Intramuscular Immunization of Mice with a Live-Attenuated Triple Mutant of *Yersinia pestis* CO92 Induces Robust Humoral and Cell-Mediated Immunity To Completely Protect Animals against Pneumonic Plague

Bethany L. Tiner, Jian Sha, Duraisamy Ponnesamy, Wallace B. Baze, Eric C. Fitts, Vsevolod L. Popov, Christina J. van Lier, Tatiana E. Erova, Ashok K. Chopra

Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA; Department of Veterinary Sciences, University of Texas MD Anderson Cancer Center, Bastrop, Texas, USA; Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; Center for Vaccine Development, University of Texas Medical Branch, Galveston, Texas, USA; Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas, USA

Earlier, we showed that the Δ*lpp ΔmsbB Δail* triple mutant of *Yersinia pestis* CO92 with deleted genes encoding Braun lipoprotein (Lpp), an acetyltransferase (MsbB), and the attachment invasion locus (Ail), respectively, was avirulent in a mouse model of pneumonic plague. In this study, we further evaluated the immunogenic potential of the Δ*lpp ΔmsbB Δail* triple mutant and its derivative by different routes of vaccination. Mice were immunized via the subcutaneous (s.c.) or the intramuscular (i.m.) route with two doses (2 × 10⁶ CFU/dose) of the above-mentioned triple mutant with 100% survivability of the animals. Upon subsequent pneumonic challenge with 70 to 92 50% lethal doses (LD₅₀) of wild-type (WT) strain CO92, all of the mice survived when immunization occurred by the i.m. route. Since Ail has virulence and immunogenic potential, a mutated version of Ail devoid of its virulence properties was created, and the genetically modified *ail* replaced the native *ail* gene on the chromosome of the Δ*lpp ΔmsbB* double mutant, creating a Δ*lpp ΔmsbB::ail*2 vaccine strain. This newly generated mutant was attenuated similarly to the Δ*lpp ΔmsbB Δail* triple mutant when administered by the i.m. route and provided 100% protection to animals against subsequent pneumonic challenge. Not only were the two above-mentioned mutants cleared rapidly from the initial i.m. site of injection in animals with no histopathological lesions, the immunized mice did not exhibit any disease symptoms during immunization or after subsequent exposure to WT CO92. These two mutants triggered balanced Th1- and Th2-based antibody responses and cell-mediated immunity. A substantial increase in interleukin-17 (IL-17) from the T cells of vaccinated mice, a cytokine of the Th17 cells, further augmented their vaccine potential. Thus, the Δ*lpp ΔmsbB Δail* and Δ*lpp ΔmsbB::ail*2 mutants represent excellent vaccine candidates for plague, with the latter mutant still retaining Ail immunogenicity but with a much diminished virulence potential.

*Yersinia pestis* is the causative agent of plague (1), and there has been a rise in the number of plague cases globally in recent years possibly due to climate changes and shifting of the rodent carrier range (2). The organism is classified as a tier 1 select agent (3–5), and the progression of septicemic and pneumonic forms of plague is very rapidly fatal after the first appearance of symptoms (4, 6–8). Alarmingly, antibiotic-resistant strains of *Y. pestis* have been isolated from plague patients and also have been engineered for bioweaponization (4). Therefore, vaccination is the optimal strategy for human protection against this deadly disease; however, there are currently no Food and Drug Administration (FDA)-licensed plague vaccines available in the United States (9–11).

Although a heat-killed plague vaccine composed of the *Y. pestis* 195/P strain was in use in the United States until 1999, the production of this vaccine was discontinued because of its effectiveness only against the bubonic plague and not the pneumonic form and also because it was highly reactogenic in humans (12, 13). Various live-attenuated *Y. pestis* EV76 vaccine strains, which lack the pigmentation locus (*pgm*) required for iron acquisition, provide protection against bubonic and pneumonic plague and are being used in some parts of the world where plague is endemic (9). However, these EV76-based vaccines are not genetically uniform and are also highly reactogenic (14); hence, they do not meet the standards for FDA approval. In addition, the Δ*pgm* mutants of *Y. pestis* (e.g., the KIM/D27 strain) may not be safe because of a reported case of fatal infection in an individual with hemochromatosis (15, 16).

In an effort to search for a new live-attenuated plague vaccine, we recently constructed a Δ*lpp ΔmsbB Δail* triple mutant, with deleted genes encoding Braun lipoprotein (Lpp), an acetyltransferase (MsbB), and the attachment invasion locus (Ail) (17). Lpp...
activates Toll-like receptor 2, which leads to the production of proinflammatory cytokines and septic shock (18–21). On the other hand, MsbB modifies lipopolysaccharide (LPS) by adding lauric acid to the lipid A moiety, resulting in the increased biological potency of LPS (22–27). Ail is an ~17-kDa outer membrane protein with four extracellular loops, and loop 2 (L2) has been reported to be mainly responsible for Ail-mediated bacterial serum resistance and adherence to/invasion of the host cells (17, 28–36).

In this study, to further characterize the vaccine potential of the Δlpp ΔmsbB Δail triple mutant, we evaluated its effectiveness when administered by the most common subcutaneous (s.c.) or the intramuscular (i.m.) route (37). Since Ail also has immunogenic potential in addition to its role as a virulence factor (38), we aimed at mutating the corresponding nucleotides in the ail gene that encode the essential amino acid residues required for virulence of L2 instead of deleting the whole ail gene from the Δlpp ΔmsbB strain of CO92 (36, 39). Indeed, the generated Δlpp ΔmsbB::ailL2 mutant was severely impaired in Ail-associated virulence traits, e.g., serum resistance, host cell adhesion, and invasion. Most importantly, immunization of mice with the Δlpp ΔmsbB Δail or the Δlpp ΔmsbB::ailL2 mutant via the i.m. or the s.c. route elicited robust humoral and cellular immune responses, which conferred up to 100% protection in animals at a high pneumonia challenge of 70 to 92 50% lethal doses (LD50) of wild-type (WT) CO92. Therefore, Δlpp ΔmsbB Δail and Δlpp ΔmsbB::ailL2 mutants represent excellent plague vaccine candidates. In addition, such vaccines can be effectively administered via different routes, providing flexibility during immunization.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Y. pestis and recombinant Escherichia coli strains were grown as described by us previously (17, 27, 40, 41). All of our studies were performed in a tier 1 select agent facility with the Galveston National Laboratory (GNL), University of Texas Medical Branch (UTMB). The molecular biological reagents were purchased from Promega (Madison, WI), Clontech (Palo Alto, CA), and Qiagen, Inc. (Valencia, CA). HeLa cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA).

