Published in final edited form as:

Vaccine. 2011 April 5; 29(16): 2986–2998. doi:10.1016/j.vaccine.2011.01.099.

A live attenuated strain of *Yersinia pestis* KIM as a vaccine against plague

Wei Sun¹, David Six², Xiaoying Kuang¹, Kenneth L Roland¹, Christian R.H. Raetz², and Roy Curtiss III^{*,1}

¹Center for Infectious Disease and Vaccinology, The Biodesign Institute, Arizona State University, Tempe, AZ, 85287, USA

²Department of Biochemistry, Duke University Medical Center, Box 3711 DUMC, Durham, NC 27710, USA

Abstract

Yersinia pestis, the causative agent of plague, is a potential weapon of bioterrorism. *Y. pestis* evades the innate immune system by synthesizing tetra-acylated lipid A with poor Toll-like receptor 4 (TLR4)-stimulating activity at 37°C, whereas hexa-acylated lipid A, a potent TLR4 agonist, is made at lower temperatures. Synthesis of *Escherichia coli* LpxL, which transfers the secondary laurate chain to the 2'-position of lipid A, in *Y. pestis* results in production of hexa-acylated lipid A at 37°C, leading to significant attenuation of virulence. Previously, we described a *Y. pestis* vaccine strain in which *crp* expression is under the control of the arabinose-regulated *araC* P_{BAD} promoter, resulting in a 4-5 log reduction in virulence. To reduce the virulence of the *crp* promoter mutant further, we introduced *E. coli* lpxL into the *Y. pestis* chromosome. The χ 10030(pCD1Ap) (Δ lpxP32::P_{lpxL} lpxL Δ P_{crp21}::TT *araC* P_{BAD} *crp*) construct likewise produced hexa-acylated lipid A at 37°C and was significantly more attenuated than strains harboring each individual mutation. The LD₅₀ of the mutant in mice, when administered subcutaneously or intranasally was >10⁷-times and >10⁴-times greater than wild type, respectively. Mice immunized subcutaneously with a single dose of the mutant were completely protected against a subcutaneous challenge of 3.6 × 10⁷ wild-type *Y. pestis* and significantly protected (80% survival) against a pulmonary challenge of 1.2 × 10⁴ live cells. Intranasal immunization also provided significant protection against challenges by both routes. This mutant is an immunogenic, highly attenuated live *Y. pestis* construct that merits further development as a vaccine candidate.

Keywords

Yersinia pestis; lipid A; regulated delayed attenuation; plague vaccine

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding Author: Roy Curtiss III, The Biodesign Institute, Center for Infectious Diseases and Vaccinology, Arizona State University, PO Box 875401, 1001 S. McAllister Avenue, Tempe, AZ 85287-5401. Tel 480-727-0445. Fax 480-727-0466. Tempe, Arizona 85287. rcurtiss@asu.edu..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest: all authors declare none.

1. Introduction

Yersinia pestis, the etiologic agent of plague, has been responsible for high mortality in several epidemics throughout human history and remains a current threat as a potential biological warfare agent [1,2]. Infection by *Y. pestis* can result in three forms of the disease, bubonic, septicemic and/or pneumonic plague. Pneumonic plague is highly contagious and easily transmitted person to person through airborne droplets, resulting in a rapid onset of disease and a mortality rate approaching 100% if treatment is delayed more than 24 h post-exposure [1-6]. There are several thousand reported cases of the disease worldwide annually [7] with a fatality rate between 5% and 15% [8]. Additionally, there has been an emergence of *Y. pestis* strains resistant to multiple drugs [9,10]. Plague has been classified as a re-emerging disease by the World Health Organization and *Y. pestis* is considered a potential bioweapon owing to its extreme virulence, its low infectious dose, and the ease of its transmission [11]. Therefore, prophylactic vaccination against this disease holds the brightest prospect for its control in the long term.

Currently, there are no licensed plague vaccines available in the United States. Although several active vaccine candidates are being developed, most require multiple administrations to achieve protective immunity [12-19]. The live EV76 vaccine is a spontaneous *pgm* mutant that has been successfully used in humans for more than 70 years in the former Soviet Union and other parts of Asia, with reported efficacy against both bubonic and pneumonic plague, and it is still in use today in the former Soviet Union and other parts of Asia [20,21]. The EV76 vaccine strain confers greater protection against bubonic and pneumonic plague than killed vaccines and subunit vaccines in animals. However, EV76 can cause local and systemic reactions and retains virulence in some non-human primate species, preventing it from gaining worldwide acceptance [16,22-28]. In spite of the drawbacks with EV76, Smiley has indicated that research toward the development of new, improved live-attenuated vaccines should continue and be strongly encouraged [29].

We have been investigating strategies for developing a live *Y. pestis* vaccine. We recently described the attributes of a *Y. pestis* strain engineered for arabinose-regulated *crp* expression. The concept behind this strategy was originally worked out in *Salmonella* and has been called regulated delayed attenuation [30]. During in vitro growth, arabinose is added to the growth medium and *crp* is expressed. Upon inoculation of the host, the bacteria invade host tissues where arabinose is not available, *crp* expression ceases and the strain becomes attenuated as the amount of Crp decreases by half at each cell division. Our results did demonstrate that this strain was somewhat attenuated, immunogenic and protective against pneumonic plague [31]. However, because this strain still retained some virulence, we continued to explore other means to achieve attenuation.

The *Y. pestis* LPS plays a role in immune evasion. *Y. pestis* does not carry the *lpxL* gene, which encodes an enzyme that transfers the secondary laurate chain to the 2'-position of lipid A at 37°C [32,33]. The absence of *lpxL* leads to production of tetra-acylated lipid A at 37°C that does not bind to host toll like receptor 4 (TLR4), thus failing to stimulate the typical inflammatory responses induced by lipid A [34,35]. *Y. pestis* does produce a form of hexa-acylated lipid A at low temperatures, through temperature-sensitive expression of *lpxP*, which encodes an enzyme similar to LpxL [35,36]. A *Y. pestis* strain expressing *E. coli lpxL* from a multicopy plasmid directs formation of hexa-acylated lipid A at 37°C, stimulates TLR4, promotes DC IL-12(p40)2 production and induction of DC migration, attenuates *Y. pestis* dramatically and confers protection against subcutaneous challenge with virulent *Y. pestis* [34,35].

In this report, we examine the virulence and immunogenicity of *Y. pestis* strains that express *E. coli lpxL* from the chromosome either alone or in combination with a Δ *pgm* or arabinose-regulated *crp* mutation and show that the χ 10030(pCD1Ap) mutant is highly attenuated and immunogenic. Mice immunized subcutaneously or intranasally with this mutant were protected against both subcutaneous (s.c.) and intranasal (i.n.) challenge with virulent *Y. pestis* KIM6+(pCD1Ap).

2. Materials and methods

2.1. Media and reagents

Tryptose blood agar (TBA) and heart infusion broth (HIB) were from Difco. HIB Congo red agar plates were used to confirm the pigmentation (Pgm) phenotype of *Y. pestis* strain (24). Ampicillin, chloramphenicol and L-arabinose were from Sigma (St. Louis, MO). Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs unless indicated otherwise. *Taq* DNA polymerase (New England Biolabs) was used in all PCR tests. *Vent* DNA polymerase (New England Biolabs) was used to amplify fragments for cloning. Qiagen products (Hilden, Germany) were used to isolate plasmid DNAs, gel-purify fragments or purify PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase were from Promega.

2.2. Bacterial strains, plasmids, and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 1. All strains were stored at -70°C in phosphate-buffered glycerol. *Y. pestis* cells were grown routinely at 28°C on Congo red agar from glycerol stocks and then grown in heart infusion broth (HIB, Difco) or on tryptose-blood agar base (TBA, Difco) [37]. All *E. coli* strains were grown routinely at 37°C in LB broth [38] or LB solidified with 1.5% Bacto Agar (Difco).

2.3. Construction of plasmids

All primers used in this paper are listed in Table S1. Primer sets of *lpxP*-U1/*lpxP*-U2 and *lpxP*-D1/*lpxP*-D2 were used for amplifying *lpxP*-U (upstream gene sequence of *lpxP*, from -330 to -843 bp), and *lpxP*-D (downstream gene sequence of *lpxP*, from $+1028$ to $+1517$ bp) fragment, respectively. The *lpxP*-U and *lpxP*-D fragments were ligated by overlapping PCR using primers *lpxP*-U1 and *lpxP*-D2, which contain regions of complementarity. The resulting PCR product contained engineered *Pst*I and *Sac*I restriction sites near the center of the fragment. The PCR product was digested with *Eco*RI and *Hind*III and ligated into pUC18 digested with the same enzymes to construct the plasmid pYA4576. The P_{lpxL} *lpxL* gene fragment was amplified from χ 6212 using *lpxL* primer1 and *lpxL* primer2. Then, the P_{lpxL} *lpxL* fragment was ligated with *Eco*RI and *Hind*III-digested pYA4576 to construct the plasmid pYA4577. The *cat-sacB* fragment in plasmid pYA4373 was amplified using primers *cat-sacB* 1 and *cat-sacB* 2. Plasmid pYA4577 was digested with *Pst*I, dephosphorylated by shrimp alkaline phosphatase and ligated with the *cat-sacB* cassette to form plasmid pYA4578. The DNA sequence of the insert in pYA4578 was confirmed by DNA sequencing.

