Deletion of Braun lipoprotein gene (*lpp*) and curing of plasmid pPCP1 dramatically alter the virulence of *Yersinia pestis* CO92 in a mouse model of pneumonic plague

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Deletion of the murein (Braun) lipoprotein gene, *lpp*, attenuates the *Yersinia pestis* CO92 strain in mouse models of bubonic and pneumonic plague. In this report, we characterized the virulence of strains from which the plasminogen activating protease (*pla*)-encoding pPCP1 plasmid was cured from either the wild-type (WT) or the Δlpp mutant strain of *Y. pestis* CO92 in the mouse model of pneumonic infection. We noted a significantly increased survival rate in mice infected with the *Y. pestis* pPCP[−]/ Δlpp mutant strain up to a dose of 5000 LD₅₀. Additionally, mice challenged with the pPCP[−]/ Δlpp strain had substantially less tissue injury and a strong decrease in the levels of most cytokines and chemokines in tissue homogenates and sera when compared with the WT-infected group. Importantly, the *Y. pestis* pPCP[−]/ Δlpp mutant strain was detectable in high numbers in the livers and spleens of some of the infected mice. In the lungs of pPCP[−]/ Δlpp mutant-challenged animals, however, bacterial numbers dropped at 48 h after infection when compared with tissue homogenates from 1 h post-infection. Similarly, we noted that this mutant was unable to survive within murine macrophages in an *in vitro* assay, whereas survivability of the pPCP[−] mutant within the macrophage environment was similar to that of the WT. Taken together, our data indicated that a significant and possibly synergistic attenuation in bacterial virulence occurred in a mouse model of pneumonic plague when both the *lpp* gene and the virulence plasmid pPCP1 encoding the *pla* gene were deleted from *Y. pestis*.

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INTRODUCTION

Yersinia pestis is one of three species of yersiniae that cause serious infections in humans, and the disease it manifests,

Abbreviations: H&E, haematoxylin and eosin; i.n., intranasal; p.i., post-infection; WT, wild-type; Yops, *Yersinia* outer-membrane proteins.

Three supplementary figures and six supplementary tables are available with the online version of this paper. The three supplementary figures show histopathology of mouse spleen and hearts following infection with wild-type, and various mutant strains of *Y. pestis* CO92 and type 3 secretion system-associated cytotoxicity in HeLa cells infected with wild-type and mutant *Y. pestis* CO92 strains. The six supplementary tables list cytokines and chemokines analysed in this study, and cytokine and chemokine levels in the lung, liver, spleen and heart homogenates and sera of mice 48 h p.i. with wild-type and mutant *Y. pestis* CO92 strains.

plague, has historically killed populations to disastrous proportions (Perry & Fetherston, 1997). All three human pathogenic species of *Yersinia* (*Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) share the presence of a 70 kb virulence plasmid, pCD1, that harbours genes encoding the type 3 secretion system (T3SS) and its effectors, referred to as *Yersinia* outer-membrane proteins (Yops). Yops are delivered into the cytoplasm following contact of the bacterium with a host cell and are required to cause disease in animals and humans (Perry & Fetherston, 1997). *Y. pestis* has two additional plasmids encoding virulence factors for the organism. Plasmid pMT1 has genes encoding the antiphagocytic capsular antigen F1 (Du *et al.*, 2002) and murine toxin, the latter of which is important in establishing *Y. pestis* colonization in the midguts of fleas (Hinnebusch *et al.*, 1998). In all, at

least 115 genes are present on the 110 kb pMT1 plasmid in the KIM5 strain of *Y. pestis* (Lindler *et al.*, 1998).

The smallest of the three virulence plasmids, pPCP1, is 9.5 kb in size and contains three genes encoding proteins with distinctly different functions. Pesticin, encoded by the *pst* gene, causes cell wall lysis and the death of neighbouring bacteria by hydrolysing the bond between *N*-acetylglucosamine and *N*-acetylmuramic acid, thus degrading the glycan backbone of murein (Braun) lipoprotein (Ferber & Brubaker, 1979). It has been suggested that *Y. pestis* uses this bacteriocin to kill neighbouring cells in which the pPCP1 plasmid is absent, while at the same time protecting itself with pesticin immunity protein, encoded by the *pim* gene on the pPCP1 plasmid (Sodeinde *et al.*, 1992).

Also present on the pPCP1 plasmid is plasminogen activating protease (Pla), a surface protein consisting of two subunits, α - and β -, with molecular sizes of 37 and 35 kDa, respectively (Kutyrev *et al.*, 1999; Sodeinde & Goguen, 1988). Pla is a member of the omptin family of aspartate proteases expressed on the surface of many members of the family *Enterobacteriaceae* (McDonough & Falkow, 1989; Sodeinde & Goguen, 1989). The function of Pla has been well characterized, and its role in the invasiveness and dissemination of *Y. pestis* extensively studied *in vitro* and *in vivo* (Beesley *et al.*, 1967; Brubaker *et al.*, 1965; Lathem *et al.*, 2007; Sebbane *et al.*, 2006). Pla subverts the host innate immune response by degrading complement proteins, thus reducing the ability of phagocytes to opsonize and phagocytize *Y. pestis*, and by preventing the chemotaxis of other inflammatory cells to the site of infection (Sodeinde *et al.*, 1992). Pla also proteolytically activates plasminogen and anchors it to the bacterial surface (Beesley *et al.*, 1967). Human plasminogen activators convert plasminogen into plasmin, which *Y. pestis* uses to gain access to deeper tissue layers and to colonize and disseminate through the host (Sodeinde *et al.*, 1992).

Since Pla plays an important role in human infections and avoidance of the immune system, significant research has been performed to better understand the role of both the *pla* gene and the pPCP1 plasmid in the pathogenesis of *Y. pestis* infections. For example, the loss of the pPCP1 plasmid or the *pla* gene attenuates *Y. pestis* administered by the subcutaneous route (Brubaker *et al.*, 1965; Samoilova *et al.*, 1996; Sodeinde *et al.*, 1992; Welkos *et al.*, 2002, 1997) in mice or guinea pigs, and by the intradermal (Sebbane *et al.*, 2006) and intranasal (i.n.) (Lathem *et al.*, 2007) routes in mice, although the bacterial dissemination that occurred in intranasally infected mice was not noted in subcutaneously infected animals. No change in the LD₅₀ was observed in mice inoculated with the wild-type (WT) versus the *pla*-minus mutant by either the intraperitoneal (Welkos *et al.*, 2002, 1997) or the intravenous (Sodeinde *et al.*, 1992) route of infection. Reports on the virulence of strains lacking this plasmid and delivered via the aerosol route of infection are not consistent and are possibly

dependent upon the strains of *Y. pestis* used in the studies (Samoilova *et al.*, 1996; Welkos *et al.*, 2002, 1997). Other studies have indicated that *pla* mutant strains evoke an earlier inflammatory response than the WT, and animals are able to clear the infection sooner, resulting in a higher survival rate in mutant versus WT bacteria-infected mice (Lathem *et al.*, 2007; Sodeinde *et al.*, 1992; Welkos *et al.*, 1997).