Mutation of the ail gene. Four amino acid residues (lysine-88, aspartate-91, aspartate-95, and phenylalanine-94) in L2 of Ail were changed to alanine by using PCRs. Briefly, the primer pairs Aup5-mAup3 and mA5m-A5m (see Table S2 in the supplemental material) were used to introduce specific mutations within the L2 region of the ail gene as well as to amplify the mutated ail gene with its upstream and downstream DNA sequences, respectively. The mutated ail gene, designated ailL2, and its upstream and downstream flanking DNA sequences were joined together by PCR with the primer pair Aup5-A5m (see Table S2). The above-mentioned PCR product was subsequently cloned into the suicide vector pDM197 (42), which generated the recombinant plasmid pDM197-ailL2 (see Table S1 in the supplemental material).

Previously, we constructed an intermediate lpp, msbB, and ail triple gene deletion mutant (Δlpp ΔmsbB Δail) of Y. pestis CO92 (see Table S1 in the supplemental material) that carried a kanamycin resistance (Km)’ gene cassette (43) in place of the ail gene (17). Therefore, the recombinant suicide vector pDM197-ailL2 was electroporated into the Δlpp ΔmsbB Δail strain (Gene Pulser Xcell; Bio-Rad, Hercules, CA) (17). The transformants that were sensitive to kanamycin (Km)’ and resistant to 5% sucrose were picked up and screened by PCR with the primer pair Up5-Dn3 (see Table S2 in the supplemental material) (17) to ensure genomic replacement of the Km’ cassette with the ailL2 gene. Genomic DNA sequencing with the primer SqAil (see Table S2) (17) was used to further confirm replacement and to ensure no alteration in the ailL2 surrounding regions in the Δlpp ΔmsbB::ailL2 mutant compared to those of the native ail gene in the Δlpp ΔmsbB double mutant.

Production of AilL2 and Pla protease in the Δlpp ΔmsbB::ailL2 mutant of Y. pestis CO92. The Δlpp ΔmsbB, Δlpp ΔmsbB Δail, and Δlpp ΔmsbB::ailL2 mutants were grown overnight in heart infusion broth (HIB) at 28°C with shaking at 180 rpm, and the resulting bacterial cells (representing similar CFU) were dissolved in SDS-PAGE sample buffer. An aliquot of the samples was then resolved by SDS-PAGE, and the Western blots were analyzed with polyclonal antibodies to Ail and the plasminogen activator (Pla) (38). As a loading control, the presence of DnaK in the bacterial pellets was assessed by using anti-DnaK monoclonal antibodies (Enzo, Farmingdale, NY).

Serum resistance, adherence, and invasion of Y. pestis CO92 mutants. Various Y. pestis strains were grown overnight, harvested, and then diluted in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD600) of 0.2 (~1 × 10⁸ CFU/ml). A 50-µl volume of the diluted bacteria (~5 × 10⁶ CFU) was mixed with either 200 µl of undiluted normal (unheated) or heat-inactivated (56°C/30 min) human serum (Sigma-Aldrich, St. Louis, MO). After incubation at 37°C for 2 h, the number of surviving bacteria (CFU) in each sample was determined by serial dilutions and by plating on sheep blood agar (SBA) plates (17, 27). The percentage of bacterial survival was calculated by dividing the average CFU in the samples incubated in normal serum by the average CFU in the samples incubated in the heat-inactivated serum and multiplying by 100.

Human HeLa cervical epithelial cells (4 × 10⁵) were seeded in 12-well plates as described previously (17) and were infected with various mutants of Y. pestis CO92 at a multiplicity of infection (MOI) of 100. The plates were centrifuged at 1,200 rpm for 10 min to facilitate bacterial contact with the HeLa cells. After 2 h of incubation, the adherence and invasion of bacteria were evaluated as we recently described (17, 44).

Animal studies. Six- to eight-week-old female Swiss-Webster mice (17 to 20 g) were purchased from Taconic Laboratories (Germantown, NY). All of the animal studies were performed in the Animal Biosafety Level 3 (ABSL-3) facility under an approved Institutional Animal Care and Use Committee protocol.

(i) Immunization. Mice were immunized by the i.m. route with one or two doses of 2 × 10⁵ CFU/100 µl of the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB::ailL2 mutant. The mutants were administered in a 50-µl volume in each of the hind legs. When two doses of each vaccine strain were administered, they were injected 21 days apart (on days 0 and 21). Mice receiving only one dose were injected on the same day when the other animals received their second vaccine dose (on day 21). Another set of mice was immunized by the s.c. route with two doses of 2 × 10⁶ CFU/100 µl of the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB::ailL2 mutant strain. Subcutaneous doses were injected at one site 21 days apart (on days 0 and 21). Mice were assessed for morbidly and/or mortality over the duration of vaccination.

(ii) Antibody responses. Blood was collected by the retro-orbital route from vaccinated mice 2 weeks after each immunization (on days 14 and 35). Preimmunization blood samples served as a control. Serum samples were separated and filtered by using Costar 0.1-µm centrifuge tube filters (Corning Inc., Corning, NY). Enzyme-linked immunosorbent assay (ELISA) plates were coated with the F1-V fusion protein (1 ng/µl; BEI Resources, Manassas, VA) (17, 27, 41). Total IgG and antibody isotypes against F1-V (capsular antigen F1 and a type 3 secretion system [T3SS] component low-calcium response V antigen [LcrV]) in the serum samples (1:5 serially diluted) of animals immunized with the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB::ailL2 mutant were then determined as we previously described (45).