2.4. Strain constructions

The Δ *lpxP32::P_{lpxL} lpxL* mutation was introduced into *Y. pestis* strains KIM6+, KIM6 (Pgm $-$) or χ 10017 (Δ *P_{crp21::TT araC P_{BAD} crp}*) to construct strains χ 10015, χ 10028 and χ 10030, respectively, using the two-step recombination method as described previously [39]. Construction of Δ *lpxP32::P_{lpxL} lpxL* *Y. pestis* strain χ 10015, Pgm $-$ Δ *lpxP32::P_{lpxL} lpxL* *Y. pestis* strain χ 10028 and Δ *lpxP32::P_{lpxL} lpxL* Δ *P_{crp21::TT araC P_{BAD} crp}* *Y. pestis* strain χ 10030 was briefly described as follow: Briefly, the linear *lpxP*-U-*cat-sacB*-*P_{lpxL}::lpxL*-

lpxP-D fragment was isolated from plasmid pYA4578 using *Eco*RI and *Hind*III and used to electroporate KIM6+, KIM6 or χ 10017 competent cells containing pKD46. Electroporants were plated onto TBA + Cm (10 μ g/ml) plates and incubated at 26°C overnight. Integrants were verified using PCR to confirm that the *lpxP*-U-*cat-sacB*-P_{lpxL}*lpxL*-*lpxP*-D linear DNA fragment was integrated into the correct chromosomal site. Mutant colonies were streaked onto TBA+ Cm (10 μ g/ml) + 5% sucrose plates and HIB Congo Red + Cm (10 μ g/ml) plates to test and isolate sucrose-sensitive colonies for the second-step recombination, respectively. Next, a linear DNA fragment carrying *lpxP*-U-P_{lpxL}*lpxL*-*lpxP*-D was isolated from pYA4577 after digestion with *Eco*RI and *Hind*III and used to electroporate *lpxP*-U-*cat-sacB*-P_{lpxL}*lpxL*-*lpxP*-D KIM6+(pKD46), *lpxP*-U-*cat-sacB*-P_{lpxL}*lpxL*-*lpxP*-D KIM6(pKD46) and *lpxP*-U-*cat-sacB*-P_{lpxL}*lpxL*-*lpxP*-D χ 10017(pKD46) and competent cells, respectively. Colonies grew on TBA + 5% sucrose plates were confirmed to be sensitive to chloramphenicol by streaking onto HIB plates containing 10 μ g/ml chloramphenicol and then tested by PCR to validate that the P_{lpxL} *lpxL*-*lpxP* fragment was inserted into the correct chromosomal site. Finally, plasmid pKD46 was eliminated from KIM6+, KIM6 and χ 10017 (Δ P_{crp21}::TT *araC* P_{BAD} *crp*) derivatives by growing them at 37°C to yield strains χ 10015, χ 10028 and χ 10030, respectively (Fig.S1). Under BLS-3 containment, plasmid pCD1Ap was then introduced by electroporation into these strains, yielding χ 10015(pCD1Ap), χ 10028(pCD1Ap) and χ 10030(pCD1Ap).

2.5. Lipid A isolation and structural analysis

Strains were streaked out on Bacto HIB (Becton, Dickinson, and Company) agar plates supplemented with Congo red (0.1 mg/ml) and galactose (0.2% w/v) at 26°C. Strains were grown in Bacto HIB at 26°C or 37°C as indicated. Typically, 200-ml cultures were inoculated from overnight cultures to A₆₀₀ 0.02-0.03 and grown to A₆₀₀ 0.5-1.0. The cells were pelleted by centrifugation at 3,500 x g for 30 min at 4°C. The supernatant was aspirated to avoid disturbing the pellet. The pellet was resuspended in phosphate-buffered saline (PBS), and pelleted again at 2,500 x g for 30 min at 4 °C. As much of the supernatant was aspirated as possible without disturbing the pellet. The cell pellets were resuspended in 20 mL of PBS and transferred to chloroform-safe Nalgene bottles. Chloroform and methanol were added to a final ratio of 1:2:0.8 (chloroform:methanol:PBS, v/v), forming a single-phase Bligh/Dyer mixture [40]. The cell suspensions were agitated by stirring for 1 h at room temperature, followed by centrifugation at 2,500 x g for 30 min. The supernatants containing phospholipids were discarded, and the pellets were resuspended by vortexing in 40 ml of a single-phase Bligh/Dyer mixture and transferred to a 50-ml Pyrex tube with a teflon-lined cap. The tubes were centrifuged again and the supernatants discarded. The air-dried pellets were resuspended in 25 ml of 1% acetic acid (v/v, pH 2.8) by vortexing and probe sonication. The tubes were then incubated at 100°C for 1 h to release the lipid A from the lipopolysaccharide (LPS). After cooling and vortexing, the solution was transferred to a chloroform-safe Nalgene bottle. Chloroform and methanol were added to a final ratio of 2:2:1.8 (chloroform:methanol:aqueous acetic acid, v/v) to form a two-phase Bligh/Dyer mixture [40]. The mixture was vigorously shaken to extract the lipid A, and the bottles were centrifuged at 2,500 x g for 20 min at 20°C to separate the two phases. The lower (chloroform) phase from each bottle was carefully removed to a round bottom flask and dried. The remaining upper phase was extracted again with fresh pre-equilibrated lower phase from a two-phase Bligh/Dyer mixture (2:2:1.8, chloroform:methanol:PBS, v/v). After centrifugation, the lower phase was added to the same flask and dried down again. The dried lipid A species were resuspended in chloroform:methanol (2:1, v/v) by vortexing and sonication, transferred to a glass tube, dried down, sealed with a teflon-lined cap, and stored at -80°C.

Spectra for the isolated *Y. pestis* lipid A species were acquired on an ABI QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada), equipped with an electrospray ionization (ESI) source. Spectra were acquired in the negative-ion mode and were the accumulation of 60 scans (1 min) collected from m/z 60-2200. The dried lipid A species were prepared for MS by resuspension in 2 ml of chloroform:methanol (2:1, v/v). A 20- μ l aliquot of the lipid A sample was diluted 10-fold in the same solvent and spiked with 2 μ l piperidine (final concentration 1%, v/v). Immediately after preparation, each sample was directly infused into the ion source at 5 μ l/min. The negative ion ESI was carried out at -4200 V. Data acquisition and analysis were performed using the Analyst QS software (ABI/MDS-Sciex).

2.6. Virulence analysis in mice

Animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and protocols were approved by the Arizona State University Animal Care and Use Committee.

Single colonies of each strain were used to inoculate HIB cultures and grown overnight at 26°C. To select for plasmid pCD1Ap, ampicillin was added into the medium at a concentration of 25 μ g/ml. Bacteria were diluted into 10 ml of fresh HIB enriched with 0.2% xylose and 2.5 mM CaCl_2 to obtain an OD_{620} of 0.1 and incubated at 26°C for s.c. infections (bubonic plague) or at 37°C for i.n. infections (pneumonic plague). Both cultures were grown to an OD_{620} of 0.6. The cells were then harvested and the pellet resuspended in 1 ml of isotonic PBS.

Female 7-week-old Swiss Webster mice from Charles River Laboratories were inoculated by s.c. injection with 100 μ l of bacterial suspension or i.n. with 20 μ l of bacterial suspension. Actual numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto TBA agar. To determine 50% lethal dose (LD_{50}), five groups of six mice were infected with serial dilutions of bacteria. Mice were monitored twice daily for 21 days, and the LD_{50} was calculated as described [41].

For colonization/dissemination analysis, groups of mice were inoculated either s.c. or i.n. At the indicated times after infection, 3 mice for each time point were euthanized, and samples of blood, lungs, spleen and liver were collected and weighed. The bacterial load for each organ was determined by plating dilutions of the homogenized tissues onto TBA plates containing 25 μ g/ml ampicillin and reported as CFU per gram of tissue or CFU per ml blood. Infections were performed in at least two independent experiments.

2.7. Histopathological analysis

Lung and spleen samples from *Y. pestis*-infected mice were collected at various time points and were fixed by immersion into 10% neutral buffered formalin in screw-cap containers. After 3 days, the tissues were moved to new containers with fresh formalin, processed, sectioned at 5 μ m, mounted on glass slides, and stained with hematoxylin and eosin (HE). The sterility of the fixed tissues was confirmed by plate counts before histopathological examination. We analyzed tissues from three animals per group for histopathology, and data from representative tissues are presented.

