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***Yersinia pestis* CO92 mutant deleted for the genes encoding Braun lipoprotein and plasminogen activator protease: Characterization of a potential live-attenuated vaccine candidate**

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# *Yersinia pestis* CO92 mutant deleted for the genes encoding Braun lipoprotein and plasminogen activator protease: Characterization of a potential live-attenuated vaccine candidate

**by**

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**DISSERTATION**

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# Dedication

This body of research was inspired by my grandfather, Peter Bubna, nurtured by my Mom and Dad, and supported by William R. Miller, PhD.

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**Deletion of Braun lipoprotein and plasminogen activating protease-encoding genes attenuates *Yersinia pestis* in mouse models of bubonic and pneumonic plague**

Publication No.__1_____

Christina J. van Lier, PhD

The University of Texas Medical Branch, 2014

Supervisor: Ashok K. Chopra, PhD, CSc

**Abstract:**

There is no FDA-approved vaccine against *Yersinia pestis*, the causative agent of bubonic and pneumonic plague. Since both humoral- and cell- mediated immunity are essential in providing protection to the host against plague, we developed a live-attenuated vaccine strain by deleting Braun lipoprotein (*lpp*) and plasminogen-activating protease (*pla*) genes from *Y. pestis* CO92. The Δ*lpp* Δ*pla* double isogenic mutant was highly attenuated in evoking both bubonic and pneumonic plague in a mouse model. Further, animals immunized with the mutant by either the intranasal or the subcutaneous routes were significantly protected from developing subsequent pneumonic plague. In mice, the mutant poorly disseminated to peripheral organs and the production of pro-inflammatory cytokines concurrently decreased. Histopathologically, reduced damage to the lungs and liver of mice infected with the Δ*lpp* Δ*pla* double mutant was observed when compared to the WT CO92-challenged animals. The Δ*lpp* Δ*pla* mutant-immunized mice elicited a humoral immune response to the WT bacterium, as well as to CO92-specific antigens. Moreover, T cells from the mutant-immunized animals exhibited significantly higher proliferative responses

when stimulated *ex vivo* with the heat-killed WT CO92 antigens, compared to responses in mice immunized with the same sub-lethal dose of WT CO92. Likewise, T cells from the mutant-immunized mice produced more interferon (IFN)-γ and interleukin (IL)-4. These animals had an increasing number of tumor necrosis factor (TNF-α)-producing $CD4^+$ and $CD8^+$ T cells compared to WT CO92-infected mice. These data emphasized the role of TNF-α and IFN-γ in protecting mice against pneumonic plague. Overall, our studies provided evidence that deletion of *lpp* and *pla* genes acted synergistically in protecting animals against pneumonic plague, and we have demonstrated an immunological basis for this protection.

**Further characterization of a highly attenuated *Yersinia pestis* CO92 mutant deleted for the genes encoding Braun lipoprotein and plasminogen activator protease in murine alveolar and primary human macrophages**

Publication No.___2_____

Christina J. van Lier, PhD

The University of Texas Medical Branch, 2014

Supervisor: Ashok K. Chopra, PhD, CSc

**Abstract:**

We recently characterized the Δ*lpp* Δ*pla* double in-frame deletion mutant of *Yersinia pestis* CO92 molecularly, biologically, and immunologically. While Braun lipoprotein (Lpp) activates toll-like receptor-2 to initiate an inflammatory cascade, plasminogen activator (Pla) protease facilitates bacterial dissemination in the host. The Δ*lpp* Δ*pla* double mutant was highly attenuated in evoking bubonic and pneumonic plague, was rapidly cleared from mouse organs, and generated protective humoral and cell-mediated immune responses to provide subsequent protection to mice against a lethal challenge dose of wild-type (WT) CO92. Here, we further characterized the Δ*lpp* Δ*pla* double mutant in two murine macrophage cell lines as well as in primary human monocyte-derived macrophages to gauge its potential as a live-attenuated vaccine candidate. We first demonstrated that the Δ*pla* single and the Δ*lpp* Δ*pla* double mutant were unable to survive efficiently in murine and human macrophages, unlike WT CO92. We observed that the levels of Pla and its associated protease activity were not affected in the Δ*lpp* single mutant,

and, likewise, deletion of the *pla* gene from WT CO92 did not alter Lpp levels. Further, our study revealed that both Lpp and Pla contributed to the intracellular survival of WT CO92 via different mechanisms. Importantly, the ability of the Δ*lpp* Δ*pla* double mutant to be phagocytized by macrophages, to stimulate production of tumor necrosis factor-α and interleukin-6, and to activate the nitric oxide killing pathways of the host cells remained unaltered when compared to the WT CO92-infected macrophages. Finally, macrophages infected with either the WT CO92 or the Δ*lpp* Δ*pla* double mutant were equally efficient in their uptake of zymosan particles as determined by flow cytometric analysis. Overall, our data indicated that although the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 was highly attenuated, it retained the ability to elicit innate and subsequent acquired immune responses in the host similar to that of WT CO92, which are highly desirable in a live-attenuated vaccine candidate.