(iii) Challenge. Twenty-one days after the last immunization (day 42), the immunized mice were anesthetized with a mixture of xylazine-ketamine and then exposed by the intranasal (i.n.) route to 3.5 × 10⁵ to 4.6 × 10⁶ CFU/40 µl (70 to 92 LD50) of the bioluminescent WT Y. pestis CO92 luc2 strain (WT CO92 luc2), which contains the luciferase operon (lux or
luc) allowing in vivo imaging of mice for bacterial dissemination in real time (17, 46). Naïve mice of the same age were used as controls. On days 3 and 7 postinfection (p.i.), the animals were imaged by using an in vivo imaging system (IVIS) 200 bioluminescent and fluorescence whole-body imaging workstation (Caliper Corp., Alameda, CA) in the ABL-3 facility.

(iv) Histopathological analysis. Immunized mice (n = 2) that received two doses of the vaccine by either the i.m. or the s.c. route were euthanized 3 weeks after the second vaccine dose (on day 42). Similarly, three mice from each of the immunized groups that survived pneumonic challenge with 70 LD<sub>50</sub> of the WT CO92 luc2 strain were sacrificed on day 54 postchallenge. Age-matched naïve infected mice (n = 2) were also euthanized as a control. The lungs, livers, and spleens were harvested from these mice and fixed in 10% neutral buffered formalin (40, 47), and tissues were processed and sectioned at 5 μm. The samples were mounted on slides and stained with hematoxylin and eosin (H&E). Tissue lesions were scored on the basis of a severity scale, which correlated with estimates of lesion distribution and the extent of tissue involvement (minimal, 2% to 10%; mild, 10% to 20%; moderate, 20% to 50%; severe, >50%) as previously described (40, 47). The histopathological evaluation of the tissue sections was performed in a blinded fashion.

(v) Progression of infection. To monitor the progression of infection in real time, various bioluminescent strains of <i>Y. pestis</i> CO92 were constructed by using the Tn7-based system (40). The Tn7-based system integrates the target gene in a site-specific manner downstream of the conserved glmS (glucosamine-6-phosphate synthase) gene on the bacterial chromosome (48, 49). Briefly, the lux operon was removed from the plasmid pUC18-mini-Tn7T-lux-Gm by SpeI/Kpn1 restriction enzyme digestion and was subcloned into plasmid pUC18R6KT-mini-Tn7T (50), resulting in a derivative, designated pUC18R6KT-mini-Tn7T-lux (see Table S1 in the supplemental material).

Subsequently, the Km<sup>+</sup> cassette from the pUC4K plasmid (BamHI digestion) was inserted into pUC18R6KT-mini-Tn7T-lux, thus, resulting in the creation of the pUC18R6KT-mini-Tn7T-lux-Km plasmid (see Table S1 in the supplemental material). The electrocompetent cells of WT CO92 and the Δlpp ΔmsbB Δail triple mutant and Δlpp ΔmsbBΔailΔlpp mutant strains were electroporated with mixed (2 to 1) pTNS2 and pUC18R6KT-mini-Tn7T-lux-Km plasmids (40, 46, 50) and were selected for Km<sup>+</sup> and luminescence. The insertion of lux at the attT7 region was confirmed by PCR using primer pair F1–P2 (see Table S2 in the supplemental material), which specifically amplified the region between the <i>Y. pestis</i> glmS gene and the Tn7 insertion cassette (40). The luminescence intensity of each strain was determined by the relative luminescence unit (RLU) measurement (SpectraMax M5e; Molecular Devices, Sunnyvale, CA) (48). The luminescence intensity of each strain was measured at 100 μL of one of the above-generated bioluminescent strains, either the Δlpp ΔmsbB Δail-lux, Δlpp ΔmsbBΔailΔlpp-lux, or WT CO92-lux strain (see Table S1 in the supplemental material), by the i.m. route. The IVIS images were taken immediately after challenge and then every 12 h until 48 h p.i. After 48 h, mice were euthanized, and the muscles, lungs, liver, and spleen were removed immediately following animal sacrifice. The tissues were homogenized in 1 ml of PBS, and serial dilutions of the homogenates were spread on the SBA plates to assess the dissemination of the bacteria to peripheral organs (27). Portions of each organ from 3 mice at each time point were also removed for histopathological analysis (40, 47).

(vi) T-cell proliferative responses and cytokine production. Mice (n = 5) were infected by the i.m. or the s.c. route with WT <i>Y. pestis</i> KIM/D27 (pgm-locus-negative strain) (see Table S1 in the supplemental material), the Δlpp ΔmsbB Δail triple mutant, or the Δlpp ΔmsbBΔailΔlpp mutant strain of <i>Y. pestis</i> CO92 at a dose of 1 × 10<sup>6</sup> CFU/100 μL. The T-cell proliferation in response to heat-killed WT CO92 antigens (pulsed) was measured on day 21 p.i. as we previously described (27, 41). T cells from uninfected mice and unpulsed T cells served as negative controls. The T-cell culture supernatants were collected at 48 h to measure cytokine/chemokine production by using a mouse 6-plex assay kit (Bio-Rad Laboratories, Inc.). After 72 h of incubation, 1 μCi of [<sup>3</sup>H]thymidine was added into each well, and the cells were harvested 16 h later using a semi-automated sample harvester (FilterMate Harvester; PerkinElmer, Waltham, MA) followed by measurement of radioactive counts (TopCount NKT, PerkinElmer).

Statistical analysis. For a majority of the experiments, one-way analysis of variance (ANOVA) was used with the Tukey's post hoc test for data analysis except for the serum resistance assay, which was examined using the Student t test. We used Kaplan-Meier survival estimates for animal studies, and P values of ≤0.05 were considered significant for all of the statistical tests used. The standard deviations were derived from three independently performed experiments with three replicates per experiment for in vitro assays.

RESULTS

Evaluation of the protection provided by intramuscular immunization of mice with the Δlpp ΔmsbB Δail triple mutant in a pneumonic plague model. Mice were i.m. vaccinated with one or two doses of the Δlpp ΔmsbB Δail triple mutant at 2 × 10<sup>7</sup> CFU/dose. As shown in Fig. 1A, similar levels of total IgG antibody titers (1:15,625) to those of F1-V were noted when animals were vaccinated with either one or two doses. In addition, mice developed balanced Th1 and Th2 responses based on IgG1, IgG2a, and IgG2b antibody titers to F1-V (1:15,625) despite the number of vaccine doses administered (Fig. 1A).