2.8. In vivo cytokine analysis

Swiss-Webster mice were inoculated s.c with 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.61×10^7 CFU of χ 10030 (pCD1Ap) or i.n. with 1.67×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.93×10^4 CFU of χ 10030 (pCD1Ap). A group of uninfected mice served as controls. Samples were taken from mice inoculated s.c. on days 2, 4 and 6 post-inoculation

and from mice inoculated i.n. at 12, 24, and 48 hours post-inoculation, three mice from each time point and group were euthanized and blood was collected. The sera were filtered through 0.22- μ m syringe filters and cultured on TBA plates to confirm their sterility. The levels of cytokines and chemokines were measured using a multiplex assay with BioPlex (Bio-Rad).

2.9. Determination of protective efficacy

Y. pestis strains were grown as described above. Two groups of Swiss Webster mice (10/group) were vaccinated s.c. with 1.4×10^7 CFU of χ 10030(pCD1Ap) cells in 100 μ l of PBS and another two groups of mice (10/group) were vaccinated i.n. with 2.5×10^5 CFU of χ 10030(pCD1Ap) cells in 20 μ l of PBS on day 0. Another two groups of mice (4/group) were injected with 100 μ l of PBS as controls. Blood was collected for antibody measurement by retro-orbital sinus puncture at 2 and 4 weeks post immunization. Mice were lightly anesthetized using a ketamine and xylazine mixture administered intramuscularly before bleeding. On day 35, animals were challenged s.c. with *Y. pestis* KIM6+(pCD1Ap) at 3.57×10^7 CFU in 100 μ l PBS or lightly anesthetized with a 1:5 xylazine/ketamine mixture and challenged i.n. with 1.24×10^4 CFU in 20 μ l PBS. Control groups were challenged with 1.3×10^3 CFU by both routes. All infected animals were observed over a 15-day period for the development of signs of plague infection.

2.10. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay serum IgG antibodies against *Yersinia* whole cell lysates (YpL) of *Y. pestis* KIM6+(pCD1Ap) [42]. Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 200ng/well of YpL antigen. The procedures were same as those described previously [42].

2.11. Statistical analysis

Log-rank test was used for analysis of the survival curves. Data are expressed as means $\pm$ SD. An ANOVA (SAS software) analysis was used to evaluate differences in antibody titer. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Strain construction and lipid A analysis

We constructed three mutant *Y. pestis* strains expressing *E. coli* *lpxL* from its native promoter either in a wild-type background, in χ 10015 (Δ *lpxP32*::*P*_{lpxL} *lpxL*), in a *Pgm*[−] background, in χ 10028 (*Pgm*[−] Δ *lpxP32*::*P*_{lpxL} *lpxL*) or in combination with a previously described attenuating mutation, Δ *P*_{crp21}::*TT araC* *P*_{BAD} *crp* [31] in χ 10030 (Δ *lpxP32*::*P*_{lpxL} *lpxL* Δ *P*_{crp21}::*TT araC* *P*_{BAD} *crp*) (Fig. 1). We used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) to characterize the different lipid A species made by *Y. pestis* KIM6+, χ 10015 and χ 10030 grown at 26°C and 37°C. At 26°C, the data for KIM6+ indicated a prominent mass peak corresponding to hexa-acyl lipid A decorated with two aminoarabinose species was observed at *m/z* 1041 in addition to minor peaks of tetra-acyl (*m/z* 832) and penta-acyl (*m/z* 924) lipid A (Fig. 2 A). The lipid A profiles of mutant strains χ 10015, χ 10028 and χ 10030 were identical and only the structure of χ 10030 is shown. The data for χ 10030 indicated prominent mass peaks of hexa-acyl lipid A with the addition of either one (*m/z* 949) or two (*m/z* 1014) aminoarabinose moieties or without any aminoarabinose (*m/z* 884) (Fig. 2 C). Note that while both KIM6+ and χ 10030 produced primarily hexa-acylated lipid A, the two lipid A structures differ slightly. *LpxP* of *Y. pestis* adds a C16 acyl group while *LpxL* from *E. coli* adds a C12 group to the lipid A, accounting for the differences in structure observed. Lipid

A isolated from *Y. pestis* KIM6+ at 37°C showed prominent mass peaks of tetra-acyl lipid A, and a minor peak of penta-acyl lipid A, and no peak was detected for hexa-acyl lipid A (Fig. 2 B), as expected. The presence of LpxL (and absence of LpxP) in χ 10030 resulted in predominantly hexa-acylated lipid A at 37°C, with no other lipid A forms detected (Fig. 2 D). These results show that strain χ 10015, χ 10028 and χ 10030 expressing *lpxL* produced hexa-acylated lipid A at both 26°C and 37°C.

LpxM (MsbB) is another protein that acylates lipid A. Previous results have shown that *lpxM* mutations have pleiotropic effects, resulting in reductions in expression and immunoreactivity of numerous major proteins and carbohydrate antigens, including F1, Pla, Ymt, V antigen, LPS, and ECA [43]. In light of these previous findings, we examined LcrV synthesis and LcrV and YopM secretion in *Y. pestis* KIM6+(pCD1Ap), χ 10015(pCD1Ap) and χ 10030(pCD1Ap). We observed no detectable differences in LcrV synthesis or on LcrV and YopM secretion, indicating that deletion of *lpxP* and expression of the *E. coli lpxL* gene does not affect synthesis or secretion of these virulence proteins (Fig. S1A & B, see supporting information).

3.2. Virulence of *Y. pestis* mutants in mice

In our initial studies, we determined the LD₅₀ of strain χ 10015(pCD1Ap) ($\Delta lpxP32::P_{lpxL}$ *lpxL*) and found it to be highly attenuated when administered subcutaneously (Table 2), comparable to what was reported for a similar strain [35], but the strain still retained virulence when administered intranasally. We obtained similar results for the *pgm* negative strain KIM6(pCD1Ap) and χ 10028(pCD1Ap) ($Pgm^- \Delta lpxP32::P_{lpxL}$ *lpxL*) (Table 2). Mice immunized s.c. with a high dose of KIM6(pCD1Ap), χ 10015(pCD1Ap) or χ 10028(pCD1Ap) developed swelling and an open wound at the injection site. Mice immunized with the highest doses of either strain were challenged either s.c. or i.n. and significant protection was observed (Fig. S2). Additional information on our protection experiments with KIM6(pCD1Ap), χ 10015(pCD1Ap) and χ 10028(pCD1Ap) are provided in supporting materials. However, because of the reactogenicity of these strains (Table 2) and their virulence when administered i.n., we deemed them in need of further modification. We therefore introduced the previously described $\Delta P_{crp21::TT}$ *araC* P_{BAD} *crp* mutation [31] into χ 10015 to create strain χ 10030.

Strain χ 10030(pCD1Ap) was highly attenuated when administered s.c. or i.n. (Table 2). Unlike our results with KIM6(pCD1Ap), χ 10015(pCD1Ap) and χ 10028(pCD1Ap), we observed only minimal swelling at the injection site of s.c. inoculated mice. The results indicate an increase in the LD₅₀ of χ 10030(pCD1Ap) by s.c. and i.n. inoculation of more than 1.5×10^7 and 3.4×10^4 -fold, respectively, compared to the wild-type virulent *Y. pestis* KIM6+(pCD1Ap).

We evaluated the ability of the χ 10030(pCD1Ap) to disseminate systemically compared with *Y. pestis* KIM6+(pCD1Ap) by monitoring the growth of both strains in the lungs, spleen and liver of infected mice over time after s.c. or i.n. inoculation. Because the LD₅₀s were different for each strain, we inoculated mice s.c. with different doses of each, 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.61×10^7 CFU of χ 10030(pCD1Ap). By day two, both wild-type KIM6+(pCD1Ap) and the mutant χ 10030(pCD1Ap) had reached titers of $1 \times 10^4 - 1 \times 10^5$ CFU in all organs (Fig. 3A). The numbers of KIM6+(pCD1Ap) steadily increased on days 4 and 6, while the numbers of χ 10030(pCD1Ap) decreased over that same time period, indicating that the bacteria were being cleared by the host immune system.