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**List of Abbreviations**

| | |
|---|---|
| UTMB | University of Texas Medical Branch |
| GSBS | Graduate School of Biomedical Sciences |
| TDC | Thesis and Dissertation Coordinator |
| WHO | World Health Organization |
| CDC | Centers for Disease Control and Infection |
| FDA | Food and Drug Administration |
| LPS | Lipopolysaccharide |
| Lpp | Braun lipoprotein |
| Pla | Plasminogen activator protease |
| Pst | Pesticin activity protein |
| Pim | Pesticin immunity protein |
| GsrA | Global stress requirement protein |
| LcrV | Low calcium response V antigen |
| CFR | Case fatality rate |
| CFU | Colony forming unit |
| $LD_{50}$ | Minimum lethal dose that kills 50% of animals |
| WT CO92 | Wild-type *Yersinia pestis* CO92 strain |
| HMDM | Human monocyte-derived macrophages |
| T3SS | Type three secretion system |
| LB | Luria-Bertani medium |
| HIB | Heart infusion broth |
| APC | Antigen presenting cells |
| PMA | Phorbol 12-myristate 13-acetate |
| KC | Keratinocyte-derived chemokine |
| IFN | Interferon |
| IL | Interleukin |
| TNF | tumor necrosis factor |
| G-SCF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte macrophage-colony-stimulating factor |
| TEM | Transmission Electron Microscopy |
| OD | Optical Density |
| ATCC | American Type Culture Collection |
| MOI | Multiplicity of Infection |
| FBS | Fetal Bovine Serum |
| DMEM | Dulbecco modified medium |
| NHP | non-human primate |
| AGM | African Green Monkeys |
| H&E | Hemotoxylin & eosin |
| s.c. | Subcutaneous |
| i.n. | Intranasal |
| SBA | sheep blood agar |
| ANOVA | analysis of variance |
| DMSO | dimethyl sulfoxide |
| NO | Nitric Oxide |

# Chapter 1: An Introduction to *Yersinia pestis*

## What is *Yersinia pestis*?

*Yersinia pestis,* a Gram-negative bacterium of the *Enterobacteriaceae* family, is the causative agent of bubonic, septicemic, and pneumonic plague. Pneumonic plague is the deadliest form in humans, with a 100% case fatality rate (CFR), if left untreated, and a 70% CFR with treatment (Prentice and Rahalison, 2007). Plague was once thought to be a disease of the past with over 200 million deaths that were attributed to three pandemics during which the plague microbe was spread by trade routes to nearly every continent (Perry and Fetherston, 1997; Huang *et al.*, 2006; Ligon, 2006; WHO, 2006; Prentice and Rahalison, 2007; Wagner *et al.*, 2014). However, with the expansion of international trade routes and global climate change, *Y. pestis* has been classified by the World Health Organization as a re-emerging human pathogen (WHO, 2006; CDC, 2008). In addition, the plague bacterium has gained notoriety as a bio-threat agent and is now categorized as a Tier-1 select agent by the Centers for Disease Control and Prevention (CDC) (Pearson, 1998; Inglesby *et al.*, 2000; CDC, 2008).

The plague bacterium is spread by the infected fleas of rodents, which can pass the infection to humans, accidental hosts, through a flea bite (Keeling and Gilligan, 2000). The organism is endemic in a wide variety of rodents and their fleas, thus making eradication impractical (Keeling and Gilligan, 2000). *Y. pestis* has also been known to naturally re-establish itself (possibly due to a third life cycle) that enables the bacterium to persist in a dormant state in dead hosts (WHO, 2006; Easterday *et al.*, 2011). For example, outbreaks occurred in Algeria (2003) after 48 years of silence, and in India (1994) after 28 years, each

outbreak had an extremely high number of plague-associated deaths, as the public health sector was unprepared for an outbreak (WHO, 2006; Easterday *et al.*, 2011). The availability of a vaccine as a prophylaxis would negate possible plague threats, either natural or intentional. Thus, human vaccination is highly desirable in endemic regions where plague is prevalent and in the case of a possible act of terrorism, as observed during the 2001 anthrax attack through the U.S. Postal Service and the recent use of ricin in letters sent to government officials (April 2013) (CDC, 2008).

**Why should we be concerned today about plague?**

There is no Food and Drug Administration (FDA)-licensed vaccine against plague. Although levofloxacin was recently approved by the FDA solely based on animal efficacy studies against all forms of plague for adults and children (FDA, 2012), the antimicrobials must be administered within 20-24 h after exposure for adequate protection (CDC, 2008; Quenee *et al.*, 2011). Promising recombinant subunit plague vaccines, which are under clinical investigation, consist of a low calcium response V (LcrV or V) antigen, a component of the type 3 secretion system (T3SS) as well as an effector, and the capsular antigen, F1. These vaccines elicit high antibody titers in rodents; however, their potential as efficacious vaccines has been debated because of varied responses in non-human primates (NHPs) (Smiley, 2008; Easterday *et al.*, 2011; Quenee *et al.*, 2011; Williamson *et al.*, 2011; FDA, 2012). Specifically, African green monkeys (AGMs), which mimic human symptoms and represent an ideal model to study *Y. pestis* infection (Meyer, 1970; Williamson *et al.*, 2005; Lin *et al.*, 2011; Rosenzweig *et al.*, 2011; Feodorova and Motin, 2012), exhibit highly varied protective responses to pneumonic plague (despite similar

antibody titers). These findings suggest that antibody titers to F1 and LcrV may not correlate with protection, and, more importantly, these antigens are poorly immunogenic in humans (Smiley, 2008).