No disease symptoms were observed in mice during the above immunizations. All of the mice immunized with two doses of the triple mutant survived the i.n. challenge with 92 LD<sub>50</sub> of the WT CO92 luc2 strain when administrated on day 21 after vaccination (Fig. 1B). While slightly lower, a still impressive level of protection (78%) was achieved when animals were vaccinated with only one dose of the triple mutant, and this protection was not statistically different than that of the group of animals receiving two doses of the vaccine (Fig. 1B). In contrast, all naïve mice succumbed to infection by day 4 p.i., and they exhibited clinical symptoms such as ruffled fur, hunched back, and lethargy and they were unable to groom and tended to huddle together.

The infected mice mentioned above were also imaged on day 3 p.i. to monitor the progression of infection. As shown in Fig. 1C, WT CO92 luc2 bacilli disseminated from the lungs to the whole body in 7 out of 8 naïve animals, and they all eventually succumbed to infection. On the contrary, of the animals receiving one immunization dose of the Δlpp ΔmsbB Δail triple mutant, only 2/9 mice were positive for bioluminescence on day 3 postchallenge (Fig. 1C). These two animals succumbed to infection, resulting in an overall 78% survival rate (Fig. 1B). It was also noted that the bioluminescent strain was confined to the initial infection site (lungs) in those two bioluminescence-positive animals compared to results for the naive but infected control animals (Fig. 1C).

In the immunized group of mice receiving two doses of the vaccine, none of the animals were positive for bioluminescence after pneumonic challenge (Fig. 1C), with 100% of the animals surviving (Fig. 1B). No clinical symptoms of the disease were apparent in mice receiving two doses of the vaccine and then subsequently challenged. Since none of the surviving animals became bioluminescent positive by day 7 (0/10), these data indicated a clearance of the WT CO92 luc2 strain by day 7 (Fig. 1C). To confirm, no bacilli were detected in the organs (lungs, liver, and the spleen) of the survivors on day 54 after WT CO92 luc2 challenge based on bacterial enumeration by plating (data not shown).

In vitro characterization of the Δlpp ΔmsbBΔailΔlpp mutant of <i>Y. pestis</i> CO92. The replacement of native ail with the ailΔlpp gene
in the Δpp ΔmsbB double mutant of Y. pestis CO92 was confirmed by PCR analysis. Further genomic DNA sequencing revealed no unexpected alterations in the ailL2 gene or in its adjacent regions on the chromosome compared to that of its parental strain (data not shown). In addition, we examined the expression of the ailL2 gene using Western blotting. As shown in Fig. 2A, Ail-specific antibodies detected the correct size protein with essentially similar intensities in all of the examined strains except for the Δpp ΔmsbB Δail triple mutant. Importantly, the expression levels of the pla gene were also similar across all strains examined, indicating that neither deletion of the native ail gene nor replacement of it with the ailL2 gene in the Δpp ΔmsbB double mutant affected production of the other tested bacterial membrane protein, i.e., Pla (Fig. 2A).

Due to the ability of Ail to impart serum resistance to Y. pestis (17, 28, 31–33), the WT CO92 and its Δpp ΔmsbB double, Δpp ΔmsbB Δail triple, and Δpp ΔmsbB::ailL2 mutants were evaluated for their ability to be killed by the complement cascade. Although all of the tested strains survived similarly in heat-inactivated serum samples after 2 h of incubation with CFU in the range of 1.6 to 2.0 × 10⁷/ml, less than 10% of the Δpp ΔmsbB Δail triple mutant isolates survived when exposed to the normal serum (Fig. 2B). On the other hand, WT CO92 and its Δpp ΔmsbB Δail double mutant strain exhibited slightly better or similar survival rates in the normal serum compared to those in the heat-inactivated serum. The survival rate of the Δpp ΔmsbB::ailL2 mutant strain was ~35% in the normal serum, and as expected, it did not reach the level of the Δpp ΔmsbB double mutant (Fig. 2B).

Since Ail also functions in the adherence and subsequent invasion of bacteria in the host cells, these virulence phenotypes of WT CO92 and their various mutants were examined in HeLa cells. The adherence (Fig. 2C, panel I) and invasive abilities (Fig. 2C, panel II) of the Δpp ΔmsbB Δail triple mutant were significantly decreased compared to those of the WT CO92 and its Δpp ΔmsbB double mutant. The Δpp ΔmsbB::ailL2 mutant behaved very similarly to the Δpp ΔmsbB Δail triple mutant in terms of its ability to adhere to and invade HeLa cells.

Evaluation of protection provided by intramuscular or subcutaneous immunization of mice with the Δpp ΔmsbB Δail triple mutant or the Δpp ΔmsbB::ailL2 mutant in a pneumonic plague model. Since our data (presented in Fig. 1B) indicated that two doses of immunization in mice provided optimal protection against WT CO92 challenge, we used the same vaccination regi-
men for the i.m. and s.c. routes of immunization and compared the protection conferred by the Δlpp ΔmsbB::ailL2 mutant with that of the Δlpp ΔmsbB ΔailI triple mutant. Irrespective of the route of immunization of either of the two mutant strains, 100% survivability of animals was noted with no clinical symptoms. All of the mice that were immunized intramuscularly were protected against a lethal pneumonic challenge at a dose of 3.5 × 10^8 CFU (70 LD50) of the WT CO92 luc2 strain (Fig. 3A), while 67% and 88% protection was achieved in mice subcutaneously immunized with the Δlpp ΔmsbB::ailL2 mutant or the Δlpp ΔmsbB ΔailI mutant, respectively (Fig. 3B). Although the level of protection provided by the s.c. route of vaccination did not reach the same level as noted for the i.m. route of immunization (Fig. 3A), the difference in protection afforded by the two mutants was statistically insignificant. All naive and unimmunized mice succumbed to infection by 4 days after challenge with WT CO92 luc2 (Fig. 3A and B).