We performed a similar experiment after inoculating groups of mice intranasally. For this experiment, we used roughly the same dose of each strain, 1.67×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.93×10^4 CFU of χ 10030(pCD1Ap). Both strains reached levels of

about 1×10^5 CFU/g in the lungs by 12 h post-inoculation (Fig 3B). The wild-type strain increased in numbers over time, while the mutant was cleared. All mice inoculated with KIM6+(pCD1Ap) succumbed to the infection before 72 h, so there are no data for the 72 h time point. No bacteria were isolated from liver or spleen of mice inoculated with $\chi 10030$ at 12 h post-infection. Low numbers of bacteria were found in these tissues by 48 h (~ 10 CFU/g) and appeared to decrease by 72 h. These results indicate that, although highly attenuated for virulence, strain $\chi 10030$ (pCD1Ap) retains the ability to transiently colonize host tissues

3.3. Histopathology following infection

To examine the histopathological effects of each strain, groups of mice (3 per group) were inoculated either subcutaneously with 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.61×10^7 CFU of $\chi 10030$ (pCD1Ap) or intranasally with 1.67×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.93×10^4 CFU of $\chi 10030$ (pCD1Ap). Tissue samples were taken 2, 4 and 6 days following s.c. infection or 12, 24 and 48 h following i.n. infection. Uninfected mice inoculated to sterile PBS were used as controls. Lesion scores determined from histological evaluation of tissues obtained from these animals are presented in Table 3. Mice infected with *Y. pestis* KIM6+(pCD1Ap) developed severe lesions in organs at 6 days or 48 h after s.c. (Fig. 4A) or i.n. infection (Fig. 4B), respectively. Spleens harvested from mice s.c. inoculated with *Y. pestis* KIM6+(pCD1Ap) exhibited neutrophilic infiltrates with congestion and cellular loss, while spleens from mice infected $\chi 10030$ (pCD1Ap) appeared to be similar to those of uninfected mice (Fig. 4A). Lungs (Fig. 4B) harvested from mice infected *Y. pestis* KIM6+(pCD1Ap) had more significant diffuse vascular congestion in alveolar septa, with more polymorphonuclear leukocytes in the interstitium, and patchy areas of bronchopneumonia with microabscess formation and necrosis than that of $\chi 10030$ (pCD1Ap). Our observations for tissues derived from mice inoculated with KIM6+(pCD1Ap) were very similar to previously reported results for mice inoculated with *Y. pestis* CO92 [44,45].

Some mice infected by either route with KIM6+(pCD1Ap) succumbed to the infection before the final time point, while all $\chi 10030$ (pCD1Ap)-infected mice appeared healthy. We also noted that the color of lungs harvested from mice infected i.n. with KIM6+(pCD1Ap) at 48h post-infection were grayish white, while lungs harvested from mice infected with $\chi 10030$ (pCD1Ap) showed a normal pinkish appearance. The weight of lungs from mice infected KIM6+(pCD1Ap) was greater than that of control mice (Table 3).

Y. pestis KIM5 (Pgm⁻), a strain that is highly attenuated in mice when administered subcutaneously (Table 2) [31,46] but retains some virulence by i.n. administration [47], is similar to the EV76, a Pgm-deficient vaccine strain developed for human use nearly a century ago [21,48]. Mice immunized s.c. with a high dose ($\sim 10^7$ CFU) of KIM5 (Pgm⁻) or $\chi 10015$ (pCD1Ap) (Pgm⁻ $\Delta lpxP32::P_{lpxL} lpxL$) developed swelling and an open wound at the injection site, with severe inflammatory symptoms. Further, the histopathological analysis at 6 days showed that mice inoculated with *Y. pestis* KIM5 (Pgm⁻) and $\chi 10015$ (pCD1Ap) developed mild splenic lesions similar to mice inoculated with $\chi 10030$ (pCD1Ap), but when inoculated i.n., the mice developed more severe lung lesions than mice inoculated with $\chi 10030$ (pCD1Ap) (data not shown).

3.4. In vivo cytokine analysis

The production of pro- and anti-inflammatory cytokines by immunomodulatory cells is a crucial factor of the host response to bacterial infection. Therefore, we compared the levels of cytokines and chemokines in sera from mice infected with wild-type strain KIM6+(pCD1Ap) to mice infected with $\chi 10030$ (pCD1Ap) at various times.

In s.c.-infected mice, induction of pro-inflammatory cytokines IL-1 β , IL-6 and IFN- γ followed similar kinetics, although the level of each cytokine at day 6 in mice inoculated with χ 10030(pCD1Ap) was lower than in mice inoculated with KIM6+(pCD1Ap) (Fig. 5). Interestingly, TNF- α was induced on days 2 and 4 in mice inoculated with χ 10030(pCD1Ap), while no induction was seen in mice inoculated with KIM6+(pCD1Ap) until day 6 when TNF- α levels were similar for both groups of mice. This result is consistent with previous observations by Montminy et al, who observed that LPS obtained from wild-type *Y. pestis* grown at 37°C were deficient at inducing TNF- α in human peripheral blood mononuclear cells and peritoneal mouse macrophages compared LPS obtained from *Y. pestis* cells grown at 26°C, conditions that promote the synthesis of hexa-acyl lipid A [35]. They found that the deficiency in TNF- α induction could be restored when the LPS was isolated from a *Y. pestis* strain expressing LpxL. Peak levels of granulocyte colony-stimulating factor (G-CSF) occurred at day 2 in mice infected with either strain, but the levels of G-CSF were greater in mice inoculated with χ 10030(pCD1Ap) than in mice inoculated with KIM6+(pCD1Ap). G-CSF levels decreased rapidly in mice infected with χ 10030(pCD1Ap), probably a reflection of the fact that the strain was being cleared. The levels of anti-inflammatory cytokine IL-10 continued to rise in mice infected with KIM6+(pCD1Ap), while the levels of IL-10 peaked at day 2 in mice infected with χ 10030(pCD1Ap) and had decreased by day 6.

In intranasally infected mice, little or no pro-inflammatory cytokine induction was observed at 12 h (Fig. 6). In mice inoculated with χ 10030(pCD1Ap), some increase in TNF- α and IL-1 β levels were observed at 24 h. By 48 h, the levels of all pro-inflammatory cytokines had either dropped to very low levels or, in the case of IL-1 β , were similar to the 24 h levels. In mice inoculated with KIM6+(pCD1Ap), the levels of pro-inflammatory cytokines remained low until 48 h, when they rose dramatically. The anti-inflammatory cytokine IL-10 was induced by 12 hours in all mice and continued to rise at 24 h. Strain χ 10030(pCD1Ap) induced higher IL-10 levels than KIM6+(pCD1Ap). By 48 h, IL-10 levels had dropped in mice inoculated with the mutant strain and continued to rise in mice inoculated with KIM6+(pCD1Ap).

3.5. Protective efficacy and serum immune responses

To evaluate immunogenicity, groups of mice were immunized with a single dose of χ 10030(pCD1Ap) and challenged 35 days later. For these experiments, we wanted to use the highest possible immunizing dose for each strain. We based our choice of immunizing doses on the LD₅₀ for each strain. Therefore, we immunized with a dose of 1.4×10^7 CFU of χ 10030(pCD1Ap) by s.c. administration and a dose of 2.5×10^5 CFU of χ 10030(pCD1Ap) by i.n. administration. Mice were challenged 35 days after immunization. Our results after challenge show that a single dose of χ 10030(pCD1Ap) administered s.c. or i.n. provided complete or 70% protection against an s.c. challenge of 3.6×10^7 CFU of KIM6+(pCD1Ap), respectively (Fig. 7A). The level of protection against a 1.3×10^4 CFU i.n. challenge was slightly less, but still significant, with 80% and 50% protection in mice immunized s.c. or i.n., respectively (Fig. 7B). None of the mice immunized with PBS survived challenge by either route (Fig. 7A and B).

To evaluate the *Y. pestis*-specific immune responses, serum IgG responses to *Yersinia* whole cell lysates (YpL) from immunized mice were measured by ELISA. Two weeks after immunization, the reciprocal anti-*Y. pestis* serum IgG titers were >500 and increased to >1,000 by 4 weeks (2^{10}) (Fig. 6C). Antibody responses to *Y. pestis* KIM6(pCD1Ap), χ 10015(pCD1Ap) and χ 10028(pCD1Ap) showed a similar trend (Fig. S2C). Mice vaccinated with χ 10030(pCD1Ap) produced similar IgG titers as mice vaccinated with *Y. pestis* KIM6(pCD1Ap) and χ 10028(pCD1Ap), but were lower than in χ 10015(pCD1Ap)-immunized mice at 2 and 4 weeks post-vaccination (Fig 6C and Fig. S2C). The serum

immune responses to YpL were further examined by measuring the levels of IgG isotype subclasses IgG1 and IgG2a. At 2 and 4 weeks post-immunization, the levels of IgG1 and IgG2a was similar, indicating a mixed Th1/Th2 response was stimulated by immunizing with any of the three mutant strains (data not shown).

3.6. Bacterial burden in vaccinated mice

To investigate the effect of immunization on clearance of wild-type KIM6+(pCD1Ap), immunized and control mice were subcutaneously and intranasally challenged with 2.65×10^7 CFU and 1.2×10^5 CFU of virulent *Y. pestis* KIM6+(pCD1Ap), respectively.

Groups of 3 mice were euthanized at 2, 4 and 6 days post-challenge for s.c.-challenged mice and at 24, 48 and 72h post-challenge for i.n. challenged mice. Blood, lung, liver, and spleen were harvested for bacteria counting. For mice challenged s.c., the challenge organism was rapidly cleared from host tissues, with complete clearance from blood by day 6 (Fig. 8). All challenged control mice succumbed to the infection by day 2.