We recently published a detailed characterization of how the absence of Braun lipoprotein (Lpp) attenuates *Y. pestis* CO92 in the i.n. mouse model of infection and how bacterial dissemination, host tissue damage and cytokine/chemokine production are decreased in mice infected with the Δ *lpp* mutant of *Y. pestis* compared with those infected with the WT bacterium (Sha *et al.*, 2008). In the light of the other studies describing the attenuation of pPCP⁻ and Δ *pla* mutants of *Y. pestis* (Brubaker *et al.*, 1965; Samoilova *et al.*, 1996; Sodeinde *et al.*, 1992; Welkos *et al.*, 2002, 1997) and recent research characterizing the importance of Pla in establishing pneumonic infection in mice (Lathem *et al.*, 2007), we undertook studies to delineate how the absence of the pPCP1 plasmid would attenuate our Δ *lpp* mutant of *Y. pestis* CO92. We noted a significant increase in the LD₅₀ in mice that were infected with the double mutant pPCP⁻/ Δ *lpp* of *Y. pestis*, and a dramatic decrease in cytokine/chemokine release compared with animals that were infected with either the pPCP⁻ or the Δ *lpp* mutant strain of *Y. pestis* alone. Our data suggested that Pla and Lpp synergistically affect the survivability and differentially affect the disseminative capacity of *Y. pestis* in a mouse model of pneumonic plague.

METHODS

Bacterial strains. Virulent WT *Y. pestis* CO92 strain was acquired from the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). The *Y. pestis* CO92 strain deficient in the *lpp* gene was created by us previously and is described elsewhere (Sha *et al.*, 2008). Strains lacking the virulence plasmid pPCP1 (pPCP⁻) were created by growing either WT or Δ *lpp* *Y. pestis* at 4 °C on trypticase soy agar plates with 5% sheep blood (SBA, Becton, Dickinson and Company) for five passages (Straley & Brubaker, 1982). Single colonies were tested via PCR for the presence of genes on each of the three virulence plasmids: *lcrV* (981 bp, low calcium response antigen present on pCD1); *pla* (939 bp, plasminogen activating protease present on pPCP1); and *cafI* (513 bp, capsular antigen present on pMT1). Colonies cured of the *pla*-encoding plasmid only were those in which PCR indicated that *pla* was absent, while verifying the presence of *lcrV* and *cafI* genes. The presence or absence of plasmids in the cured strains was also ascertained by agarose gel electrophoresis (Sha *et al.*, 2008). *Y. pestis* strains were grown in heart infusion broth (HIB) medium (Difco, Voigt Global Distribution) at 28 °C with constant shaking at 180 r.p.m. in our CDC-approved, restricted-entry, biosafety level (BSL)-2 laboratory.

Complementation of the Δ *lpp* mutant strain of *Y. pestis* CO92.

The Tn7 transposon-based broad-range vector pUC18R6K-mini-Tn7T was used in this study (Choi *et al.*, 2005). The transposon Tn7 can insert at a high frequency into bacterial chromosomes in a site- and orientation-specific manner at the Tn7 attachment (*attTn7*) site.

This site is located downstream of the highly conserved *glmS* gene, which encodes an essential glucosamine-6-phosphate synthetase (Craig, 1996; Peters & Craig, 2001). This system provides a site-specific insertion of a single copy of the target gene into the genome without interrupting any other genes, and is an ideal system for complementation studies. Briefly, primers *lpp5* (5' ACGAATTCGCGCACCTTACAAATAAGAC 3', *EcoRI* site underlined) and *lpp3* (5' AGCTGCAGTTACTTCTTGTAAGCTTGAG 3', *PstI* site underlined) were used to PCR-amplify the *lpp* coding and promoter (200 bp upstream of the start codon) regions from the WT *Y. pestis* genome. The amplified DNA fragment was cloned into the pUC18R6K-mini-Tn7T vector at the *EcoRI/PstI* sites, which generated the recombinant plasmid pUC18R6K-mini-Tn7T/*lpp*. Subsequently, a kanamycin-resistance (Km^r) gene cassette [with Flippase (FLP) recombinase recognition target site at both ends], which was PCR-amplified from plasmid pKD13 by using specific primers *Km5* (5' ATTCCGGGGATCCGTCGACC 3', *BamHI* site underlined) and *Km3* (5' TTGGATCCGTGTAGGCTGGAGCTGCTTC 3', *BamHI* site underlined), was inserted into the plasmid pUC18R6K-mini-Tn7T/*lpp* at the *BamHI* site, thus generating the recombinant plasmid pUC18R6K-mini-Tn7T- Km^r /*lpp*.

This newly generated recombinant plasmid and the helper plasmid pTNS2 (Choi *et al.*, 2005), which expresses the transposase complex to facilitate efficient transposition, were mixed at a ratio of 1:1 and transformed into the Δlpp *Y. pestis* mutant strain via electroporation by using a Gene Pulse Xcell (Bio-Rad). The transformants were selected on the kanamycin plate, and the antibiotic-resistant colonies were picked up. As the helper plasmid pTNS2 also contains the R6K origin, it is not able to replicate inside *Y. pestis* and was lost during bacterial propagation. The site-specific Tn7 insertion in these *Y. pestis* colonies was subjected to further PCR analyses with specific primer sets as described by Choi *et al.* (2005). The *Y. pestis* colony that contained the insertion of the Km^r and *lpp* gene with its promoter downstream of the *glmS* gene in the genome due to Tn7 site-specific integration was considered to be the intermediate form of the Δlpp *Y. pestis* complemented strain. Subsequently, the Km^r gene cassette was removed by transforming this intermediate form strain with plasmid pFlp2, which expresses the FLP recombinase. Plasmid pFlp2 was then cured with 5% sucrose in the growth medium, and the final form of the Δlpp *Y. pestis* complemented strain was sensitive to kanamycin and free of plasmids pTNS2 and pFlp2. The expression of the *lpp* gene in the complemented strain was further confirmed by Western blot analysis using antibodies to Lpp (Sha *et al.*, 2008).