The bioluminescence images further showed that the organism disseminated from the lungs to the whole body of all naive but infected control mice (10/10) by day 3 p.i. (Fig. 3C to I). Only one animal from the Δlpp ΔmsbB ΔailI i.m. immunized group was positive for bioluminescence, and the infection was confined to the throat region (Fig. 3C, panel II). However, the infection cleared from this animal by day 7 p.i. and, hence, was not fatal. In the s.c. immunized group of mice postchallenge, one animal from each of the mutant-immunized groups was positive for bioluminescence on day 3 p.i. (Fig. 3C, panel III), albeit only at the original infection site of the lungs; these two mice eventually succumbed to infection by day 4 to 5 p.i. (Fig. 3B). Surprisingly, 2 additional mice that were initially negative for bioluminescence on day 3 p.i. in the Δlpp ΔmsbB::ailI2 mutant s.c. immunized group also died by day 5 to 6 p.i. Upon necropsy, the death of these two mice was confirmed to be due to Y. pestis infection, suggesting that the level of bioluminescence in these animals was below the threshold of detection when imaged on day 3 (46). However, by day 7 p.i., none of the remaining mice, regardless of the mutant and route used for immunization, were positive for bioluminescence, and they were healthy throughout the experiment. Importantly, 54 days after WT CO92 luc2 challenge, organs (lungs, livers, and spleens) harvested from three randomly selected survivors of each group (immunized via either the i.m. or the s.c. route) were free of the bacilli as evaluated by plate counting (data not shown).

To gauge the immunogenicity of the vaccine strains via different routes of immunization, serum samples were collected from all mice 14 days after each immunization. We noted a boost in antibody titers between the first and the second doses when vaccination was performed via the s.c. route. However, this phenomenon was not observed via the i.m. route of immunization, as the

FIG 2 Ail-associated virulence activities in the Δlpp ΔmsbB::ailL2 mutant. (A) Overnight Y. pestis cultures grown at 28°C were collected and analyzed by immunoblotting using antibodies to Ail and Pla, respectively. Anti-DnaK antibodies were used as a loading control for the Western blots. (B) Various Y. pestis strains were incubated separately with the normal serum and the heat-inactivated human serum at 37°C for 2 h. The percentage of bacterial survival

in normal serum over the heat-inactivated serum was plotted. P values shown are based on one-way ANOVA. **, P < 0.005 compared to WT CO92 and the Δlpp ΔmsbB double mutant. *, Statistical significance (P < 0.05) between the two indicated groups. (C) HeLa cells were infected at an MOI of 100 with various Y. pestis strains. After 2 h of incubation, the host cells were gently washed and the adherent bacteria were collected, and the percentage of adhesion (I) was calculated. In another set of wells, the gentamicin protection assay was followed and the percentage of invasive bacteria (II) was calculated. P values shown are based on one-way ANOVA. **, P < 0.001 compared to both WT CO92 and the Δlpp ΔmsbB mutant. The arithmetic means ± standard deviations are plotted.
Immunity conferred by the ∆lpp ∆msbB ∆ail and the ∆lpp ∆msbB::ailL2 mutants to mice via intramuscular and subcutaneous routes of immunization. Mice (n = 8 to 10 per group) were immunized intramuscularly (A) or subcutaneously (B) with 2 doses of 2 × 10^6 CFU/100 µl of the ∆lpp ∆msbB ∆ail triple mutant or the ∆lpp ∆msbB::ailL2 mutant on days 0 and 21. Mice were challenged intranasally on day 42 with 3.5 × 10^4 CFU (70 LD50; 1 LD50 = 500 CFU) of the WT Y. pestis CO92 luc2 strain. Naive control, infected naive mice. The P values were in comparison to the naive control and were based on Kaplan-Meier curve analysis. (C) The infected mice (I, naive; II, i.m. immunized; and III, s.c. immunized) were imaged on days 3 and 7 postchallenge for bioluminescence, and the scale within the figure ranged from most intense (red) to least intense (violet).
peak antibody titers were achieved after only one vaccine dose (data not shown). The two above-mentioned mutants triggered higher levels of antibody responses (IgG titers of 1:46,875) to the F1-V antigen when vaccination occurred via the i.m. route compared to the s.c. route of immunization, which showed IgG titers to the naive serum. *** with horizontal lines, statistical significance by one-way ANOVA with the Bonferroni correction. (A) ***, P < 0.001 compared to that of the IgG1 of mice immunized subcutaneously with the Δlpp ΔmsbB: ailL2 mutant.

Histopathological analysis of mouse tissues after intramuscular immunization with the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB: ailL2 mutant and after exposure to WT Y. pestis CO92 in a pneumonia plague model. Prior to WT CO92 challenge and after two doses of vaccination, organs (muscles, lungs, livers, and spleens) were harvested from mice (n = 2) in each group for histopathological analyses. Muscles from mice immunized with the Δlpp ΔmsbB: ailL2 mutant were within the normal limits histopathologically and were similar to the muscles obtained from naive, unimmunized mice (Fig. 5). Muscles from mice immunized with the Δlpp ΔmsbB Δail triple mutant were also within the normal limits except for mild focal inflammation (Fig. 5). Irrespective of the two above-mentioned mutants used for immunization, the lungs, livers, and spleens of animals did not exhibit any abnormal histopathologies and were comparable to the organs of naive, unimmunized mice (Fig. 5).

As all i.m. immunized animals survived exposure to 70 LD₅₀ of the WT CO92 luc2 strain (Fig. 3A), organs (lungs, livers, and spleens) from 3 of these immunized mice were excised on day 54 p.i. to examine histopathological lesions and bacterial clearance. All of the naive mice succumbed to infection, and organs from three of them were harvested at time of death. In the lungs, all of the WT CO92-infected, unimmunized control mice had mild-to-moderate neutrophilic inflammation (see Fig. S1, arrow, in the supplemental material), present bacteria (arrowhead), and mild and diffused congestion. Also, the alveoli of these mice had a moderate level of hemorrhage with few alveolar spaces observed (see Fig. S1 in the supplemental material). All of the livers from WT CO92-infected, unimmunized mice had bacteria, some necrosis, and neutrophilic infiltration (see Fig. S1, arrow). All of the spleens of WT CO92-infected, unimmunized mice had bacteria (see Fig. S1, arrowhead), mild lymphoid depletion of the marginal zone in the white pulp (asterisk), and mild-to-marked diffuse rarefaction of normal cell population of the red pulp with fibrin present (see Fig. S1). On the contrary, all of the tissues from mice immunized with one of the two mutants (the Δlpp ΔmsbB Δail triple or the Δlpp ΔmsbB: ailL2) and then challenged with WT CO92 exhibited histopathologies within normal limits (see Fig. S1) and were similar to tissues from uninfected naive animals (Fig. 5).