In mice challenged i.n., only low levels of organisms were detected in the blood. High numbers of organisms were detected in the lungs, consistent with the i.n. route of challenge (Fig. 9). The numbers steadily decreased over the time course studied. Numbers of *Y. pestis* in the spleen and liver were low at 24 h, but increased by 48 h and showed only a modest decline by 72 h, suggesting that the time course for clearance was longer than 72 h (Fig. 9).

4. Discussion

Previous work performed by the Lien and Smiley groups showed that *Y. pestis* expressing *lpxL* from a pBR322 plasmid derivative was attenuated and immunogenic by the s.c. [35] and i.n. [49] routes of administration. While these were important findings, from the point of view of vaccine development, expressing *lpxL* from a medium to high-copy number plasmid is not optimal due to potential stability issues, since loss of the plasmid would result in virulence. In this work, we constructed a strain that expressed *lpxL* from the chromosome in a manner that resulted in essentially all the lipid A in the cell to be hexa-acylated at 37°C (Fig. 2). To enhance safety and reduce reactogenicity, we also introduced an arabinose-regulated *crp* mutation into the strain, resulting in strain $\chi 10030(\text{pCD1Ap})$. We demonstrated that strain $\chi 10030(\text{pCD1Ap})$ (ΔP_{crp} TT *araC* P_{BAD} *crp* $\Delta lpxP::P_{\text{lpxL}}$ *lpxL*) synthesized hexa-acylated lipid A at 37°C and was highly attenuated in a mouse model when administered s.c. and i.n. (Fig. 2, Table 2). Strain $\chi 10030(\text{pCD1Ap})$ was able to colonize host tissues but was unable to establish a lethal infection when administered subcutaneously or intranasally at high doses. Previous work indicated immunization with live attenuated strains such as $\chi 10004(\text{pCD1Ap})$ (ΔrelA ΔspoT) [42], $\chi 10017(\text{pCD1Ap})$ (ΔP_{crp} TT *araC* P_{BAD} *crp*) [31], and *Y. pestis* KIM6(pCD1Ap), analogous to EV76 [48] and, in this work, $\chi 10015(\text{pCD1Ap})$ ($\Delta lpxP32::P_{\text{lpxL}}$ *lpxL*) can provide immunity against both bubonic and pneumonic plague. Szaba et al also reported that D27-pLpxL, an avirulent strain of *Y. pestis*, primes T cells that protect against pneumonic plague [49]. We constructed a similar strain, $\chi 10028(\text{pCD1Ap})$ (Δpgm $\Delta lpxP32::P_{\text{lpxL}}$ *lpxL*) and confirmed their results. However, $\chi 10028(\text{pCD1Ap})$ retained virulence by i.n. administration. In our hands, Swiss Webster mice immunized with a high dose of any of these strains ($\chi 10017(\text{pCD1Ap})$, $\chi 10015(\text{pCD1Ap})$, $\chi 10028(\text{pCD1Ap})$, or KIM6(pCD1Ap)) developed severe swelling and an open wound at the injection site and severe inflammatory symptoms. Mice inoculated with $\chi 10030(\text{pCD1Ap})$ developed only minor swelling and inflammation (Table 3 and unpublished observations), indicating that this combination of mutations is more suitable for use as a vaccine.

A previous study showed that tetra-acylated lipid A from *Y. pestis*, but not LpxL-mediated hexa-acylated lipid A, was defective in inducing TNF- α in human peripheral blood mononuclear cells (PBMCs) [35]. Our results were in agreement. The levels of TNF- α we observed in mice immunized with KIM6+(pCD1Ap) were significantly less than in mice immunized s.c. with χ 10030(pCD1Ap) in early infectious stages (Fig. 5D). In contrast, our results differed from the previous study with regard to IL-6 induction. In the previous study, human PBMCs were exposed to purified *Y. pestis* lipid A [35]. The tetra-acylated lipid A produced by wild-type *Y. pestis* did not induce IL-6 production, while hexa-acylated lipid A produced in a *Y. pestis* strain expressing *lpxL* did induce IL-6. In our study, mice immunized with either KIM6+(pCD1Ap) or χ 10030(pCD1Ap) produced similar levels of IL-6 (Fig. 5B) when inoculated s.c.. However, when administered i.n., the results were different (Fig. 6). In that case, mice immunized with KIM6+(pCD1Ap) produced more TNF- α and more IL-6, by 48 h, than mice immunized with χ 10030(pCD1Ap) (Fig. 6B and D). It is apparent from these results that in addition to lipid A, there may be other factors influencing in vivo cytokine levels. Our cytokine results from mice inoculated i.n. with KIM6+(pCD1Ap) are consistent with previous results [50,51] showing biphasic cytokine induction during disease progression in which “the infection begins with an anti-inflammatory state in the first 12 to 24 h, rapidly progressing to a highly pro-inflammatory state by 48 h and death by 3 days” [44]. While pro-inflammatory cytokines from mice intranasally infected with χ 10030(pCD1Ap) remained low at all time points tested.

Perhaps the most striking result was our observation that inoculation with χ 10030(pCD1Ap) induced high levels of IL-10, which is thought to suppress the immune response against *Y. pestis* [52]. IL-10 levels decreased in χ 10030(pCD1Ap)-inoculated mice by day 6 for the s.c. group, while levels continued to increase in mice inoculated with KIM6+(pCD1Ap) (Fig. 5C). The drop in IL-10 levels coincided with the clearance of χ 10030(pCD1Ap). The mechanism underlying this relationship is not clear. In the case of mice inoculated with KIM6+(pCD1Ap), IL-10 levels and CFU in tissues both increased over the same time period, leading to the death of the animal. Conversely, in the lungs of mice inoculated i.n. with wild-type *Y. pestis*, IL-10 induction is low [51], consistent with our result that the mutant strain χ 10030(pCD1Ap) induced higher IL-10 levels in serum than KIM6+(pCD1Ap) (Fig. 6C). The results also indicate that there is a mechanism for IL-10 induction remaining in χ 10030(pCD1Ap). Although the kinetics of IL-10 induction by χ 10030(pCD1Ap) and KIM6+(pCD1Ap) are clearly different (Figs 5C,6C), because of the observed link between IL-10 induction early in *Y. pestis* infection and its pathogenicity [53], the remaining mechanism of IL-10 induction by χ 10030(pCD1Ap) may not be ideal. This mechanism and its effect on vaccine efficacy will need to be investigated in future work.

Strains *Y. pestis* KIM6(pCD1Ap) and χ 10015(pCD1Ap) were more reactogenic than χ 10030(pCD1Ap) in mice inoculated s.c., but no differences in histopathology at sites distal to the site of injection were observed among the three strains (data not shown). When administered i.n., severe histopathological symptoms in the lungs were observed in mice infected with *Y. pestis* KIM6(pCD1Ap) and χ 10015(pCD1Ap), but only mild symptoms for χ 10030(pCD1Ap) (data not shown). In addition, although somewhat attenuated, strains *Y. pestis* KIM6(pCD1Ap) and χ 10015(pCD1Ap) retain substantial virulence when administered i.n. to mice (Table 2). Therefore, we conclude that χ 10030(pCD1Ap) is safer than *Y. pestis* KIM6(pCD1Ap) and χ 10015(pCD1Ap) regardless of infection route.

Mice immunized s.c. achieved a greater level of protection than mice immunized i.n. (Fig. 7A and 7B). One likely reason for this observation is that we used a lower immunizing dose for intranasal immunization than we did for subcutaneous immunization, which may have had an effect on the level of protective immunity achieved. This view is supported by the observed higher antibody titers in mice immunized s.c. compared to mice immunized i.n.

(Fig. 7). We also cannot rule out that this effect may also be due to differences in the route-dependent strategies used by *Y. pestis* for establishing infection. *Y. pestis* has evolved to be very efficient at establishing itself when introduced by the s.c. route and is less efficient when delivered by the intranasal route as evidenced by the 100-fold difference in LD₅₀s between routes (Table 1). This could result in the requirement for a higher effective dose when immunizing via the i.n. route to elicit protective immunity comparable to an effective s.c. dose.

Importantly, mice immunized with strain χ 10030(pCD1Ap) developed antibody responses and protective immunity comparable to or greater than mice immunized with the more reactogenic strains *Y. pestis* KIM6(pCD1Ap) and χ 10015(pCD1Ap) ($\Delta lpxP32::P_{lpxL} lpxL$) (Fig. S2).

Immunization with χ 10030(pCD1Ap) elicits anti-*Y. pestis* serum IgG (Fig. 7C). While the induction of pathogen-specific antibodies is important for combating *Y. pestis* infection [54-56], induction of cellular arm of the immune system is also important for robust immunity [57]. Induction of anti-*Y. pestis* cellular immunity by χ 10030(pCD1Ap) is supported by the observed increase in TNF- α in s.c.-immunized mice (Fig. 5D) and by the balanced Th1/Th2 response we observed as indicated by the IgG1/IgG2a ratios. Thus, χ 10030(pCD1Ap) appears to stimulate both humoral and cellular immunity. Taken together, our results indicate that immunization with χ 10030(pCD1Ap) elicits highly protective immunity against bubonic and pneumonic plague with minimal reactogenicity in mice, attributes consistent with our goal of designing a live safe *Y. pestis* vaccine. The safety and efficacy of this mutant strain may be further enhanced with additional defined attenuating mutations, including a *pgm* deletion and evaluated on different animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Susan Straley for providing anti-YopM antibodies and Dr. Praveen Alamuri for his valuable assistance in performing animal experiments.