Animals. All of our mice studies were performed in the animal BSL (ABSL)-3 facility under the protocol approved by the UTMB Institutional Animal Care and Use Committee. Five- to six-week-old female Swiss-Webster mice were purchased from Charles River Laboratories and challenged via the i.n. route with various doses of WT *Y. pestis* or the Δlpp , pPCP⁻ and pPCP⁻/ Δlpp mutant strains, as previously described (Sha *et al.*, 2008). To generate the survival curves, mice were assessed twice daily for morbidity or mortality over a period of 40 days. For the histopathology, cytokine/chemokine and bacterial dissemination analyses, the sera and organs of animals were harvested at the indicated time points.

Histopathology. Mice (five per group) infected with 8.5×10^4 (250 LD₅₀) c.f.u. by the i.n. route, with either WT *Y. pestis* or one of the mutant strains, were euthanized at 1 and 48 h post-infection (p.i.) using a mixture of ketamine (90 mg kg⁻¹) and xylazine (10 mg kg⁻¹). At these time points, portions of the lung, liver, spleen and heart tissues were placed in screw-capped containers and immersion-fixed in 10% neutral buffered formalin, as described previously (Agar *et al.*, 2008a, b; Sha *et al.*, 2008). The tissues were transferred to new containers with fresh formalin 3 days later, and then processed and sectioned at 5 μ m. Tissue sections were mounted on glass slides,

stained with haematoxylin and eosin (H&E), and evaluated by light microscopy in a blinded fashion. Tissue lesions were scored based on a severity scale of minimal, mild, moderate and marked; the scale correlated with estimates of lesion distribution and extent of tissue involvement (i.e. minimal = 2–10%; mild >10–20%, moderate >20–50%, severe >50%), as previously described (Agar *et al.*, 2008a, b; Sha *et al.*, 2008). Inflammation indicated the presence of neutrophils usually associated with oedema and fibrin. In some sections, bacteria consistent with *Y. pestis* infection were present, although bacteria-specific staining was not performed.

Measurements of cytokines and chemokines in tissue homogenates and sera. Concurrently with the collection of tissues for histopathology from infected mice, at 1 and 48 h p.i., groups of animals were killed, blood was collected and portions of the organs were homogenized. Uninfected mice served as a control group. Blood was centrifuged for 5 min at 1500 r.p.m. and the sera were extracted and filtered using 0.22 μ m pore-size centrifugal filters (Millipore). The organs were weighed and homogenized by using tissue grinders (Kendall), and tissue homogenates were filtered using both 0.45 and 0.22 μ m pore-size syringe filters. The levels of 32 cytokines and chemokines were simultaneously measured using a Milliplex multiplex assay (Millipore).

Survival of WT and mutant strains in the peripheral organs of mice. Using the tissue homogenates and blood collected as described above, serial dilutions were made and cultured on SBA plates to assess the dissemination of the bacteria. Bacterial colonies were counted following 28 °C incubation for 48–72 h.

Survival of WT and mutant strains in murine RAW 264.7 macrophages. WT and mutant *Y. pestis* strains were grown in HIB overnight at 28 °C. RAW 264.7 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) and 1% L-glutamine (Cellgro) were seeded in six-well plates, so that after 24 h they were 60–70% confluent. Bacteria at an m.o.i. of 1 were centrifuged, and the pellets were resuspended in DMEM before being transferred into duplicate wells of RAW cells, as previously described (Sha *et al.*, 2008). In brief, plates were incubated at 37 °C in 5% CO₂ for 30 min, after which the wells were washed twice with PBS. Extracellular bacteria were killed by adding 100 μ g gentamicin ml⁻¹ to each well for 1 h. One set of plates was harvested immediately (0 h time point), while DMEM containing 10 μ g gentamicin ml⁻¹ was added to the other wells for the duration of the experiment. At each time point (4, 8 and 24 h p.i.), RAW cells were washed twice with PBS before being lysed with 300 μ l sterile water. Lysed host cells were serially diluted 10-fold and cultured on SBA plates. After 48 h at 28 °C, bacterial colonies were counted to determine the percentage survival of each *Y. pestis* strain at the corresponding time point.

Statistical analyses. To generate the survival curves, 10 mice per group were infected with multiple doses of WT and mutant strains of *Y. pestis*, and the Kaplan–Meier survival estimate was used for data analyses. Either Student's *t* test or ANOVA with Bonferroni correction were used for cytokine/chemokine analysis, bacterial dissemination studies in mice, and intracellular survival of bacteria in macrophages.

RESULTS

The *Y. pestis* pPCP⁻/ Δlpp mutant strain is less lethal than the WT or its single mutants

Mice were inoculated intranasally with various doses of WT, Δlpp , pPCP⁻ or pPCP⁻/ Δlpp *Y. pestis* and were

monitored for morbidity and mortality (Fig. 1). Animals infected with a 250 LD₅₀ dose [1 LD₅₀ was calculated to be 340 bacteria (Sha *et al.*, 2008)] of either WT or the Δlpp mutant of *Y. pestis* died by days 3 and 5, respectively. As expected, the mean time to death was increased in mice infected with the Δlpp mutant (Sha *et al.*, 2008). Notably, animals infected with the pPCP⁻/ Δlpp mutant strain showed much higher survival rates at all doses tested than did those animals infected with the pPCP⁻ mutant strain alone at the same doses. For example, at the lowest tested dose, those mice infected with a 250 LD₅₀ dose of the pPCP⁻/ Δlpp mutant strain showed 100 % survival, while those infected with the pPCP⁻ mutant strain exhibited only a 30 % survival rate. In the 2500 LD₅₀ dose group, 100 % of the animals infected with the pPCP⁻/ Δlpp mutant strain survived, while 20 % of mice infected with the pPCP⁻ mutant strain survived. Finally, at the highest dose of 5000 LD₅₀, 50 % of the animals infected with the pPCP⁻/ Δlpp mutant strain survived. All mice infected with the pPCP⁻ mutant strain at this dose died by day 5 p.i. This indicated to us that curing of the pPCP1 plasmid and deleting the *lpp* gene synergistically attenuated the virulent *Y. pestis* CO92 strain.

WT-infected mice have more severe tissue injury than do mice infected with the mutant strains

Based on the data presented in Fig. 1, we chose a time point of 48 h for collection of tissues and blood for our subsequent analyses, as by day 3 all of the animals infected with the WT bacterium died due to the high dose (250 LD₅₀) used in this study. Portions of the mouse tissues were processed and stained with H&E to assess the level of tissue damage associated with the bacterial infection (Fig. 2 and Supplementary Figs S1 and S2). At 1 h p.i., no significant tissue damage was noted in the lungs, livers, spleens or hearts of mice infected with WT, Δlpp , pPCP⁻ or pPCP⁻/ Δlpp mutant strains (data not shown). These tissues exhibited histopathology similar to those sections from uninfected mice (anaesthetized and given PBS) serving as negative controls (data not shown).