In addition, F1-minus strains of *Y. pestis* that occur naturally are as virulent as the encapsulated organism; moreover, different strains of the plague bacterium possess LcrV with divergent amino acid sequences (Perry and Fetherston, 1997; Huang *et al.*, 2006; Anisimov *et al* 2009). Consequently, F1-LcrV-based vaccines may not be effective against all *Y. pestis* strains that exist in nature (Perry and Fetherston, 1997; Huang *et al.*, 2006; WHO 2006). Recent studies also indicated that a cell-mediated immune response, i.e., the necessity of $CD4^+$, $CD8^+$, and $T_H17$ cells, is crucial in host protection against pneumonic plague (Meyer, 1970; Williamson *et al.*, 2005; Smiley, 2008; Lin *et al.*, 2011; Rosenzweig *et al.*, 2011; Rosenzweig *et al.*, 2011; Feodorova and Motin, 2012), underscoring the need for developing a live-attenuated plague vaccine. *Y. pestis* EV76 vaccine strain, lacking the pigmentation locus, is currently used for immunizing people in the states of former Soviet Union (Meyer, 1970; WHO 2006; Feodorova and Motin, 2012). However, this vaccine is not approved by the FDA because of its high reactogenicity due to the presence of lipopolysaccharide (LPS), and the lack of characterization of this vaccine strain (Meyer, 1970; Feodorova and Motin, 2012; FDA, 2012) . Therefore, the goal of this study was to develop a viable live-attenuated vaccine candidate which would be able to protect mice against both pneumonic and bubonic plague by triggering humoral- and cell- mediated immune responses. This candidate strain might serve as a starting point from which other virulence genes could be deleted to develop a safe and efficacious vaccine for human use. We chose to focus on the genes encoding Braun lipoprotein (*lpp*) and plasminogen

activator (*pla*) protease. These two genes are located on different parts of the genome (chromosome versus the plasmid, respectively), and deletion of these genes would lead to the development of a stable mutant with minimal probability of reversion to the wild-type (WT) phenotype.

**The genes deleted in our live-attenuated vaccine candidate**

Lpp represents one of the components of the bacterial cell wall, and one copy of this gene is encoded on the 4.65 Mb chromosome of *Y. pestis* CO92 (Perry and Fetherston, 1997). Lpp links the peptidoglycan layer to the outer bacterial membrane, and our earlier studies have shown that its absence decreases the ability of *Y. pestis* to survive in macrophages (Sha *et al.*, 2008; Liu *et al.*, 2010). Further, Lpp triggers toll-like receptor (TLR)-2 leading to an inflammatory response in the host cells (Aliprantis *et al.*, 1999; Sha *et al.*, 2008). Specifically, Lpp and LPS purified from *Y. entercolitica* synergize to induce the production of pro-inflammatory cytokines both *in vitro* and *in vivo* leading to septic shock; one of the hallmarks of a severe infection with *Y. pestis* (Aliprantis *et al.*, 1999; Sha *et al.*, 2008).

The *pla* gene is encoded on the 9.5 kb pPCP-1 plasmid at approximately 180 copies per bacterial cell (Sodeinde and Goguen, 1988). Two other functional proteins, namely Pst (pesticin activity protein) and Pim (pesticin immunity protein) are also encoded on pPCP-1 plasmid, and provide a selective growth advantage to virulent *Y. pestis* (Sodeinde and Goguen, 1988; Lahteenmaki *et al.*, 1998). Pla is a surface protease of the omptin family which functions by cleaving plasminogen to plasmin and by degrading α2-anti-plasmin, a plasmin inhibitor (Sodeinde and Goguen, 1988; Lahteenmaki *et al.*, 1998; Perry R, 2007).

This function aids in the dissolution of fibrin clots, allowing deep tissue dissemination of the bacteria during bubonic plague (Sodeinde *et al.*, 1992; Sebbane, Jarrett, *et al.*, 2006). Fibrin deposits represent an important first step in combating *Y. pestis* infections by preventing bacterial dissemination and killing of the microbes through the recruitment of neutrophils and potentially $T_H17$ cells via production of interleukin (IL)-6 by thrombin (Szaba and Smiley, 2002; Lathem *et al.*, 2007; Rosenzweig *et al.*, 2011). Pla also plays a role in pneumonic plague, as the Δ*pla* mutant fails to colonize lung tissue in high numbers (Szaba and Smiley, 2002; Lathem *et al.*, 2007; Caulfield and Lathem, 2012; Luo *et al.*, 2013).

In an earlier study, we showed that the Δ*lpp* single mutant was attenuated in both bubonic and pneumonic mouse models of plague (Sha *et al.*, 2008). We also observed that curing of the pPCP-1 plasmid from the Δ*lpp* isogenic mutant resulted in a highly attenuated disease phenotype for pneumonic plague in a mouse model (Agar *et al.*, 2009). These studies led us to construct a double isogenic mutant, Δ*lpp* Δ*pla,* to directly discern the extent of bacterial attenuation by targeting two genes, without affecting other gene products encoded by the pPCP-1 plasmid. Consistent with our hypothesis, the Δ*lpp* Δ*pla* double mutant exhibited synergistic attenuation of virulence in evoking pneumonic plague, and it triggered protective humoral- and cell- mediated immunity in mice, which represent the hallmarks of an effective vaccine.