When comparing histopathological changes, essentially similar data were obtained when immunization occurred via the s.c. route with either of the two mutants and after challenge of the immunized mice by the pneumonic route with WT CO92 (data not shown).

**Progression of infection and histopathological lesions in mice intramuscularly infected with the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB: ailL2 mutant of Y. pestis CO92.** Mice (n = 3) were either challenged with 2 × 10⁶ CFU of either the WT CO92-lux, the Δlpp ΔmsbB Δail-lux, or the Δlpp ΔmsbB:: ailL2-lux strain. Using IVIS, animals were imaged at 0, 12, 24, 36, and 48 h p.i. At 0 h, all mice were positive for bioluminescence that was localized to the injection site in the muscle (Fig. 6A, panel I). By 12 h p.i., all mice infected with the Δlpp ΔmsbB Δail-lux or the Δlpp ΔmsbB:: ailL2-lux strain were positive, but the intensity of bioluminescence in the muscle had decreased compared to 0 h (Fig. 6A, panel II). From 24 to 48 h p.i., none of the mice infected with the Δlpp ΔmsbB Δail-lux or the Δlpp ΔmsbB:: ailL2-lux strain were positive for bioluminescence (Fig. 6A, panels III to V).

However, mice infected with the WT CO92-lux strain had increased bioluminescence localized to the muscle at 12 h (Fig. 6A, panel II), and further dissemination of bacteria was observed in 1/3 of the mice at 24 h p.i. (Fig. 6A, panel III). This dissemination pattern became more prominent and was noted in the other two animals at 36 h p.i. (Fig. 6A, panel IV). At 48 h p.i., the level of bioluminescence was reduced in 1/3 of the mice (Fig. 6A, panels III to V). This is
attributed to the death of this animal, which causes bioluminescence to decrease due to diminished oxygen levels and temperature (46).

To further examine the bacterial load in mice, after in vivo imaging at 48 h p.i., all of the animals were sacrificed, and the organs (muscles, lungs, livers, and spleens) were harvested and subjected to bacterial count determination. As shown in Fig. 6B, mice infected with the WT CO92 had a high bacterial load in each of these organs (range from $8 \times 10^4$ to $8.7 \times 10^7$ CFU per organ or per gram of muscle) (Fig. 6B). The animal with a relatively lower bacterial load in various organs correspondingly exhibited weak bioluminescence at 36 and 48 h p.i. (Fig. 6A, panels IV and V), indicating bacterial counts to be below the threshold of bioluminescence detection for the WT CO92-lux strain during in vivo imaging.

All of the animals infected with the WT CO92-lux strain had severe plague symptoms and were on the verge of death. In contrast, mice infected with either the $\Delta lpp \Delta msbB \Delta ail-lux$ triple mutant or the $\Delta lpp \Delta msbB::ailL2$ mutant had minimal to no detectable bacterial load in any of the organs examined at 48 h p.i. (Fig. 6B). All of the tissues (muscle, liver, spleen, and lung) of WT CO92-infected animals had the presence of necrosis, hemorrhage, inflammation, edema, and bacteria (see Fig. S2 in the supplemental material). However, all the tissues from mice infected with the $\Delta lpp \Delta msbB \Delta ail-lux$ triple mutant or the $\Delta lpp \Delta msbB::ailL2-lux$ mutant were within normal limits histopathologically (see Fig. S2).

Activation of T cells by the $\Delta lpp \Delta msbB \Delta ail$ triple mutant and the $\Delta lpp \Delta msbB::ailL2$ mutant of Y. pestis CO92 after intramuscular or subcutaneous immunization of mice. To investigate T-cell responses, mice were i.m. or s.c. infected/immunized with a $1 \times 10^7$ CFU dose of either KIM/D27, the $\Delta lpp \Delta msbB \Delta ail$ triple mutant, or the $\Delta lpp \Delta msbB::ailL2$ mutant strain. Y. pestis KIM/D27 is a pgm-locus-negative mutant with characteristics similar to the live-attenuated Y. pestis EV76 vaccine strain (51), and, thus, it served as an appropriate control. No clinical symptoms were observed in mice immunized with either the $\Delta lpp \Delta msbB \Delta ail$ triple mutant or the $\Delta lpp \Delta msbB::ailL2$ mutant strain.

Although none of the KIM/D27-infected mice succumbed to infection at this low dose ($1 \times 10^7$ CFU), they all had ruffled fur and were lethargic up to 7 days postimmunization. On day 21 p.i., T cells isolated from these mice were restimulated with the heat-killed WT Y. pestis CO92 ex vivo, and T-cell proliferation (in terms of cpm) and cytokine production were evaluated.

As shown in Fig. 7, except for the naive group, all pulsed T cells (black bars) proliferated robustly compared to their corresponding unpulsed controls (gray bars). In addition, T cells from mice immunized by either the i.m. or the s.c. route significantly proliferated compared to T cells isolated from naive mice. These data indicated successful priming and restimulation during the experiment. Importantly, T cells isolated from mice immunized intramuscularly by either the $\Delta lpp \Delta msbB \Delta ail$ triple mutant or the $\Delta lpp \Delta msbB::ailL2$ mutant robustly proliferated at comparable levels, which were significantly higher than those of T cells isolated from the KIM/D27-immunized mice (Fig. 7A). During s.c. immunization, the T cells from the KIM/D27-infected mice proliferated similarly to the T cells isolated from the $\Delta lpp \Delta msbB \Delta ail$ triple mutant- or the $\Delta lpp \Delta msbB::ailL2$ mutant-infected mice (Fig. 7B).
Interestingly, the T-cell proliferation was significantly higher in mice immunized (by the s.c. route) with the \(H9004\) \(\Delta lpp\) \(\Delta msbB\) \(\Delta ail\) triple mutant than it was in animals vaccinated with the \(H9004\) \(\Delta lpp\) \(\Delta msbB::ailL2\) mutant strain (Fig. 7B).