At 48 h p.i., the lungs (Fig. 2a) of WT-infected mice (A and E) showed mild alveolar oedema (asterisks), as well as mild neutrophilic inflammation. Bacteria (arrows) were present in the alveoli of all mice and in the perivascular region in two out of five mice. The lungs of all five Δlpp -infected mice (B and F) showed moderate oedema (asterisks) and

Fig. 1. Survival analysis of mice infected with WT and mutant *Y. pestis* strains. Following i.n. inoculation with various doses of WT, Δlpp , pPCP⁻ and pPCP⁻/ Δlpp mutants of *Y. pestis*, animals were monitored for morbidity and mortality. Those infected with a 250 LD₅₀ dose of either WT (●) or Δlpp (○) *Y. pestis* died by days 3 and 5, respectively. However, mice infected with either a 250 LD₅₀ (△) or a 2500 LD₅₀ (□) dose of the pPCP⁻/ Δlpp strain showed a 100 % survival rate, while those infected with the pPCP⁻ strain showed 30 % (▼) and 20 % (■) survival rates, respectively. At the highest dose tested, 5000 LD₅₀, 50 % of the mice infected with the pPCP⁻/ Δlpp strain survived (◇), while no mice infected with the pPCP⁻ strain survived (◆). By Kaplan–Meier analysis, only the group infected with a 5000 LD₅₀ dose of the pPCP⁻ strain was not significantly different from the WT- and Δlpp -infected groups. All other treatment groups were significantly different ($P < 0.001$).

Fig. 2. Histopathology of mouse tissues following infection with WT and mutant strains. Groups of five mice were infected with WT *Y. pestis* or Δlpp , $pPCP^-$ or $pPCP^-/\Delta lpp$ mutant strains and euthanized at 1 and 48 h p.i. The lungs (a), livers (b), and spleens or hearts (Supplementary Figures S1–S2 and Supplementary Tables S2–S5) were harvested at these time points. No significant tissue damage was noted in mice infected with WT *Y. pestis* at 1 h p.i. (results not shown). Tissue sections of WT-infected mice at 48 h p.i. are shown in (A) and (E), those from Δlpp -infected mice are shown in (B) and (F), those from $pPCP^-$ -infected mice are shown in (C) and (G), and those from $pPCP^-/\Delta lpp$ -infected mice are shown in (D) and (H). Bacteria in these sections are indicated by arrows, oedema is indicated by asterisks, inflammation is outlined by chevrons, and necrosis is indicated with an arrowhead. All sections were stained with H&E and scale bars are present on each image.

mild neutrophilic inflammation in the alveoli. Bacteria (arrows) were present in most alveoli associated with fibrin. The interstitium had small areas of necrosis in two of the five mice, and one mouse had multiple areas of moderate perivascular oedema with mild perivascular fibrin. The lungs of mice infected with the $pPCP^-$ mutant strain (C and G) showed mild neutrophilic inflammation

(outlined by chevrons) in both the alveolar and perivascular regions. Three of five mice had mild alveolar fibrin, one of five mice had alveolar oedema, and one of five had mild haemorrhaging. The lungs of all five mice infected with the $pPCP^-/\Delta lpp$ mutant (D and H) had perivascular neutrophilic inflammation, while only three had perivascular oedema. Two of five mice had mild peribronchiolar

inflammation (outlined by chevrons), and one mouse had mild neutrophilic inflammation in the bronchioles.

The livers (Fig. 2b) of two of the five WT-infected mice (A and E) showed small areas of necrosis (arrowhead) and minimal neutrophilic inflammation in the hepatic sinusoids, which also contained occasional bacteria (arrows) and fibrin. Two of five Δlpp -infected mice had minimal neutrophilic inflammation in the sinusoids of the liver (B and F), while one mouse had bacteria (arrows) in the sinusoids. One of five mice infected with the pPCP⁻ mutant strain had bacteria in the liver sinusoids (C and G, arrow). Two of five mice had minimal and mild neutrophilic liver inflammation (outlined by chevrons). Three of five mice infected with the pPCP⁻/ Δlpp mutant (D and H) had mild neutrophilic inflammation (outlined by chevrons), and one of these had a small area of necrosis in the sinusoids. The livers of the other two mice in this group were within normal limits.

Four out of five spleens (Supplementary Fig. S1) in the WT-infected mice group (A and E) had bacteria (arrows) and fibrin in the red pulp, while one mouse had lymphoid depletion in the white pulp. One of five mice infected with the Δlpp mutant had bacteria (arrows) and fibrin in the red pulp and moderate lymphoid depletion in the white pulp of the spleen (B and F). No mice infected with the *Y. pestis* pPCP⁻ or pPCP⁻/ Δlpp mutant strains exhibited any lesions in the spleen (C, G and D, H, respectively). These histopathology data corresponded with gradual and reduced mortality in mice infected with the pPCP⁻ or pPCP⁻/ Δlpp mutant strains compared with that found in the WT and Δlpp mutant strains (Fig. 1).

The histopathology for the hearts of mice is also shown in Supplementary Fig. S2, and appeared unremarkable. Overall, the inflammatory lesions and bacteria seen in the WT-infected mouse organs were consistent with *Y. pestis* infection. In general, lesions tended to diminish in occurrence, severity and/or the amount of bacteria present in the mice infected with the Δlpp , pPCP⁻ and pPCP⁻/ Δlpp mutants as compared with the WT-infected mice, with the most minimal changes occurring in the latter two groups. The pathology seen in various organs of mice infected with the WT and the Δlpp mutant strain of *Y. pestis* appeared similar. However, we noted that in the organs of mice infected with the Δlpp mutant, there were less severe histopathological changes when compared with those tissues from WT-infected animals at 48 h p.i., which coincided with a longer mean time to death with the Δlpp mutant (Fig. 1).