# Chapter 2: Materials and Methods

## METHODOLOGIES USED FOR *IN VIVO* MURINE EXPERIMENTS

### Bacterial strains and plasmids

All of the bacterial strains and plasmids used in this study are listed in **Table 1**. *Y. pestis* was grown in heart infusion broth (HIB) (Difco, Voigt Global Distribution Inc., Lawrence, KS) at 26 to 28$^{o}$C with constant agitation (180 rpm). On the solid surface, *Y. pestis* was grown on either HIB agar or 5% sheep blood agar (SBA) plates (Teknova, Hollister, CA). Luria-Bertani (LB) medium was used for growing recombinant *Escherichia coli* at 37$^{o}$C with agitation. All of our studies were performed in a Tier-1 select agent BSL2 laboratory located in the Galveston National Laboratory.

Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, WI). Advantage cDNA PCR kits were purchased from Clontech (Palo Alto, CA). All digested plasmid DNA or DNA fragments from agarose gels were purified by using QIAquick kits (Qiagen, Inc., Valencia, CA).

**Table 1: Bacterial strains and plasmids used in this study**

| Strain/plasmid | Description and Source | Reference |
|---|---|---|
| **Strains** | | |
| *Y. pestis* strain CO92 | Virulent WT *Y. pestis* isolated in 1992 from a fatal pneumonic plague case, biovar Orientalis, and naturally resistant to polymyxin B | (Doll *et al.*, 1994; Parkhill *et al.*, 2001) |
| CO92 [pBR322] | WT *Y. pestis* transformed with pBR322 ($Tc^s$) | (Agar *et al.*, 2009; Sha *et al.*, 2008) |
| Δ*lpp* CO92 | *lpp* gene deletion mutant of CO92 | (Sha *et al.*, 2008; Agar *et al.*, 2009) |
| Δ*lpp* Tn*7*-*lpp* | *lpp* gene deletion mutant of CO92 complemented with the *lpp* gene in *cis* by using the targeted Tn*7* | (Agar *et al.*, 2009) |
| Δ*lpp* CO92 [pBR322] | *lpp* gene deletion mutant of CO92 transformed with pBR322 ($Tc^s$) | (Galindo *et al.*, 2010) |
| Δ*lpp* CO92 [pBR322*YgsrA*] | *lpp* gene deletion mutant of CO92 complemented with the *gsrA* gene via plasmid pBR322*YgsrA* ($Tc^s$) | (Galindo *et al.*, 2010) |
| Δ*pla* CO92 | *pla* gene in-frame deletion mutant of *Y. pestis* CO92 | (van Lier *et al.*, 2014) |
| Δ*pla* CO92 [pBR322] | *pla* gene deletion mutant of CO92 transformed with pBR322 ($Tc^s$) | (van Lier *et al.*, 2014) |
| Δ*pla* CO92 [pBR322*YgsrA*] | *pla* gene deletion mutant of CO92 complemented with the *gsrA* gene via plasmid pBR322*YgsrA* ($Tc^s$) | This study |
| Δ*lpp* Δ*pla* CO92 | *lpp* and *pla* gene double deletion mutant of *Y. pestis* CO92 | (van Lier *et al.*, 2014) |
| Δ*lpp* Δ*pla* [pBR322] | Δ*lpp* Δ*pla* CO92 double mutant transformed with pBR322 ($Tc^s$) | (van Lier *et al.*, 2014) |
| Δ*lpp* Δ*pla* [pBR322*Ypla*] | Complemented *Y. pestis lpp* and *pla* -double deletion mutant via plasmid pBR322*Ypla;* ($Tc^s$) | (van Lier *et al.*, 2014) |
| Δ*lpp* Δ*pla* CO92 [pBR322*YgsrA*] | *lpp* and *pla* double gene deletion mutant complemented with the *gsrA* gene via plasmid pBR322*YgsrA* ($Tc^s$) | This study |
| *E. coli* SM10 | Contains the *λpir* gene (lysogenized with *λpir* phage) and it is designed for cloning and propagation of plasmid with R6K origin of replication | (Miller and Mekalanos, 1988) |
| **Plasmids** | | |
| pBR322 | Cloning vector for complementation ($Tc^r$ & $Ap^r$) | GE Healthcare |
| pKD13 | Template plasmid for PCR amplification of the $Km^r$ gene cassette flanked by FLP sites | (Datsenko and Wanner, 2000) |
| pFlp2 | Produces the FLP recombinase to remove the $Km^r$ gene cassette from the mutant | (Choi and Schweizer, 2005) |
| pDMS197 | Suicide vector with a conditional R6K origin of replication (*ori*) and a levan sucrase gene (*sacB*) from *Bacillus subtilis* used for homologous recombination | (Edwards *et al.*, 1998) |
| pBR322*Ypla* | Recombinant plasmid containing the *pla* gene coding region and its putative promoter in vector pBR322 used to complement the Δ*pla* mutant of *Y. pestis* ($Tc^s$) | (van Lier *et al.*, 2014) |
| pDMS197*pla*UDKm | The suicide vector pDMS197 based recombinant plasmid containing the *Y. pestis* CO92 *pla* gene up- and downstream flanking DNA fragments with the $Km^r$ cassette was used for homologous recombination. | (van Lier *et al.*, 2014) |
| pBR322*YgsrA* | Recombinant plasmid containing the *gsrA* gene coding region and its putative promoter in vector pBR322 used to complement the Δ*pla* mutants | This study |