Supernatants collected from the above-mentioned T cells were then assessed for cytokine production by using a Bio-Rad mouse 6-plex assay kit. Robust cytokine/chemokine production (i.e., gamma interferon \([IFN-\gamma]\), tumor necrosis factor alpha \([TNF-\alpha]\), interleukin-6 \([IL-6]\), IL-1\(\beta\), IL-10, and IL-17A) was observed in T cells obtained from mice immunized intramuscularly across all of the above-mentioned \(Y. pestis\) mutant strains tested in response to restimulation with the heat-killed \(Y. pestis\) CO92 (Fig. 8). A similar trend was noted for the production of IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-10 in T cells isolated from mice vaccinated subcutaneously with the above-mentioned mutant strains, except for levels of IL-17A production, which were similar in pulsed and unpulsed T cells (data not shown). Overall, cytokines were either very low or undetectable from T cells isolated from unimmunized, naive mice (data not shown).

**DISCUSSION**

The resurgence of plague in many parts of the world, the existence of natural or intentionally generated antibiotic-resistant strains, and the lack of a current FDA-approved plague vaccine in the United States necessitate the development of an effective vaccine.

Currently, the most promising clinical trials are testing recombinant subunit vaccines consisting of F1 and LcrV antigens. These F1-V-based vaccines are efficacious against pneumonic plague in rodents and macaques (52–56); however, protection was variable in African green monkeys (9, 12, 55, 57, 58). Further, F1 capsular antigen is dispensable for virulence (59, 60) and the LcrV amino acid sequence has diverged among \(Y. pestis\) strains (1, 61). Therefore, the F1-V-based subunit vaccines most likely will not provide optimal protection across all plague-causing strains in humans, specifically those that have been intentionally modified for possible use in terrorist attacks (62, 63).

The live-attenuated vaccines that promote humoral and cell-mediated immune responses may represent a better option for overcoming the above-mentioned shortcomings of the subunit vaccines (9, 12). Recently, our laboratory and others reported the development of mutant strains of \(Y. pestis\) that have shown vaccine potential (17, 27, 41). For example, a single dose of our \(\Delta lpp\) \(\Delta msbB\) \(\Delta ail\) triple mutant conferred dose-dependent protection in mice against developing subsequent pneumonic plague when immunization occurred by the intranasal route (17). Our data showed that up to a 3.4 \(\times 10^6\) CFU dose of this vaccine strain was unable to kill mice, and the animals developed balanced Th1 and Th2 antibody responses, which provided subsequent protection (70%) to mice when challenged with 28 LD50 of WT CO92 (17). Although the experiments were not performed in parallel, our intramuscular immunization data with a single dose of this mutant (Fig. 1) provided comparable protection (78%) in mice but...
under a much more stringent challenge dose of WT CO92 (92 \(LD_{50}\)). These data suggested that vaccination by the i.m. route might be superior to the i.n. route of immunization with this triple mutant in a mouse model.

It is generally believed that intranasal immunization has an advantage, as it results in the development of mucosal immunity and the systemic immune response. However, during mucosal immunization, the vaccine must be able to penetrate the epithelial barrier and to survive luminal host innate defenses. On the contrary, intramuscular immunization enables the vaccine to easily access blood vessels to reach blood circulation to directly stimulate the immune system (64). However, irrespective of the vaccination route, the \(\Delta lpp \Delta msbB \Delta ail\) triple mutant (at doses of 2 \(\times 10^6\) to 3.4 \(\times 10^6\) CFU) was unable to confer 100% protection to immunized mice in a single dose against developing subsequent pneumonic plague (17) (Fig. 1). Since Ail also has immunogenic potential in addition to its role as a virulence factor (38), we generated the \(\Delta lpp \Delta msbB::ail\) mutant strain with the intention of further improving its immunogenicity.

The Ail protein has eight transmembrane domains with four extracellular loops and eight amino acid residues in loops 2 and 3 of \textit{Yersinia enterocolitica}, which are responsible for imparting serum resistance and the microbe’s ability to adhere to/infiltrate host cells (39). Likewise, 3 amino acid residues within loop 1 have been predicted to play an important role in bacterial adherence (36). Loop 2 of Ail in \textit{Y. enterocolitica} and that in \textit{Y. pestis} share 70% homology, and mutations in 4 amino acid residues resulted in drastically altering the ability of the \(\Delta lpp \Delta msbB::ail\) mutant to...
adhere to, invade, and/or exhibit serum resistance compared to that of WT CO92 and other tested mutants (Fig. 2).

A slight increase in the serum resistance of the Δlpp ΔmsbB::ailL2 mutant compared to that of the Δlpp ΔmsbB Δail triple mutant was possibly related to the contribution of other Ail loops in serum resistance that were intact in the Δlpp ΔmsbB::ailL2 mutant. Indeed loops 1 and 3 of Ail in Y. pestis are important for adherence and invasion, with up to 50% reduction in the above-mentioned phenotypes when these two loops were mutated (65). However, similar levels of adherence and invasion of the Δlpp ΔmsbB::ailL2 and the Δlpp ΔmsbB Δail triple mutants in HeLa cells signified the important role of loop 2 in Ail-associated virulence phenotypes and also suggested that a conformational association between various loops of Ail might be necessary for efficient bacterial adherence to and invasion of the host cells.

Comparable levels of AilL2 production in the Δlpp ΔmsbB::ailL2 mutant and its parental Δlpp ΔmsbB strain (Fig. 2A) and diminished virulence of Ail in the Δlpp ΔmsbB::ailL2 mutant (Fig. 2B and C) indicated successful creation of a potentially better vaccine candidate than the Δlpp ΔmsbB Δail triple mutant. In addition, a partial restoration of serum resistance in this Δlpp ΔmsbB::ailL2 mutant (Fig. 2B) might lead to a better recognition by the host immune system. However, our data indicated that the Δlpp ΔmsbB Δail triple mutant and the Δlpp ΔmsbB::ailL2 mutant triggered similar humoral and cell-mediated immune responses in mice immunized via the i.m. route (Fig. 4 and 7A) and provided 100% protection to vaccinated animals when exposed to a high challenge dose of WT CO92 in a pneumonic plague model (Fig. 3).