Curing of the pPCP1 plasmid or deleting the *lpp* gene results in differential bacterial survivability in mouse peripheral organs

Organs retrieved for histopathological analysis were also homogenized and cultured to assess the survivability of the two pPCP mutant strains (pPCP⁻ and pPCP⁻/ Δlpp) as

compared with the WT and Δlpp mutant strains. The organs of five mice infected with each of the four bacterial strains were homogenized at 1 and 48 h p.i. and cultured on SBA plates. Colonies were counted and data plotted on a log scale to easily visualize the increases and decreases in bacterial numbers. Importantly, the numbers of bacteria in the lungs (Fig. 3a) of WT- and Δlpp mutant-infected mice increased by 1–2 logs at 48 h p.i. when compared with 1 h p.i. control lung tissues. However, the number of bacteria cultured from WT- and Δlpp mutant-infected mice was not statistically different at 48 h p.i. Conversely, in mice infected with either the pPCP⁻ or the pPCP⁻/ Δlpp mutant, the levels of bacteria present in the lungs at 48 h p.i. dropped when compared with the levels in the lungs at 1 h p.i. This drop was significant ($P < 0.05$) when we compared the findings with those in the WT-infected mice, but not when compared with the levels in the Δlpp mutant-infected mice at 48 h p.i. This indicated an inability of these mutant bacteria (pPCP⁻ or pPCP⁻/ Δlpp mutant) to survive within the lung.

Bacteria recovered from the livers of mice (Fig. 3b) infected with either the WT or its Δlpp mutant strain increased by 4–5 logs from 1 to 48 h p.i. These increases in the numbers of bacteria recovered from the livers of Δlpp -infected mice were similar to what we reported previously (Sha *et al.*, 2008). However, only one of five mice in either the pPCP⁻ or the pPCP⁻/ Δlpp mutant-infected group had an appreciable number of bacteria in the liver. In the remaining four animals in each group, no detectable numbers of bacteria were noted in the livers when compared with those findings in the WT-infected mice. The limit of detection of bacteria in various organs in mice was ~80 c.f.u.

In the spleen (Fig. 3c), there were no significant differences between the numbers of bacteria that disseminated in WT-infected mice compared with findings in the Δlpp -, pPCP⁻- or pPCP⁻/ Δlpp mutant-infected animals at 48 h p.i. The trend, however, indicated an inability of Δlpp mutant bacteria (only one out of five mice having an appreciable number of bacteria) to survive within spleen cells, as we previously reported (Sha *et al.*, 2008). Interestingly, in three and two out of five mice, respectively, appreciable numbers of the pPCP⁻ and pPCP⁻/ Δlpp mutant bacteria were noted in the spleen. These data were in contrast to what was observed in the lungs and livers of mice infected with the pPCP⁻ and pPCP⁻/ Δlpp mutant strains, as they did not survive in these tissues after 48 h p.i. (Fig. 3a, b). These data suggested to us that both of these mutants could disseminate to the liver and spleen but were possibly differentially killed in these two organs.

No pPCP⁻ or pPCP⁻/ Δlpp mutant bacteria were detected in the hearts of mice (Fig. 3d), whereas significantly ($P < 0.05$) higher levels of WT and Δlpp strains were noted in the heart at 48 h p.i. Likewise, WT bacteria were detected in the blood of mice (Fig. 3e) at 48 h p.i. Only one out of five mice showed appreciable numbers of Δlpp mutant bacteria in the blood. Although no pPCP⁻/ Δlpp

Fig. 3. Survival of WT and mutant *Y. pestis* strains at 1 and 48 h p.i. in peripheral organs of mice. Groups of five animals were infected with a 250 LD₅₀ dose of WT and mutant *Y. pestis* strains. At 1 and 48 h p.i., mice were euthanized, blood was drawn and organs were harvested, homogenized, serially diluted and cultured on SBA plates. Bacterial counts per gram of lung (a), liver (b), spleen (c) and heart (d) and per millilitre of blood (e) were determined for 1 h p.i. (●) and 48 h p.i. (○). Solid bars indicate the average value at 1 h p.i., while hatched bars indicate the average value at 48 h p.i. The dashed line parallel to the *x* axis is the limit of bacterial detection. Significant differences (Student's *t* test) between groups are noted.

mutant was detected in the blood at 48 h p.i., three out of five mice had low levels of the pPCP⁻ mutant in the blood.

Mice infected with strains cured for the pPCP1 plasmid show lower levels of cytokines and chemokines

In parallel with the histopathology and dissemination experiments, blood was drawn via cardiac puncture from mice at 1 and 48 h after i.n. inoculation with either WT *Y. pestis* or one of the mutant strains. The sera from uninfected mice were used as controls. The levels of 32 cytokines and chemokines were evaluated by using the Millipore Milliplex assay. A maximum of 23 of these cytokines/chemokines showed a response and are listed and defined in Supplementary Table S1.

The levels of cytokines and chemokines (pg ml⁻¹) for all tissue homogenates and in the sera can be found in Supplementary Tables S2–S6. Most remarkable were the drops in the levels of cytokines and chemokines noted in the pPCP⁻/Δ*lpp*-infected animals compared with the WT-infected mice. These data have been summarized based on their percentage differences in Table 1. Generally speaking, WT-infected mice had an overwhelming inflammatory response at 48 h p.i., and the levels of cytokines and chemokines from mice infected with the Δ*lpp* mutant were similar to those for WT-infected animals. This is not surprising given the high dose used for this study. The levels of cytokines/chemokines in mice infected with the pPCP⁻ mutant strain, however, had dropped by 48 h p.i. when we compared them with the levels in WT-infected animals. For most of these cytokines and chemokines, the decrease was substantially greater in mice infected with the pPCP⁻/Δ*lpp* mutant when compared with either of the single mutants. This indicated to us that a synergistic effect occurred in these cytokine/chemokine responses attributable to deletion of the *lpp* gene and curing of the pPCP1 plasmid in the mouse model of infection. In fact, in the lungs of mice (Supplementary Table S2), 16 of these cytokines and chemokines decreased between 90 and 100 % as compared with those levels from the WT-infected animals, and five more decreased between 50 and 89 % at 48 h p.i. Eotaxin levels decreased by 17 % when compared with the levels in WT-infected mice, while IL-10 levels decreased by 32 %.

In mice infected with the pPCP⁻/Δ*lpp* mutant strain, significant decreases ($P < 0.05$, indicated by an asterisk) were noted in the levels of 14 cytokines and chemokines in the liver (Supplementary Table S3), with G-CSF, IFN- γ , IL-1 α , KC, LIF, MCP-1, M-CSF and MIP-2 dropping between 90 and 100 %, when compared with the levels in WT-infected animals. IL-1 β , IL-6, MIG, MIP-1 α , and TNF- α levels decreased between 50 and 90 %, while LIX decreased by only 16 % compared with levels in the livers of WT-infected mice at 48 h p.i. The levels of Eotaxin and IL-12p70 increased compared with those for the WT at 48 h p.i., while neither the IL-10 nor MIP-1 β levels changed at

48 h p.i. in the livers of animals infected with the pPCP⁻/Δ*lpp* mutant strain. We again noted a synergistic effect in the decrease of many of the cytokine and chemokine levels in the livers of mice infected with the pPCP⁻/Δ*lpp* mutant as compared with mice infected with either of the single mutant strains by 48 h p.i.