**Construction of Δ*pla* single- and Δ*lpp* Δ*pla* double- in-frame deletion mutants of *Y. pestis* CO92**

The Δ*pla* single- and Δ*lpp* Δ*pla* double- in-frame deletion mutants were generated by homologous recombination (Sha *et al.*, 2008). Based on the genome sequence of WT CO92 (GenBank accession number NC_003143), the up- and downstream DNA sequences flanking the *pla* gene were polymerase chain reaction (PCR) amplified using the primer sets $Pla_{up}5$-$Pla_{up}3$ and $Pla_{dn}5$-$Pla_{dn}3$, respectively (**Table 2**). A kanamycin resistance ($Km^r$) gene cassette with flippase (FLP) recombinase recognition target sites at both ends was PCR amplified from plasmid pKD13 using a specific primer set Km5-Km3 (**Tables 1&2**). The above-mentioned DNA fragments were ligated together through their compatible restriction enzyme sites (**Table 2**). The resulting *pla* upstream flanking region-$Km^r$ gene cassette-and the *pla* downstream flanking region containing DNA fragment was subsequently cloned into the pDMS197 suicide vector at the appropriate restriction enzyme sites (Datsenko and Wanner, 2000) using *E. coli* SM10 **(Table 1)**. The recombinant plasmid pDMS197*pla*UDKm **(Table 1)** was then transformed by electroporation (Genepulser Xcell; Bio-Rad, Hercules, CA) (Sha *et al.*, 2008) into the WT or the Δ*lpp* single mutant of CO92, generating either a Δ*pla* single- or a Δ*lpp* Δ*pla* double mutant, respectively. The transformants were plated onto HIB agar plates containing 5% sucrose and 100 µg/ml kanamycin. The $Km^r$ colonies were PCR screened using primer sets Pla5-Pla3 and Up5-Dn3 (**Table 2**). The colonies showing genomic replacement of the *pla* gene with the $Km^r$ gene cassette (intermediate form) were transformed with the plasmid pFlp2, which expresses the gene encoding FLP recombinase, to remove the $Km^r$ gene cassette. The plasmid pFlp2 was then cured with 5% sucrose; the final Δ*pla* mutants were sensitive

to kanamycin ($Km^s$) and free of plasmid pFlp2. An in-frame deletion of the *pla* gene was further confirmed by genomic sequencing with the primer Spla (**Table 2**).

**Table 2: Sequences of the primers used in this study**

| Primer | Primer sequences 5'-3'(restriction enzymes)* | Purpose |
|---|---|---|
| $Pla_{up}5$-$Pla_{up}3$ | AATGAGCTCCGGCCAGGCAGATCCACATAATG (*Sac*I), ATCGGATCCTCATTAGACACCCTTAATC (*Bam*HI) | PCR amplification of the upstream flanking fragment to the *pla* gene of *Y. pestis* CO92 |
| $Pla_{dn}5$-$Pla_{dn}3$ | ATCGAATTCTGAAAAATACAGATCATATC (*Eco*RI), TTCGGTACCTATAACTCGTAGGTTATATTG (*Kpn*I) | PCR amplification of the downstream flanking fragment to the *pla* gene of *Y. pestis* CO92 |
| Km5-Km3 | ATTCCGGGGATCCGTCGACC(*Bam*HI), CTTGAATTCTGTAGGCTGGAGCTGCTT(*Eco*RI) | PCR amplification of the $Km^r$ gene cassette with FLP recombinase recognition target sites at both ends from plasmid pKD13 |
| Pla5-Pla3 | TTACAGTTGCAGCCTCCACC, ATTCTTATCAATGGTCTGAG | PCR amplification of the coding region of the *pla* gene of *Y. pestis* CO92 |
| Up5-Dn3 | TCCATCTCCGTATCAATCGG, TTGCCGTGATCGCGCTGAAC | *pla* mutant verification primers located on the bacterial chromosome outside the flanking DNA sequences that were used to generate the *pla* mutants of *Y. pestis* CO92 |
| Spla | CCACCGTTTCTTATGTGAGC | Primer located upstream of the *pla* gene; used to confirm the in-frame deletion of the *pla* gene by chromosomal DNA sequencing |
| $Cpla_{up}$-$Cpla_{dn}$ | ATGCATGCGGCTCAACGCTCGTTGTCG (*Sph*I) ACGTCGACTCAGAAGCGATATTGCAGAC (*Sal*I) | Primers used to amplify the *pla* gene and its promoter region for the complementation studies |

*Underlining indicates restriction enzyme sites in the primers

**Complementation of the Δ*pla* mutant strains of *Y. pestis* CO92**

The coding region of the *pla* gene, along with its promoter, was PCR amplified using the following primers: $Cpla_{up}$ and $Cpla_{dn}$ **(Table 2)**, and the amplified DNA fragment was cloned into the pBR322 plasmid vector, thus generating the recombinant plasmid pBR322*Ypla*. The pBR322*Ypla* recombinant plasmid was then transformed into Δ*pla* single- and Δ*lpp* Δ*pla* double- *Y. pestis* mutant strains via electroporation (**Table 1**) (Sha *et al.*, 2008; Agar *et al.*, 2009). The complemented *Y. pestis* strains Δ*pla* [pBR322*Ypla*] and Δ*lpp* Δ*pla* [pBR322*Ypla*] were sensitive to tetracycline due to inactivation of the $Tc^r$ gene on the plasmid. As appropriate controls, $Tc^s$ variant of the pBR322 vector (Agar *et al.*, 2009) was also electroporated in WT CO92, Δ*pla* single- and Δ*lpp* Δ*pla* double- mutants of *Y. pestis* (**Table 1**).