A biased Th2 antibody response was observed in mice when vaccinated with the Δlpp ΔmsbB::ailL2 mutant by the s.c. route, which provided a slightly lower protection rate in immunized mice (67%) during subsequent pneumonic challenge. In comparison, an 88% protection rate with a balanced Th1 and Th2 antibody response and a higher T-cell proliferation were noticed in mice when they were vaccinated by the s.c. route with the Δlpp ΔmsbB Δail triple mutant (Fig. 3B, 4B, and 7B).

The reason for this phenomenon is not clear; however, additional animal models, such as rat or nonhuman primate models (NHP), may be needed to fully evaluate the immunogenic potential of the Δlpp ΔmsbB::ailL2 mutant. Indeed, studies have shown that Ail plays an even more important role in the pathogenesis of Y. pestis infection in a rat model of pneumonic plague compared to the mouse model (28, 31–33). In addition, a correlation between distinct IgG antibody subclasses and the Th1/Th2 profile seen in mice may differ in humans (64).

Of the two vaccination routes examined and based on the protection rates and the antibody and T-cell responses generated by the Δlpp ΔmsbB Δail triple mutant and the Δlpp ΔmsbB::ailL2 mutant in the mouse model, the i.m. route was certainly optimal compared to the s.c. route (Fig. 3, 4, 7, and 8). Our observation that a robust IL-17A recall response was only observed in T cells from mice that were immunized intramuscularly but not subcutaneously with the above-mentioned mutants was important.
(Fig. 8). A study has shown that injection of a vaccine into the layer of subcutaneous fat, where vascularization is poor, may result in slow mobilization and processing of the antigen (66). Compared to intramuscular administration, subcutaneous injection of hepatitis B vaccine leads to significantly lower seroconversion rates compared to intramuscular administration, subcutaneous injection of hepatitis B vaccine leads to significantly lower seroconversion rates and more rapid decay of antibody response (66, 67). A similar phenomenon was reported with the rabies and influenza vaccines (68).

Recently, it was reported that intradermal inoculation of Y. pestis in C57BL/6J mice resulted in faster kinetics of infection compared to that of the subcutaneous route of inoculation due to the organisms’ greater ability to access the vascular and lymphatic vessels in the dermis (69). Studies have shown that the dermis of the skin is enriched in terminal lymphatic vessels, which facilitate antigen uptake and the infiltration of immune cells to mount a stronger immune response compared to that of the subcutaneous layer (70–72). Therefore, future studies examining the intradermal route of immunization with our mutants will be undertaken.

IL-17A is a signature cytokine of Th17 cells that has been recently shown to provide an antibody-independent heterogeneous protection and also has been implicated in protecting the host against many pathogenic bacterial infections, including Y. pestis (73, 74). Interestingly, production of IL-17A from Th17 cells was also observed in our previous study with the Δlpp ΔmsbB Δail double mutant of WT CO92 when mice were intranasally immunized (27). Similarly, IL-17A was induced by the intranasal immunization of mice with the Y. pestis strain D27-pLpxL KIM/D27 that was engineered to express E. coli LpxL (75), which contributed significantly to the cell-mediated defense against pulmonary Y. pestis infection (74). Therefore, the induction of the Th17 response in addition to the Th1 and Th2 responses provided by the Δlpp ΔmsbB Δail triple mutant and the Δlpp ΔmsbB:ailL2 mutant might be beneficial in live-attenuated plague vaccines and need to be further studied.

The vaccine dose we used for each of our mutants (2 × 10⁶ CFU/dose) was considerably lower than the 8 × 10⁹ CFU/dose of the live-attenuated EV76 vaccine strain given to humans by the i.m. route in some countries and the 1 × 10⁷ CFU/dose that has been used in murine studies (76–78). In addition, up to a 3 × 10⁹ CFU/dose of EV76 has been used to immunize humans by the cutaneous route (79). The EV76 vaccine strain causes severe local and systemic reactions in animals and humans (80–83), and, more importantly, deaths have been reported in NHPs (82). In addition, a similar pgm-negative strain of Y. pestis retains virulence in mice and NHPs when administered by the intranasal (i.n.) and intravenous (i.v.) routes (57, 82, 84), raising serious questions about its suitability as a human vaccine (85). Indeed, a fatal laboratory-acquired infection with the pgm-negative KIM/D27 strain in an individual with hemochromatosis was reported recently (15), and mice infected intramuscularly with 10⁵ CFU of KIM/D27 in our study also showed ruffled fur and lethargy for up to 7 days postinfection. However, mice immunized with up to 2 to 3.4 × 10⁶ of either the Δlpp ΔmsbB Δail triple mutant or Δlpp ΔmsbB:ailL2 mutant via various immunization routes (i.n., i.m., and s.c.) did not display any local or systemic reactions or any adverse histopathological lesions (Fig. 5; see also Fig. S2 in the supplemental material) (17).

In summary, both of our Δlpp ΔmsbB Δail triple mutant and Δlpp ΔmsbB:ailL2 mutant have rationally designed in-frame deletions and, therefore, trigger a minimal inflammatory response. Most importantly, T cells isolated from mice immunized with either the Δlpp ΔmsbB Δail triple mutant or the Δlpp ΔmsbB:ailL2 mutant via the i.m. route displayed stronger proliferative responses than those of the KIM/D27-vaccinated mice (Fig. 7A). Therefore, the above-mentioned mutants might represent better plague vaccine candidates than the pgm-negative mutants for further development and testing in higher animal models.

ACKNOWLEDGMENTS

This research was supported by NIH/NIAID grants AI064389 and NO1-AI-30065 awarded to A. K. Chopra. We also acknowledge a UC7 grant (AI070083), which facilitated our research in the Galveston National Laboratory, UTMB, Galveston, TX. B. L. Tiner was supported through a predoctoral fellowship from the Sealy Center for Vaccine Development, Galveston, TX, and the McLaughlin Endowment Scholar Program, Galveston, TX.

We thank members of the Electron Microscopy Core Laboratory for their assistance in performing electron microscopy studies and members of W. B. Baze’s Histopathology Laboratory for rendering their help in histopathological examination of tissue specimens.

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