In the spleen (Supplementary Table S4) at 48 h p.i., we noted changes in the levels of 17 cytokines and chemokines in mice infected with the pPCP⁻/Δ*lpp* mutant strain compared with the levels in WT-infected animals, with 13 of these showing a decrease between 90 and 100 %, three between 50 and 89 %, and one with a 21 % decrease. Many of these showed a synergistic decrease when compared with mice infected with the single mutants. Only RANTES increased significantly ($P < 0.05$, indicated by a dagger) in mice infected with the pPCP⁻/Δ*lpp* mutant strain, compared with RANTES levels found in WT-infected animals at 48 h p.i.

We noted significant decreases in the levels of eight cytokines and chemokines in the heart homogenates (Supplementary Table S5) of mice infected with the pPCP⁻/Δ*lpp* mutant strain at 48 h p.i. Levels of five of these cytokines and chemokines were synergistically lower than the levels seen in either of the single mutant-infected animals (pPCP⁻ or Δ*lpp*). The levels of LIX and M-CSF remained unchanged in the hearts of pPCP⁻/Δ*lpp* mutant-infected mice.

In the sera (Supplementary Table S6) of mice infected with the pPCP⁻/Δ*lpp* mutant strain at 48 h p.i., the levels of G-CSF, GM-CSF, IL-1 α , IL-6, IL-9, IL-12p70, IP-10, MIP-1 α , MIP-1 β , RANTES and TNF- α were all significantly decreased by 80–100 % compared with those in WT-infected animals. Many of these were also synergistically lower than the levels seen in the sera of single mutant-infected mice groups. Eotaxin levels decreased by 67 %, and no cytokine or chemokine levels increased in the sera of mice infected with the pPCP⁻/Δ*lpp* mutant compared with those in WT-infected mice.

Survivability of *Y. pestis* mutants in murine macrophages is dependent upon the *lpp* gene rather than the pPCP1 plasmid

RAW 264.7 murine macrophages were infected with each of the *Yersinia* strains, and infected macrophages were harvested after a 1 h gentamicin treatment (0 h time point), and at 4, 8 and 24 h post treatment. At each time point, macrophages were lysed and cultured on SBA plates to determine the percentage intracellular survival of *Y. pestis* strains (Fig. 4). Data were normalized to the 0 h time points.

At 4 h p.i. (Fig. 4a), the numbers of WT cells recovered from macrophages decreased slightly, compared with those at the 0 h time point, while the numbers of mutant bacteria recovered from macrophages were significantly decreased ($P < 0.001$). The numbers of the pPCP⁻/Δ*lpp* mutant strain

Table 1. Percentage changes in cytokine and chemokine levels in tissue homogenates 48 h p.i. with *Y. pestis* pPCP⁻/Δ*lpp* when compared with WT CO92

BDL, Below detectable limit.

Cytokine or chemokine	Lung			Liver		
	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP ⁻ /Δ <i>lpp</i> compared with WT	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP ⁻ /Δ <i>lpp</i> compared with WT
Eotaxin	209 ± 47	173 ± 36	17 %	37 ± 24	92 ± 46*	45 %†
G-CSF	109 350 ± 18 765	1540 ± 2736*	99 %	53 607 ± 34 666	209 ± 204*	100 %
GM-CSF	607 ± 446	9 ± 9*	99 %	BDL	BDL	BDL
IFN-γ	8612 ± 8874	70 ± 55	99 %	382 ± 348	8 ± 10*	98 %
IL-1α	7603 ± 5424	1226 ± 210*	84 %	1691 ± 1050	146 ± 58*	91 %
IL-1β	1117 ± 456	69 ± 12*	94 %	186 ± 103	70 ± 42*	62 %
IL-6	77 588 ± 31 992	204 ± 102*	100 %	293 ± 154	74 ± 29*	75 %
IL-10	19 ± 3	13 ± 3*	32 %	40 ± 26	55 ± 21	Unchanged
IL-12p70	138 ± 23	12 ± 7*	91 %	39 ± 20	115 ± 67*	92 %
IL-17	374 ± 192	7 ± 13*	98 %	BDL	BDL	BDL
IP-10	5943 ± 990	440 ± 414*	93 %	1491 ± 1626	169 ± 114	89 %
KC (IL-8)	19 490 ± 6928	1696 ± 1777*	91 %	10 496 ± 3264	883 ± 1004*	92 %†
LIF	1014 ± 665	2 ± 2*	100 %	22 ± 16	BDL*	100 %
LIX	798 ± 232	318 ± 286*	60 %	304 ± 43	256 ± 89*	16 %
MCP-1	6731 ± 4184	219 ± 188*	97 %	1591 ± 1111	136 ± 83*	91 %
M-CSF	403 ± 141	72 ± 11*	82 %	378 ± 192	40 ± 22*	90 %
MIG	110 006 ± 101 343	5113 ± 4622	95 %	115 125 ± 63 867	33 656 ± 45 249*	71 %
MIP-1α	1916 ± 768	162 ± 73*	92 %	168 ± 66	80 ± 45*	53 %
MIP-1β	2097 ± 1138	145 ± 62*	93 %	49 ± 53	64 ± 71	Unchanged
MIP-2	16 499 ± 9576	128 ± 101*	99 %	3481 ± 1817	146 ± 102*	96 %
RANTES	215 ± 16	62 ± 15*	71 %	BDL	BDL	BDL
TNF-α	285 ± 109	4 ± 3*	98 %	41 ± 18	7 ± 3*	84 %
VEGF	204 ± 84	91 ± 21*	56 %	BDL	BDL	BDL
	Spleen			Serum		
	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP ⁻ /Δ <i>lpp</i> compared with WT	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP ⁻ /Δ <i>lpp</i> compared with WT
Eotaxin	261 ± 147	44 ± 8*	83 %	840 ± 525	278 ± 116*	67 %
G-CSF	10 480 ± 4902	86 ± 50*	99 %	147 789 ± 14 893	4038 ± 4734*	97 %
GM-CSF	BDL	BDL	BDL	52 ± 12	1 ± 3*	98 %
IFN-γ	765 ± 1040	2 ± 4	100 %	7499 ± 13 413	17 ± 33	100 %
IL-1α	298 ± 73	13 ± 27*	96 %	275 ± 133	47 ± 55*	83 %
IL-1β	51 ± 33	25 ± 9*	52 %	138 ± 151	BDL	100 %
IL-6	481 ± 502	BDL	100 %	29 833 ± 12 847	393 ± 466*	99 %
IL-10	20 ± 6	10 ± 5*	53 %	11 ± 5	3 ± 1*	74 %
IL-12p70	15 ± 6	1 ± 1*	94 %	114 ± 82	20 ± 30*	82 %
IL-17	BDL	BDL	BDL	433 ± 499	2 ± 3	100 %
IP-10	5690 ± 2084	160 ± 56*	97 %	4435 ± 2834	178 ± 91*	96 %
KC (IL-8)	11 519 ± 3907	251 ± 157*	98 %	26 500 ± 25 849	133 ± 62	99 %
LIF	6 ± 4	BDL*	100 %	BDL	BDL	BDL
LIX	BDL	BDL	BDL	BDL	BDL	BDL
MCP-1	1080 ± 412	29 ± 26*	97 %	13 144 ± 13 258	49 ± 49	100 %
M-CSF	59 ± 57	46 ± 20*	21 %	9 ± 7	BDL	96 %
MIG	103 723 ± 69 003	9048 ± 3431*	91 %	42 714 ± 63 895	258 ± 211	99 %
MIP-1α	211 ± 102	12 ± 17*	94 %	297 ± 46	58 ± 49*	80 %
MIP-1β	534 ± 270	163 ± 95*	69 %	393 ± 179	BDL *	100 %