***In vitro* characterization of the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92**

**Functionality of the type 3 secretion system (T3SS)**

The low calcium response (LCR) is a distinctive trait of the *Yersinia* T3SS in which effectors designated as *Yersinia* outer membrane proteins (Yops), such as YopE and YopH, are secreted in response to a low calcium signal. To demonstrate functionality of the T3SS, the secretion of Yops from *Y. pestis* cultures (WT as well as Δ*lpp*-, Δ*pla*-, and Δ*lpp* Δ*pla* mutants of CO92) was induced by the addition of EGTA (Sigma-Aldrich, St. Louis, MO) as we previously described (Sha *et al.*, 2008; Agar *et al.*, 2009). Briefly, aliquots (500 µl) from different cultures were removed after 5 min of EGTA addition, followed by separation of bacterial cells from the supernatants. The presence of Yops in cell pellets and

supernatants was analyzed by immunoblotting using antibodies to YopE (Santa Cruz Biotechnology, Santa Cruz, CA) and YopH (Agrisera, Stockholm, Sweden) (Sha *et al.*, 2008; Agar *et al.*, 2009).

**Measurement of the minimum inhibitory concentration (MIC) of gentamicin**

The MICs of gentamicin for WT *Y. pestis* CO92 and its double mutant Δ*lpp* Δ*pla* were determined by using the E-test (BioMérieux Inc., Durham, NC). Briefly, the overnight cultures were diluted (1:4) with fresh HIB and continued to grow at 28°C for 2 h ($OD_{600}$ of 0.6). The bacterial cultures were then spread evenly onto SBA plates, and the pre-defined gentamicin E-strips (range 0.016 to 256 μg/ml) were placed in the center of the plates, which were then incubated for 48 h at 28°C or 37°C, and the MICs were recorded.

**Serum resistance and membrane blebbing**

WT CO92 and its Δ*lpp* Δ*pla* double mutant were incubated with sera pooled from 5 naïve mice. The heat inactivated sera and phosphate-buffered saline (PBS) served as controls. After 1 h incubation at 37°C, the number of surviving bacteria (cfu) in each sample was determined by serial dilutions and plating on SBA plates, as we previously described (Sha *et al.*, 2008; Agar *et al.*, 2009).

To evaluate membrane integrity, both the WT CO92 and the Δ*lpp* Δ*pla* double mutant were grown to exponential phase at 28°C ($OD_{600}$ of 0.8). The cells were washed, pelleted, fixed, and subjected to transmission electron microscopy (Sha *et al.*, 2004).

**Survival of WT *Y. pestis* CO92 and its mutant strains in murine macrophages**

RAW 264.7 cells were infected with the WT CO92 and its mutant strains at a multiplicity of infection (MOI) of 1. RAW 264.7 cells were grown in Dulbecco modified eagle medium (DMEM) supplemented with 10% FBS. The infected macrophages were incubated at 37°C with 5% $CO_2$ for 30 min followed by 1 h of treatment with 100 μg/ml gentamicin to kill extracellular bacteria. The surviving bacteria inside the macrophages were enumerated immediately after the gentamicin treatment (0 h time point) and 4 h later by serial dilution and plating, as we previously described (Sha *et al.*, 2008, Agar *et al.*, 2009).

**Animal studies**

All of the mice studies were performed in the ABSL-3 facility under the protocol approved by the UTMB Institutional Animal Care and Use Committee. Six to eight-week-old Swiss Webster female mice were purchased from Taconic Laboratories (Germantown, NY) and challenged by either the subcutaneous (s.c.) or intranasal (i.n.) route to induce bubonic or pneumonic plague, respectively, with WT *Y. pestis* CO92, Δ*lpp*-, Δ*pla*-, or Δ*lpp* Δ*pla*- mutant strains as we previously described (Sha *et al.*, 2008). The inoculation of bacterial-suspension into nares would lead to animals inhaling bacterial droplets resulting in developing pneumonic plague. To generate survival curves, mice were assessed for mortality over a period of 30 days. For histopathology, cytokine/chemokine analysis, ELISA for antibody isotyping, and bacterial dissemination, the sera and organs of animals were harvested at the indicated time points (see below).