Funding for research: This work was supported by National Institutes of Health grant 5R01 AI057885 to R. C. and by grant GM51310 to C. R. H. R. The mass spectrometry facility in the Department of Biochemistry of the Duke University Medical Center is supported by the LIPID MAPS Large Scale Collaborative Grant number GM-069338 from NIH.

Reference

- [1]. Perry RD, Fetherston JD. *Yersinia pestis*--etiologic agent of plague. Clin Microbiol Rev. Jan; 1997 10(1):35-66. [PubMed: 8993858]
- [2]. Prentice MB, Rahalison L. Plague. Lancet. Apr 7; 2007 369(9568):1196-207. [PubMed: 17416264]
- [3]. Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Working Group on Civilian Biodefense. Plague as a biological weapon: medical and public health management. JAMA. May 3; 2000 283(17):2281-90. [PubMed: 10807389]
- [4]. Smego RA, Frean J, Koornhof HJ, Yersiniosis I. microbiological and clinicoepidemiological aspects of plague and non-plague *Yersinia* infections. Eur J Clin Microbiol Infect Dis. Jan; 1999 18(1):1-15. [PubMed: 10192708]
- [5]. Titball RW, Hill J, Lawton DG, Brown KA. *Yersinia pestis* and plague. Biochem Soc Trans. Feb; 2003 31(Pt 1):104-7. [PubMed: 12546664]

- [6]. Zhou D, Han Y, Yang R. Molecular and physiological insights into plague transmission, virulence and etiology. *Microbes Infect.* Jan; 2006 8(1):273–84. [PubMed: 16182593]
- [7]. Dennis, DT.; Grantz, N.; Poland, JD.; Tikhomirov, E. Plague manual: epidemiology, distribution surveillance and control. WHO. , editor. Geneva: 1999. GK
- [8]. Anonymous. Human plague in 1998 and 1999. In: WHO. , editor. *Wkly Epidemiol Rec.* Vol. 75. 2000. p. 337–44.
- [9]. Guiyoule A, Rasoamanana B, Buchrieser C, Michel P, Chanteau S, Carniel E. Recent emergence of new variants of *Yersinia pestis* in Madagascar. *J Clin Microbiol.* Nov; 1997 35(11):2826–33. [PubMed: 9350742]
- [10]. Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, et al. Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia pestis*. *Emerg Infect Dis.* Jan-Feb; 2001 7(1):43–8. [PubMed: 11266293]
- [11]. Duplantier JM, Duchemin JB, Chanteau S, Carniel E. From the recent lessons of the Malagasy foci towards a global understanding of the factors involved in plague reemergence. *Vet Res.* May-Jun; 2005 36(3):437–53. [PubMed: 15845233]
- [12]. Anderson GW Jr, Leary SE, Williamson ED, Titball RW, Welkos SL, Worsham PL, et al. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. *Infect Immun.* Nov; 1996 64(11):4580–5. [PubMed: 8890210]
- [13]. Garmory HS, Freeman D, Brown KA, Titball RW. Protection against plague afforded by immunisation with DNA vaccines optimised for expression of the *Yersinia pestis* V antigen. *Vaccine.* Feb 25; 2004 22(8):947–57. [PubMed: 15161071]
- [14]. Heath DG, Anderson GW Jr, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, et al. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine.* Jul; 1998 16(11-12):1131–7. [PubMed: 9682370]
- [15]. Leary SE, Williamson ED, Griffin KF, Russell P, Eley SM, Titball RW. Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. *Infect Immun.* Aug; 1995 63(8):2854–8. [PubMed: 7622205]
- [16]. Russell P, Eley SM, Hibbs SE, Manchee RJ, Stagg AJ, Titball RW. A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model. *Vaccine.* Nov; 1995 13(16):1551–6. [PubMed: 8578841]
- [17]. Titball RW, Williamson ED. Vaccination against bubonic and pneumonic plague. *Vaccine.* Jul 20; 2001 19(30):4175–84. [PubMed: 11457543]
- [18]. Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, et al. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine.* Sep 3; 2004 22(25-26):3348–57. [PubMed: 15308359]
- [19]. Williamson ED, Eley SM, Griffin KF, Green M, Russell P, Leary SE, et al. A new improved sub-unit vaccine for plague: the basis of protection. *FEMS Immunol Med Microbiol.* Dec; 1995 12(3-4):223–30. [PubMed: 8745007]
- [20]. Titball RW, Williamson ED. *Yersinia pestis* (plague) vaccines. *Expert Opin Biol Ther.* Jun; 2004 4(6):965–73. [PubMed: 15174978]
- [21]. Girard G. Immunity in plague. Acquisitions supplied by 30 years of work on the “*Pasteurella pestis* EV” (Girard and Robic) strain. *Biol Med (Paris).* Nov-Dec. 1963 52:631–731. [PubMed: 14095162]
- [22]. Update: human plague--India, 1994. *MMWR Morb Mortal Wkly Rep.* Oct 7; 1994 43(39):722–3. [PubMed: 8090161]
- [23]. Hallett AF, Isaacson M, Meyer KF. Pathogenicity and immunogenic efficacy of a live attenuated plague vaccine in vervet monkeys. *Infect Immun.* Dec; 1973 8(6):876–81. [PubMed: 4206343]
- [24]. Meyer KF, Cavanaugh DC, Bartelloni PJ, Marshall JD Jr. Plague immunization. I. Past and present trends. *J Infect Dis.* May; 1974 129(Suppl):S13–8. [PubMed: 4596516]
- [25]. Meyer KF, Smith G, Foster L, Brookman M, Sung M. Live, attenuated *Yersinia pestis* vaccine: virulent in nonhuman primates, harmless to guinea pigs. *J Infect Dis.* May; 1974 129(Suppl):S85–120. [PubMed: 4207627]

- [26]. Batra HV, Agarwal GS. Isolation and identification of *Yersinia pestis* responsible for the recent plague outbreaks in India. *Curr Sci.* 1996; 71:787–91. TU.
- [27]. Meyer KF. Effectiveness of live or killed plague vaccines in man. *Bull World Health Organ.* 1970; 42:653–66. [PubMed: 4988692]
- [28]. Une T, Brubaker RR. Roles of V antigen in promoting virulence and immunity in yersiniae. *J Immunol.* Oct; 1984 133(4):2226–30. [PubMed: 6381597]
- [29]. Smiley ST. Current challenges in the development of vaccines for pneumonic plague. *Expert Rev Vaccines.* Mar; 2008 7(2):209–21. [PubMed: 18324890]
- [30]. Curtiss R III, Wanda SY, Gunn BM, Zhang X, Tinge SA, Ananthnarayan V, et al. *Salmonella enterica* serovar typhimurium strains with regulated delayed attenuation in vivo. *Infect Immun.* Mar; 2009 77(3):1071–82. [PubMed: 19103774]
- [31]. Sun W, Roland KL, Kuang X, Branger CG, Curtiss R III. *Yersinia pestis* with regulated delayed attenuation as a vaccine candidate to induce protective immunity against plague. *Infect Immun.* Mar; 2010 78(3):1304–13. [PubMed: 20086087]
- [32]. Six DA, Carty SM, Guan Z, Raetz CR. Purification and mutagenesis of LpxL, the lauroyltransferase of *Escherichia coli* lipid A biosynthesis. *Biochemistry.* Aug 19; 2008 47(33):8623–37. [PubMed: 18656959]
- [33]. Deng W, Burland V, Plunkett G 3rd, Boutin A, Mayhew GF, Liss P, et al. Genome sequence of *Yersinia pestis* KIM. *J Bacteriol.* Aug; 2002 184(16):4601–11. [PubMed: 12142430]
- [34]. Robinson RT, Khader SA, Locksley RM, Lien E, Smiley ST, Cooper AM. *Yersinia pestis* evades TLR4-dependent induction of IL-12(p40)2 by dendritic cells and subsequent cell migration. *J Immunol.* Oct 15; 2008 181(8):5560–7. [PubMed: 18832714]
- [35]. Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE, et al. Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. *Nat Immunol.* Oct; 2006 7(10):1066–73. [PubMed: 16980981]
- [36]. Carty SM, Sreekumar KR, Raetz CR. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction At 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J Biol Chem.* Apr 2; 1999 274(14):9677–85. [PubMed: 10092655]
- [37]. Straley SC, Bowmer WS. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. *Infect Immun.* Feb; 1986 51(2):445–54. [PubMed: 3002984]
- [38]. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol.* Sep; 1951 62(3):293–300. [PubMed: 14888646]
- [39]. Sun W, Wang S, Curtiss R III. Highly efficient method for introducing successive multiple scarless gene deletions and markerless gene insertions into the *Yersinia pestis* chromosome. *Appl Environ Microbiol.* Jul; 2008 74(13):4241–5. [PubMed: 18487404]
- [40]. Nichols FC. Distribution of 3-hydroxy iC17:0 in subgingival plaque and gingival tissue samples: relationship to adult periodontitis. *Infect Immun.* Sep; 1994 62(9):3753–60. [PubMed: 8063390]
- [41]. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Hyg.* 1938; 27:493–7.
- [42]. Sun W, Roland KL, Branger CG, Kuang X, Curtiss R III. The role of *relA* and *spoT* in *Yersinia pestis* KIM5+ pathogenicity. *PLoS One.* 2009; 4(8):e6720. [PubMed: 19701461]
- [43]. Feodorova VA, Pan'kina LN, Savostina EP, Kuznetsov OS, Konnov NP, Sayapina LV, et al. Pleiotropic effects of the *lpxM* mutation in *Yersinia pestis* resulting in modification of the biosynthesis of major immunoreactive antigens. *Vaccine.* Apr 6; 2009 27(16):2240–50. [PubMed: 19428838]
- [44]. Sha J, Agar SL, Baze WB, Olano JP, Fadl AA, Erova TE, et al. Braun lipoprotein (Lpp) contributes to virulence of yersiniae: potential role of Lpp in inducing bubonic and pneumonic plague. *Infect Immun.* Apr; 2008 76(4):1390–409. [PubMed: 18227160]
- [45]. Agar SL, Sha J, Foltz SM, Erova TE, Walberg KG, Parham TE, et al. Characterization of a mouse model of plague after aerosolization of *Yersinia pestis* CO92. *Microbiology.* Jul; 2008 154(Pt 7):1939–48. [PubMed: 18599822]
- [46]. Une T, Brubaker RR. In vivo comparison of avirulent Vwa- and Pgm- or Pstr phenotypes of yersiniae. *Infect Immun.* Mar; 1984 43(3):895–900. [PubMed: 6365786]