Table 1. cont.

	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP/ Δ <i>lpp</i> compared with WT	WT 48 h p.i. (pg ml ⁻¹)	pPCP ⁻ /Δ <i>lpp</i> 48 h p.i. (pg ml ⁻¹)	Decrease in pPCP/Δ <i>lpp</i> compared with WT
MIP-2	483 ± 463	BDL*	100 %	4722 ± 9749	16 ± 18	100 %
RANTES	97 ± 24	135 ± 22*	40 %†	91 ± 53	9 ± 6*	91 %
TNF-α	13 ± 5	1 ± 2*	90 %	42 ± 18	BDL*	100 %
VEGF	BDL	BDL	BDL	BDL	BDL	BDL

*Significant changes as compared with WT-infected mice (Student's *t* test, *P* < 0.05).

†Increase in this cytokine or chemokine above the level in the WT.

Fig. 4. Survival of WT and mutant *Y. pestis* strains *in vitro*. Murine RAW 264.7 macrophages were infected at an m.o.i. of 1 with WT and mutant *Y. pestis* strains for 30 min. Monolayers were treated with 100 µg gentamicin ml⁻¹ for 1 h, and at the indicated time points (post gentamicin), macrophages were lysed. Lysates were serially diluted and cultured on SBA plates. Colonies were counted and data were normalized to the 0 h time point. Significant differences (ANOVA, *P* < 0.001) between groups are noted. (b) Comparison of WT- and Δ*lpp* mutant-infected macrophages with the *lpp*-complemented strain of *Y. pestis* CO92. Significant differences (Student's *t* test, *P* < 0.05) between groups are noted. The inset in (b) shows Western blot analysis data showing the presence or absence of Lpp (6.3 kDa) using specific antibodies in various strains.

recovered from macrophages decreased the most, however, to a level significantly different ($P < 0.001$) from that of the pPCP⁻ mutant strain, while maintaining a level of survival similar to that of the Δlpp mutant strain. At 4 h p.i., the absence of Lpp appeared to be the determining factor for the intracellular survival of these *Y. pestis* strains within macrophages.

At 8 h p.i. (Fig. 4a), the number of WT *Y. pestis* that survived within the macrophages decreased only slightly from the 4 h time point and again showed a significantly higher ($P < 0.001$) level of survivability when compared to findings in the other three mutant strains. A similar pattern with the mutant strains emerged at this time point, except that the number of Δlpp mutant bacteria that survived decreased to a level significantly ($P < 0.001$) below that of the pPCP⁻ or pPCP⁻/ Δlpp mutant strains recovered from macrophages.

At 24 h p.i. (Fig. 4a), the number of WT bacteria that survived inside the macrophages did not change compared with their numbers at the 8 h time point. Although the number of pPCP⁻ mutant bacteria increased from the 8 h time point, the numbers of bacteria retrieved from macrophages were significantly ($P < 0.001$) lower than that for the WT bacteria. The survivabilities of the Δlpp and pPCP⁻/ Δlpp mutant strains dropped dramatically compared with the survivability of either the WT or pPCP⁻ mutant strains and were not significantly different from each other at 24 h p.i. In addition, the strain in which the *lpp* gene was complemented showed statistically similar survivability to that of the WT bacteria in macrophages 24 h p.i. (Fig. 4b), and the complementation of the Δlpp mutant was also confirmed by Western blot analysis (inset, Fig. 4b). A 6.3 kDa band corresponding to Lpp could clearly be detected in the WT and *lpp* complemented strains but not in the Δlpp mutant of *Y. pestis*. Taken together, these data indicated that the lack of survivability of *Y. pestis* within macrophages seemed to be more dependent on the presence of Lpp rather than the pPCP1 plasmid.

DISCUSSION

We recently showed that deletion of the *lpp* gene from *Y. pestis* CO92 affects the virulence of the organism in the mouse model of pneumonic plague (Sha *et al.*, 2008). Other investigators have also reported attenuation in *Y. pestis* strains lacking either the pPCP1 plasmid or the *pla* gene (Brubaker *et al.*, 1965; Lathem *et al.*, 2007; Samoilova *et al.*, 1996; Sodeinde *et al.*, 1992; Welkos *et al.*, 2002, 1997). In fact, Lathem and co-workers concluded that Pla was responsible for establishing infection in the lung when bacteria were administered intranasally but was not necessary for *Y. pestis* to disseminate from the lungs to peripheral organs, e.g. the spleen. For this study, we passaged bacteria at a low temperature to cold-cure the smallest virulence plasmid, pPCP1, from the WT and the Δlpp mutant strain. Plasmid pCD1-dependent cytotoxicity, measured as previously described (Sha *et al.*, 2008), did not

differ between the WT and the mutant strains (Supplementary Fig. S3), and we were able to isolate the two remaining virulence plasmids and their associated virulence genes from the mutants (results not shown). Additionally, we noted that a 250 LD₅₀ dose of the pPCP⁻ strain, when administered into mice, provided minimal protection when compared with the WT bacteria, suggesting that cold-curing did not affect virulence of the mutant. Most interesting, however, was the observation that *Y. pestis* strains lacking both the *lpp* gene and the pPCP1 plasmid were highly attenuated in the mouse model of pneumonic plague, when compared with the single *lpp* gene deletion or the pPCP⁻ strain. Furthermore, such a pPCP⁻/ Δlpp mutant strain exhibited drastically reduced tissue injury and a muted cytokine and chemokine induction in tissue homogenates and sera when compared with equivalent findings in WT, Δlpp and pPCP⁻ mutant strains. We opted to cure the pPCP1 plasmid from WT and Δlpp *Y. pestis* strains rather than selectively delete the *pla* gene, as pesticin and pesticin-immunity protein do not contribute to the virulence of *Y. pestis* in the animal model (Anisimov *et al.*, 2009).