**Histopathology and bacterial dissemination**

Mice infected with $5x10^5$ cfu (representing 1,000 $LD_{50}$ of WT CO92 where 1 $LD_{50}$=500 cfu) of WT CO92, Δ*lpp*-, Δ*pla*-, and the Δ*lpp* Δ*pla*- double mutant strain by the i.n. route were euthanized post infection (p.i.) at 2, 6, 8, and 14 days. A portion of lungs, liver, and spleen from 3 representative animals was removed and immersion-fixed in 10% neutral buffered formalin (Sha *et al.*, 2008; Agar *et al.*, 2009). The tissues were sectioned at 5 μm, mounted on glass slides, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy in a blinded fashion. Tissue lesions were scored based on a severity scale, which correlated with estimates of lesion distribution and extent of tissue involvement (minimal=2-10%, mild >10-20%, moderate >20-50%, and severe >50%), as previously described (Sha *et al.*, 2008; Agar *et al.*, 2009). In some sections, bacteria consistent with *Y. pestis* infection were present, although bacteria-specific staining was not performed.

At the indicated time points, various tissues (lungs, liver, spleen and the blood) were evaluated for the dissemination of bacteria (Sha *et al.*, 2008). The bacterial colonies were counted for determining cfu/organ or cfu/ml (for the blood).

**Measurement of antibodies and cytokines/chemokines in the sera**

Concurrently with the collection of tissues for histopathology and bacterial dissemination from infected mice, blood samples were drawn on days 2, 4, 8, and 16 for mice injected by the s.c. route with the bacteria and on days 2, 4, 6, and 14 in those inoculated by the i.n. route. Uninfected naïve mice served as the control group. The sera were extracted and filtered using 0.22 μm centrifuge tube filters from Costar (Corning

Incorporates, Corning, NY). The cytokine/chemokine levels in sera samples were analyzed as we previously described (Sha *et al.*, 2008).

Specific antibody responses (IgG and various isotypes) to WT CO92 (whole bacteria) as well as to F1-LcrV (F1-V) antigens of CO92 were measured by ELISA. Briefly, ELISA plates were either coated overnight at 4$^{o}$C with F1-V (1 ng/ml, BEI Resources, Manassas, VA) or the whole plague bacterium. For preparing the latter, the WT CO92 culture was grown overnight at 28$^{o}$C in HIB. The culture was diluted and the growth temperature shifted to 37$^{o}$C until an $OD_{600}$ of 0.8 was reached. This shift in the temperature allowed F1 production and T3SS induction (Sha *et al.*, 2008). The culture was then re-suspended to a concentration of 5 x $10^{9}$ cfu/ml and used to coat plates treated with poly-L-lysine (10 μg/ml). A serial dilution (1:5) of sera was made to evaluate antibody titers, which were defined as the inverse of the highest serum dilution giving an absorbance reading of ≥0.2. Antibody classes and IgG isotypes were also examined by using specific isotype secondary antibodies as we previously described (Tao *et al.*, 2013).

**T cell responses**

A total of 60 mice were challenged by the i.n. route with 250 cfu of WT CO92 or its Δ*lpp* Δ*pla*- double mutant (30 mice per group). At 14, 21, and 60 days p.i., spleens from 5 mice in each infected group were harvested and T cells isolated from each animal by using a CD3ε MicroBead Kit from MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). T cells isolated from uninfected mice were used as controls at each time point. All isolated T cells were incubated *ex vivo* with γ-irradiated, antigen presenting cells (APCs) from naïve mice that were either pulsed with heat-killed WT CO92 or remained

un-pulsed. The proliferation of T cells was measured by their abilities to incorporate [$^3$H] thymidine as we previously described (Sha *et al.*, 2013). The culture supernatants were collected at 48 h and subjected to a multiplex assay with Milliplex (Millipore), which detected 6 cytokines/chemokines.

The T cell subsets producing interferon (IFN)-γ and tumor necrosis factor (TNF)-α were measured by flow cytometry. Briefly, T cells from animals infected with the WT CO92, Δ*lpp* Δ*pla*- double mutant, or uninfected mice were cultured as described above with pulsed APCs in 24-well plates at 37°C for 5 days. Cells were treated with ionomycin (1 μg/ml) and phorbol 12-myristate 13-acetate (PMA, 10 μg/ml), and 2 h later, incubated with Brefeldin A (1 μg/ml) for 3 h to accumulate intracellular cytokines. The T cells were then surface stained with monoclonal anti-mouse CD4-Pac blue (BioLegend, San Diego, CA), viable dye APC/Cy7 (Molecular Probes, Eugene, OR), and anti-mouse CD8-PE (BioLegend) for 30 min in the dark at 4$^o$C. Subsequently, the T cells were permeabilized with a staining buffer set (eBioscience, San Diego, CA) and stained with IFN-γ-FITC (BioLegend) and TNF-α-PECy7 (BioLegend) for 30 min. The cells were acquired on a flow cytometer (LSRII Fortessa, UTMB Core) and analyzed by using FACS diva software.

**Statistical analysis for *in vivo* murine studies**

Whenever appropriate, analysis of variance with the Bonferroni correction or Tukey's test post-hoc with one-way ANOVA was employed for data analysis. We used Kaplan-Meier survival estimates for animal studies, and $p$ values of $\leq 0.05$ were considered significant.