- [47]. Lee-Lewis H, Anderson DM. Absence of inflammation and pneumonia during infection with nonpigmented *Yersinia pestis* reveals a new role for the *pgm* locus in pathogenesis. *Infect Immun.* Jan; 2010 78(1):220–30. [PubMed: 19841077]
- [48]. Brubaker RR. The Vwa+ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca^{2+} *Rev Infect Dis.* Sep-Oct; 1983 5(Suppl 4):S748–58. [PubMed: 6195719]
- [49]. Szaba FM, Kummer LW, Wilhelm LB, Lin JS, Parent MA, Montminy-Paquette SW, et al. D27-pLpxL, an avirulent strain of *Yersinia pestis*, primes T cells that protect against pneumonic plague. *Infect Immun.* Oct; 2009 77(10):4295–304. [PubMed: 19620344]
- [50]. Cathelyn JS, Crosby SD, Lathem WW, Goldman WE, Miller VL. RovA, a global regulator of *Yersinia pestis*, specifically required for bubonic plague. *Proc Natl Acad Sci U S A.* Sep 5; 2006 103(36):13514–9. [PubMed: 16938880]
- [51]. Lathem WW, Crosby SD, Miller VL, Goldman WE. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc Natl Acad Sci U S A.* Dec 6; 2005 102(49):17786–91. [PubMed: 16306265]
- [52]. Nedialkov YA, Motin VL, Brubaker RR. Resistance to lipopolysaccharide mediated by the *Yersinia pestis* V antigen-polyhistidine fusion peptide: amplification of interleukin-10. *Infect Immun.* Apr; 1997 65(4):1196–203. [PubMed: 9119451]
- [53]. Brubaker RR. Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). *Infect Immun.* Jul; 2003 71(7):3673–81. [PubMed: 12819047]
- [54]. Hill J, Copse C, Leary S, Stagg AJ, Williamson ED, Titball RW. Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*. *Infect Immun.* Apr; 2003 71(4):2234–8. [PubMed: 12654847]
- [55]. Green M, Rogers D, Russell P, Stagg AJ, Bell DL, Eley SM, et al. The SCID/Beige mouse as a model to investigate protection against *Yersinia pestis*. *FEMS Immunol Med Microbiol.* Feb; 1999 23(2):107–13. [PubMed: 10076907]
- [56]. Williamson ED, Flick-Smith HC, Waters E, Miller J, Hodgson I, Le Butt CS, et al. Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates. *Microb Pathog.* Jan; 2007 42(1):11–21. [PubMed: 17107769]
- [57]. Smiley ST. Immune defense against pneumonic plague. *Immunol Rev.* Oct.2008 225:256–71. [PubMed: 18837787]
- [58]. Nakayama K, Curtiss R III. Construction of an Asd+ expression vector: stable maintenance and high expression of cloned genes in a Salmonella vaccine strain. *Bio/Technology.* 1988; 6:693–7. KSM.
- [59]. Gong S, Bearden SW, Geoffroy VA, Fetherston JD, Perry RD. Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. *Infect Immun.* May; 2001 69(5):2829–37. [PubMed: 11292695]
- [60]. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A.* Jun 6; 2000 97(12):6640–5. [PubMed: 10829079]

Fig. 1. Schematic diagram depicting the chromosomal structure of $\chi 10015$ ($\Delta lpxP::P_{lpxL} lpxL$) and $\chi 10030$ ($\Delta P_{crp} TT araC P_{BAD} crp \Delta lpxP::P_{lpxL} lpxL$).

Fig.2.

Mass spectrometry analysis of lipid A from *Y. pestis* KIM6 and χ 10030 grown at 26 °C or 37 °C. (A) At 26 °C, KIM6+ synthesizes at least three different species of lipid A: tetra-acyl lipid A with four C14:0 acyl groups (m/z 832) with 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety, a penta-acyl lipid A that has an additional secondary C12:0 acyl chain (m/z 924), and a hexa-acyl lipid A with additional C12:0 and C16:1 secondary acyl chains (m/z 1042). (B) When the bacteria is grown at 37°C, they produce predominantly tetra-acyl lipid A: tetra-acyl lipid A with four C14:0 acyl groups (m/z 701) without 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety, tetra-acyl lipid A with four C14:0 acyl groups (m/z 767) with a L-Ara4N moiety, tetra-acyl lipid A with four C14:0 acyl groups (m/z 823) with two L-Ara4N moiety and a penta-acyl lipid A that has an additional secondary C12:0 acyl chain (m/z 792), (C and D) Structural analysis was also carried out for the lipid A from χ 10030 expressing the acyltransferase LpxL from *E. coli*. (C) At 26°C the lipid A revealed the presence of a novel hybrid hexa-acyl species with two L-Ara4N moieties (m/z 1015), one L-Ara4N moiety (m/z 949) and without L-Ara4N moiety (m/z 884), which has a C12:0 acyl chain at the 2' secondary position rather than a C16:1 acyl chain. D) At 37 °C the mass spectra were similar to C, only variation of L-Ara4N moiety in the hexa-acyl species.

Fig. 3.

Kinetics of infection with *Y. pestis* KIM6+(pCD1Ap) (○) or χ 10030(pCD1Ap) (▲) in mouse tissues. Groups of nine mice were inoculated s.c. or i.n., and at various times CFU per organ in the lungs, spleens and livers were determined for 3 mice per group. (A) Bacteria were inoculated s.c. with 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap), 1.61×10^7 CFU of χ 10030(pCD1Ap) and at 2, 4 and 6 days CFU per organ in the lungs, spleens and livers were determined. (B) Bacteria were inoculated i.n. with 1.67×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) or 1.93×10^4 CFU of χ 10030(pCD1Ap) and at 12, 24, 48 and 72 hours CFU per organ in the lungs, spleens and livers were determined. Error bars represent standard deviation. All experiments were performed twice with similar results.

Fig. 4. Histopathology of the spleens and lungs of Swiss Webster mice inoculated s.c. with either KIM6+ (pCD1Ap) or χ 10030(pCD1Ap). (A) Histopathology of the spleens from Swiss Webster mice 6 days after s.c. inoculation with either 1.35×10^3 CFU of KIM6+(pCD1Ap) or 1.61×10^7 CFU of χ 10030(pCD1Ap). (B) Histopathology of the lungs from Swiss Webster mice 48 hours after i.n. infection with either 1.67×10^4 CFU of KIM6+(pCD1Ap) or 1.93×10^4 CFU of χ 10030(pCD1Ap). Spleens and lungs from uninfected mice served as negative controls. Tissues were fixed with 10% formalin, and 5- μ m sections were stained with hematoxylin/eosin. (Scale bars, 100 μ m). All experiments were performed twice with similar results.

Fig.5.

