an N-terminal six-histidyl tag and the rV10 coding sequence. Glycine (G) and Threonine (T) amino-acids have been introduced into the original sequence to replace the 30 native amino-acids (271–300) of wild-type *Y. pestis* LcrV protein. (B) Flow-chart of the rV10-2 manufacturing process. (C) Filtered bulk protein was separated on 10% SDS-PAGE and stained with Coomassie blue. Samples were also electro-transferred onto PVDF membrane and immunoblotted with monoclonal antibody MAb-BA5, which recognizes residues 196–225 of LcrV. (D) RP-HPLC chromatogram of 100 μg rV10-2 vaccine. (E) Quality control and residual testing performed on the rV10-2 Filtered Bulk protein.

Figure 4. Efficacy of rV10-2 plague vaccine in mice, rats and guinea pigs

Cohorts of mice (A and B, $n=30$) or rats (C and D, $n=10$) were immunized by intramuscular injection on day 0 and 21 with 25 μ g rV10-2 adsorbed to 250 μ g Alhydrogel. Cohorts of guinea pigs (E and F, $n=10$) were immunized by intramuscular injection on day 0 and 21 with 50 μ g rV10-2 adsorbed to 500 μ g Alhydrogel. Serum antibody responses specific to rV10-2 were determined by ELISA on day 42 (A, C and E). Mock immunized (PBS/Alhydrogel) and rV10-2 vaccinated animals were challenged 42 days after the first vaccine administration via intranasal instillation of 1,000 MLD of *Y. pestis* CO92 (B, D and F). For mice or guinea pigs, vaccinated animals were also challenged with 1,000 MLD *Y. pestis*

CAC1 (B and F). All animals were monitored for disease progression and survival over 14 days.

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Figure 5. Efficacy of rV10-2 plague vaccine in non-human primates

Cynomolgus macaques (CM) or African Green Monkeys (AGM) were immunized with a prime-two booster schedule by intramuscular injection with 500 μ g Alhydrogel ($n=6$, Adjuvant control) or with 50 μ g rV10-2 adsorbed to 500 μ g Alhydrogel ($n=12$) on day 0, 21 and 42. Mock as well as rV10-2 immunized animals were challenged on day 70 via aerosol exposure with approximately 50 MLD of *Y. pestis* CO92. CM (A) and AGM (B) were monitored for disease progression and survival over 20 days. Serum antibody responses to immunization in CM (C) or AGM (D) were determined by ELISA on day 7, 35, 56 and 70 and recorded as rV10-2 specific IgG titers.

Table 1
Protection of Cynomolgus macaques against pneumonic plague with clinical grade F1-V and rV10 vaccines

Vaccine group	NHP ID	F1-V ^a	rV10 ^a	rLcrV ^a	rF1 ^a	Number of survivors/number challenged ^b	MTD (days) ^b	Aerosol challenge (CFU) ^c	Status 14 days post challenge ^c	TOD (days) ^c
Group 1 PBS/Adjuvant	06723	BD	BD	BD	BD	0/5	7±2.28	1.49 × 10 ⁴	A	NA
	06949	BD	BD	BD	BD	0/5	7±3.74	1.43 × 10 ⁴	D	5
	06968	BD	BD	BD	BD	0/5	6.6±1.35	1.86 × 10 ⁴	D	4
	07412	BD	BD	BD	BD	0/5	5.8±0.74	9.22 × 10 ³	D	4
Group 2 F1-V	06750	4.46	3.87	4.17	3.69	2/5	12±2.16	1.41 × 10 ⁴	D	7
	07449	3.95	4.30	4.49	4.50	5/5	NA	9.96 × 10 ³	A	NA
	06871	4.38	4.34	4.77	4.30	5/5	NA	1.17 × 10 ⁴	A	NA
	07423	4.34	4.39	4.77	4.39	4/5	14±0.0	1.42 × 10 ⁴	A	NA
Group 3 rV10	07076	4.50	4.43	4.44	BD	4/5	9±0.0	1.38 × 10 ⁴	A	NA
	07461	4.34	4.92	4.25	BD	3/5	12.5±0.5	1.54 × 10 ⁴	A	NA
	06962	4.46	5.11	4.41	BD	4/4	NA	1.54 × 10 ³	A	NA
	07568	4.63	5.02	4.74	BD	4/4	NA	2.08 × 10 ⁴	A	NA

^a Cynomolgus macaques immunized with adjuvant alone (PBS/Alhydrogel) or with clinical grade F1-V and rV10 vaccines. Serum antibody titers against the vaccine antigens or recombinant LcrV (rLcrV) and F1 pilin (rF1) were determined by end point ELISA and reported as log10.

^b Naïve BALB/c mice were passively immunized by intraperitoneal injection with 500 µl of non-human primate serum 60 min prior plague challenge via subcutaneous injection of 20 MLD of *Yersinia pestis* CO92. Mean Time to Death (MTD) ± standard error of the means was recorded in days.

^c *Y. pestis* CO92 aerosol challenge dose for non-human primates in colony forming units (CFU). Status of non-human primates is recorded on day 14 following challenge as alive (A) or dead (D). Time-of-death (TOD) was recorded in days.

NA: not applicable; BD: Below Detection; NHP ID: non-human primate identification number.

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Table 2

Passive immunization of mice with antisera raised against clinical grade rV10-2

Challenge strain ^a	Number of animals with 14 day survival/total number challenged					
	Serum from mock immunization ^b			Serum from rV10-2 immunization ^b		
	mouse	rat	guinea pig	mouse	rat	guinea pig
<i>Yersinia pestis</i> CO92	0/10	2/10	1/10	10/10	10/10	10/10
<i>Yersinia pestis</i> CAC1	ND ^c	1/10	3/10	ND	10/10	10/10

^a Passively immunized BALB/C mice (6 to 8 weeks old) were challenged with 20 CFU of *Y. pestis* strains CO92 or CAC1 (*cafI*/A:IS/54) administered via subcutaneous injection.

^b Serum of prime-booster (day 21) immunized animals (200 µl) receiving PBS/Alhydrogel (mock) or rV10-2/Alhydrogel vaccine was administered 1 hour prior to plague challenge.

^c ND, Not Determined

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Table 3

Passive immunization of mice with rabbit rV10-2 antiserum

Challenge strain ^a	Number of animals with 14-day survival/total number challenged									
	Naïve rabbit serum ^b Dilution					rV10-2 rabbit immune serum ^b Dilution				
	UD ^d	1:2 ^c	1:4	1:8	1:10	UD	1:2	1:4	1:8	1:10
<i>Yersinia pestis</i> CO92	0/10	0/10	0/10	0/10	1/10	10/10	10/10	10/10	10/10	10/10
<i>Yersinia pestis</i> CAC1	1/10	1/10	1/10	2/10	1/10	10/10	10/10	10/10	10/10	10/10

^aPassively immunized BALB/C mice (6 to 8 weeks old) were challenged with 20 CFU of *Y. pestis* strains CO92 or CAC1 (ca71A:1S /541) administered via subcutaneous injection.

^bSerum (200 µl) from a naïve rabbit or a rabbit immunized with clinical grade rV10-2 was administered 1 hour prior to plague challenge.

^cSerum was diluted into sterile Phosphate Buffered Saline (PBS)

^dUD, Undiluted