We used a much higher dose (250 LD₅₀) of bacteria in the current report with similar results to a previous study (Sha *et al.*, 2008). Based on our previous report at a dose of 5 LD₅₀, WT-infected mice began to die between 60 and 72 h p.i., and we noted appropriate cytokine and chemokine responses and definitive pathology by 48 h p.i. (Agar *et al.*, 2008a, b; Sha *et al.*, 2008). Additionally, multiple survival/mortality experiments using the pPCP⁻ and pPCP⁻/ Δlpp mutant strains indicated that we needed very high doses to determine the LD₅₀ of these two mutant strains. Therefore, in order to use WT- and Δlpp -infected mice as a comparison with the two pPCP⁻ mutant strains with such a large amount of bacteria, we chose the latest time point as 48 h p.i. in this study.

In terms of dissemination and survivability of WT *Y. pestis* CO92 and its various mutants in a mouse model, we concluded that the Δlpp mutant could leave the confines of the lungs to replicate within the liver and the heart, but was unable to do so in the spleen or blood and hence cleared from these organs by 48 h p.i. This confirmed our previous findings in which we demonstrated a high bacterial (Δlpp) load in the lungs and liver of mice but not in the spleen and blood at 48 h p.i. (Sha *et al.*, 2008). The inability to recover mutant bacteria from the spleen could be explained by the uptake and subsequent killing of the mutant by splenic cells or by the migration of immune cells from the spleen following uptake of the mutant bacteria (Marketon *et al.*, 2005). In the lungs, the ability of Δlpp mutant bacteria to replicate indicated either that the alveolar macrophages were unable to kill the mutant efficiently or that the bacteria were also taken up by lung epithelial cells, where they survived effectively and replicated. It is also possible, as was noted above, that these bacteria in the lungs resisted phagocytosis and efficiently grew extracellularly within the lung.

The pPCP⁻ and pPCP⁻/Δ*lpp* mutant strains were not able to survive in the lung, unlike the WT and Δ*lpp* mutant strains, and those bacteria that left the lung either showed a tropism for the spleen over the liver, heart or blood, or were cleared by the last three organs by 48 h. This was confirmed by our histopathology data, with pPCP⁻ and pPCP⁻/Δ*lpp* mutant strains completely clearing from the tissues 48 h p.i. Clearly, Pla was required to establish an infection in the lungs of mice and possibly dissemination of bacteria from the lungs to other organs. However, the presence of pPCP⁻ and pPCP⁻/Δ*lpp* mutant bacteria at 48 h p.i. in the spleens of 40–60 % of the mice indicated that these mutant bacteria did disseminate, albeit in lower numbers, or that these mutant bacteria were efficiently killed in the lungs, liver, heart and blood, but not in the spleen. Alternatively, the pPCP⁻ mutant might have a tropism for the spleen, while the Δ*lpp* mutant migrated mostly to the liver. These data are very provocative and require further studies.

These data corroborated the cytokine and chemokine induction data from the tissue homogenates and the sera. Mice infected with the Δ*lpp* mutant had significantly lower levels of MIG and MIP-2 in the liver homogenates, IP-10 and MIG in the spleen homogenates, and IL-9 and MIG in the heart homogenates when compared with WT-infected mice. However, the levels of the above-mentioned cytokines and chemokines were lowered even further in those mice infected with either of the two pPCP⁻ mutants (pPCP⁻ and pPCP⁻/Δ*lpp*) when compared with the levels in the Δ*lpp* mutant-infected mice. Interestingly, MIG and IP-10 are induced by IFN-γ (Brice *et al.*, 2001), the cytokine with immunoregulatory functions important in the host response to infection. In the case of the WT- and Δ*lpp*-infected mice, however, the overwhelming induction of cytokines and chemokines led to their demise. Animals infected with either pPCP⁻ mutant, on the other hand, showed lower cytokine and chemokine responses, and evidence of tissue repair by 48 h p.i., which indicated to us that by this time point the mice had resolved the infection. In fact, a study by Lathem and co-workers showed that mice infected with a *pla* isogenic mutant had little elevation of the quantities of cytokine transcripts (IL-10, IL-1α, IL-17, IL-6, MIP-2 and TNF-α) at 24 h p.i. (Lathem *et al.*, 2007), which indicated that these mice and those in our study avoided the massive proinflammatory response that more than likely contributed to the demise of our WT- and Δ*lpp*-infected animals. Taken together, these data suggest to us that Lpp and Pla contribute to bacterial virulence by different mechanisms, but synergistically result in a more efficient clearance of the bacteria from mice and hence their higher survival rate.

These *in vivo* data corroborated our *in vitro* studies, in which we infected murine RAW 264.7 macrophages with various mutant strains. As noted, Δ*lpp* and pPCP⁻/Δ*lpp* mutant bacteria died rapidly within the macrophages, when compared with the WT or pPCP⁻ strain of *Y. pestis*. These data indicated that Lpp and not Pla contributed to

bacterial survival in macrophages. This supported an earlier study which showed that neither the pCD1 nor the pPCP1 virulence plasmid is required for *Yersinia* to efficiently grow within macrophages (Straley & Harmon, 1984). In contrast, in the liver, both the WT and Δ*lpp* mutant bacteria could efficiently survive. *Y. pestis* produces an antiphagocytic capsule and Yops at 37 °C, and as a result efficiently grows extracellularly (Cavanaugh & Randall, 1959; Pujol & Bliska, 2005; Viboud & Bliska, 2005). Another alternative is that the WT and Δ*lpp* mutant bacteria escaped killing by resisting phagocytosis and grew extracellularly in the liver.

Importantly, deletion of Lpp and Pla resulted in a greater ability of mice to recover from infection following high doses (250–2500 LD₅₀), as compared with the single-mutant strains, and this effect was synergistic. Because of the absence of Pla, chemotaxis of inflammatory cells was not impeded and complement C3 was not inactivated, resulting in a vigorous enough immune response for the mice to survive infection (Sodeinde *et al.*, 1992).

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