Serum cytokine levels in groups of mice infected s.c. with 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap) (○) or 1.61×10^7 CFU of χ 10030(pCD1Ap) (▲). Serum samples were collected from 3 mice in each group on days 2, 4 and 6 p.i. and these samples were pooled by group and day, for assay of cytokine content as described. (A) Concentration of IL-1 β in sera; (B) Concentration of IL-6 in sera; (C) Concentration of IL-10 in sera; (D) Concentration of TNF- α in sera; (E) Concentration of IFN- γ in sera; (F) Concentration of G-CSF in sera. Values were calculated as picograms cytokine per ml blood. All experiments were performed twice with similar results.

Fig.6.

Serum cytokine levels in mice infected i.n. with 1.67×10^4 CFU of *Y. pestis* KIM6+ (pCD1Ap) (○) or 1.93×10^4 CFU of $\chi 10030$ (pCD1Ap) (▲). Serum samples were collected from 3 mice in each group on hours 12, 24 and 48 p.i. and pooled by group and day, for assay of cytokine content as described. (A) Concentration of IL-1 β in sera; (B) Concentration of IL-6 in sera; (C) Concentration of IL-10 in sera; (D) Concentration of TNF- α in sera; (E) Concentration of INF- γ in sera; (F) Concentration of G-CSF in sera. Values were calculated as picograms cytokine per ml blood. All experiments were performed twice with similar results.

Fig.7.

Mouse survival after *Y. pestis* KIM6+(pCD1Ap) Challenge and serum IgG antibody responses in sera of mice vaccinated with χ 10030(pCD1Ap). All experiments were performed twice with similar outcomes and the results were pooled for presentation. (A) Swiss Webster mice vaccinated with a dose of 1.4×10^7 CFU of χ 10030(pCD1Ap) by s.c. or 2.5×10^5 CFU of χ 10030(pCD1Ap) by i.n. Mice were challenged 35 days later with 3.57×10^7 CFU of *Y. pestis* KIM6+(pCD1Ap) via the s.c. route, respectively. (B) Swiss Webster mice vaccinated with a dose of 1.4×10^7 CFU of χ 10030(pCD1Ap) by s.c. or 2.5×10^5 CFU of χ 10030(pCD1Ap) by i.n. Mice were challenged 35 days later via the i.n. route with 1.24×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap), respectively. Immunization provided significant protection against both challenge routes ($P < 0.001$). (C) A *Y. pestis* whole cell lysate was used as the coating antigen. Serum IgG responses from s.c. and i.n. vaccination were evaluated by comparing results from pre-immune sera to week 2 and week 4. *, the P value was less than 0.001. In each experiment there were 10 mice in the vaccinated group and 4 mice in the control group.

Fig. 8.

Bacteria recovered from immunized mice following *Y. pestis* KIM6+(pCD1Ap) challenge. Swiss Webster mice were subcutaneously immunized with $\chi 10030(pCD1Ap)$ or PBS on day 0. Mice were subcutaneously challenged with 2.65×10^7 CFU of *Y. pestis* KIM6+(pCD1Ap) 30 days later. Bacterial burdens in the blood, lung, liver, and spleen were determined on days 2, 4 and 6 after s.c. infection.

Fig. 9.

Bacteria recovered from immunized mice following *Y. pestis* KIM6+(pCD1Ap) challenge. Swiss Webster mice were subcutaneously immunized with $\chi 10030(pCD1Ap)$ or PBS on day 0. Mice were intranasally challenged with 1.2×10^5 CFU of *Y. pestis* KIM6+(pCD1Ap) 30 days later. Bacterial burdens in the blood, lung, liver, and spleen were determined on hours 12, 24 and 48 after i.n. infection.

Table 1

Bacterial strains and plasmids used in this study

Strain	Relevant genotype or Annotation	Source or derivation
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen
χ6212	Asd ⁻ DH5α derivative	[58]
<i>Y. pestis</i> KIM6+	Pgm ⁺ , Lcr ⁻ , pMT1, pPCP1	[59]
<i>Y. pestis</i>	Pgm ⁺ , pMT1, pPCP1, pCD1Ap KIM6+(pCD1Ap)	[59]
<i>Y. pestis</i> KIM6 (pCD1Ap)	Pgm ⁻ , pMT1, pPCP1, pCD1Ap	[48]
χ10017	ΔP _{crp21} ::TT <i>araC</i> P _{BAD} <i>crp</i> <i>Y. pestis</i> KIM6+	[31]
χ10015	Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> <i>Y. pestis</i> KIM6+	This study
χ10028	Pgm ⁻ Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> KIM5	This study
χ10030	Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> ΔP _{crp21} ::TT <i>araC</i> P _{BAD} <i>crp</i> <i>Y. pestis</i> KIM6+	This study
χ10015(pCD1Ap)	Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> with pCD1Ap	χ10015
χ10017(pCD1Ap)	ΔP _{crp21} ::TT <i>araC</i> P _{BAD} <i>crp</i> with pCD1Ap	[31]
χ10028(pCD1Ap)	Pgm ⁻ Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> with pCD1Ap	χ10028
χ10030(pCD1Ap)	Δ <i>lpxP32</i> ΩP _{lpxL} <i>lpxL</i> χP _{crp21} ::TT <i>araC</i> P _{BAD} <i>crp</i> with pCD1Ap	χ10030
Plasmid		Source
pKD46	λ Red recombinase plasmid	[60]
pYA4373	The <i>cat-sacB</i> cassette in the <i>Pst</i> I and <i>Sac</i> I sites of pUC18.	[39]
pYA4576	The <i>lpxP-U-lpxP-D</i> fragments ligated by overlapping PCR containing restriction endonuclease sites (<i>Pst</i> I and <i>Sac</i> I) near the center of the fragment were cloned into <i>Eco</i> RI and <i>Hind</i> III sites of pUC18.	pUC18
pYA4577	The P <i>lpxL</i> :: <i>lpxL</i> gene fragment cut from pYA4455 was cloned into the <i>Pst</i> I and <i>Sac</i> I sites of pYA4576	pYA4576
pYA4578	The <i>cat-sacB</i> cassette from pYA4373 was ligated into <i>Pst</i> I site of pYA4577.	pYA4577

Virulence of *Y. pestis* strains in Swiss-Webster mice

Table 2

Strains	LD ₅₀ of s.c. infection (CFU)	MTD for s.c. infection (days)	LD ₅₀ of i.n. infection (CFU)	MTD for i.n. infection (days)	Reference
<i>Y. pestis</i> KIM6 ⁺ (pCD1Ap)	<10	6.7	~100	3.1	This work
<i>Y. pestis</i> KIM6 (p g_{m}^{-}) (pCD1Ap)	>10 ⁷	ND	~10 ⁴	7.4	This work
χ 10004(pCD1Ap) (Δ relA Δ spoT)	5.8 $\times$ 10 ⁵	13.5	4.3 $\times$ 10 ³	5.3	[42]
χ 10017(pCD1Ap) (Δ P _{crp} TT <i>araC</i> P _{BAD} <i>crp</i>)	4.3 $\times$ 10 ⁵	17.2	~10 ⁵	12.3	[31]
χ 10015(pCD1Ap) (Δ lpxP::P _{lpxL} <i>lpxL</i>)	~10 ⁷	ND	2.7 $\times$ 10 ³	5.2	This work
χ 10028(pCD1Ap) (P g_{m}^{-} Δ lpxP::P _{lpxL} <i>lpxL</i>)	~10 ⁸	ND	5 $\times$ 10 ³	5.7	This work
χ 10030(pCD1Ap) (Δ P _{crp} TT <i>araC</i> P _{BAD} <i>crp</i> Δ lpxP::P _{lpxL} <i>lpxL</i>)	>10 ⁸	ND	> 3.4 $\times$ 10 ⁶	ND	This work

Note: mean time-to-death (MTD); ND, No detected.

Table 3
Histopathology of organs of Swiss Webster mice infected with *Y. pestis* KIM6+(pCD1Ap) or χ 10030(pCD1Ap)

Organ	Spleen / s.c. inoculation					Lung / i.n. inoculation				
	WT					χ 10030(pCD1Ap)				
	2 dpi	4 dpi	6 dpi	2 dpi	4 dpi	6 dpi	12 hpi	24 hpi	48 hpi	0.2
Score	++	+++	+++	-	+	+	+	+	+++	+
weight	0.23±0.06	0.32±0.08	0.33±0.06	0.13±0.06	0.2	0.23±0.06	0.2	0.33±0.06	0.37±0.08	0.18±0.03

Note: -, no lesion; +, slight lesion; ++, serious lesion; +++, very serious lesion; ND, no lesions detected. Spleens were examined from uninfected mice and mice infected subcutaneously with 1.35×10^3 CFU KIM6+(pCD1Ap) or 1.61×10^7 CFU χ 10030(pCD1Ap) at 2, 4, or 6 days. Lungs were examined from uninfected mice or mice infected with 1.67×10^4 CFU KIM6+(pCD1Ap) or 1.93×10^4 CFU χ 10030(pCD1Ap) at 12, 24, or 48 hours. No lesions were observed in tissues from PBS-inoculated controls.