## METHODOLOGIES USED FOR *IN VITRO* MACROPHAGE EXPERIMENTS

### Bacterial strains and plasmids

All of the bacterial strains and plasmids used in this study are listed in **Table 1**. *Y. pestis* was grown in heart infusion broth (HIB) (Difco, Voigt Global Distribution Inc., Lawrence, KS) at 26 to 28°C with constant agitation (180 rpm). On the solid surface, *Y. pestis* was grown on either HIB agar or 5% sheep blood agar (SBA) plates (Teknova, Hollister, CA). All of our studies were performed in a Tier-1 select agent Biosafety level (BSL)-2 laboratory located in the Galveston National Laboratory, at the University of Texas Medical Branch (UTMB).

### Complementation of the mutant strains of *Y. pestis* CO92 with *gsrA*

The coding region of the *gsrA* gene, along with its promoter, was PCR amplified, and the amplified DNA fragment was cloned into the pBR322 plasmid vector, thus generating the recombinant plasmid pBR322*YgsrA* as we previously described (Galindo *et al* 2010). The pBR322*YgsrA* recombinant plasmid was then transformed into Δ*lpp* and Δ*pla* single- and Δ*lpp* Δ*pla* double- *Y. pestis* mutant strains via electroporation (**Table 1**) (Sha *et al.*, 2008; Agar *et al.*, 2009; Galindo *et al* 2010). The *gsrA* complemented *Y. pestis* strains Δ*lpp* [pBR322*YgsrA*], Δ*pla* [pBR322*YgsrA*], Δ*lpp* Δ*pla* [pBR322*YgsrA*] were sensitive to tetracycline due to inactivation of the $Tc^r$ gene on the plasmid (**Table 1**).

### Human monocyte isolation and derivation of macrophages

Human buffy coats were obtained separately from three healthy individuals in 10 ml Vacutainer tubes without additive (Becton Dickinson Labware, Franklin Lakes, NJ)

from the UTMB blood bank under an Institutional Review Board-approved protocol. EDTA-treated blood from the donors was handled under endotoxin-free conditions; diluted 1:1 with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMC) were purified by centrifugation over a Ficoll-sodium diatrizoate solution (Ficoll-Paque$^{TM}$ PLUS, GE Healthcare Bio-sciences AB, Uppsala, Sweden). Monocytes were then purified from PBMCs by positive selection, using human CD14 microbeads and a magnetic column separation system (Miltenyi Biotec, Auburn, CA). Human monocyte-derived macrophages (HMDMs) were generated from purified $CD14^+$ monocytes.

Briefly, monocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, HEPES, sodium pyruvate, penicillin- streptomycin, plus granulocyte macrophage-colony stimulating factor (GM-CSF) at 100 ng/ml (Leukine, Genzyme, Cambridge, MA). Monocytes were seeded in 6-or 24-well tissue-culture plates at $10^6$ cells/ml. Adherent HMDMs were obtained at 6 days post culture. Viability of HMDMs was determined by Trypan blue staining, and cells were only used when viability exceeded 95%.

**Survival of WT *Y. pestis* CO92, its mutants, and the complemented strains in murine RAW 264.7 bone marrow-derived macrophages, murine MH-S alveolar macrophages, and HMDMs**

MH-S and RAW 264.7 macrophages (American Type Culture Collection [ATCC], Manassas, VA) were plated at a concentration of 1 x $10^6$ cells/well in a 12-well tissue culture plate to establish confluent monolayers. MH-S cells were grown in RPMI-1640 supplemented with 10% FBS and 0.5 mM 2-mercaptoethanol, whereas RAW 264.7 cells

were grown in Dulbecco modified eagle medium (DMEM) supplemented with 10% FBS. Both of the cell lines were infected with WT CO92, its mutants, or the complemented strains at a multiplicity of infection (MOI) of 1. The infected macrophages were incubated at 37°C with 5% $CO_2$ for 45 min followed by 1 h of treatment with 10 μg/ml gentamicin for MH-S cells and 100 μg/ml gentamicin for RAW 264.7 cells to kill extracellular bacteria. The surviving bacteria inside the macrophages were enumerated immediately after the gentamicin treatment (0 h time point) and subsequently at 4 h by serial dilution and plating on SBA plates (Sha *et al.*, 2008; Agar *et al.*, 2009).

HMDMs were infected with WT CO92 and the Δ*lpp* Δ*pla* double mutant at an MOI of 0.5. The infected macrophages were incubated at 37°C with 5% $CO_2$ for 40 min followed by 1 h of treatment with 10 μg/ml gentamicin to kill extracellular bacteria. The surviving bacteria inside the macrophages were enumerated immediately after the gentamicin treatment (0 h time point) and subsequently at 1, 2, and 4 h by serial dilution and plating (Sha *et al.*, 2008; Agar *et al.*, 2009).

**Visualization of intracellular WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant in HMDMs by confocal microscopy**

Post-infection (40 min of infection followed by 1 h of gentamicin treatment) with WT CO92 or its Δ*lpp* Δ*pla* double mutant at an MOI of 0.5, 4% freshly prepared paraformaldehyde and 0.5% Triton X-100 were used to fix and permeabilize the HMDMs. The fixed macrophages were then stained with primary mouse anti-F1 antibodies (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary Alexa Fluor 488 (green) donkey anti-goat antibodies (Molecular Probes, Carlsbad, CA). Uninfected

macrophages were used as negative controls. Mounting medium with DAPI (blue) (Vector Laboratories, Burlingame, CA) and Alexa Fluor 568 (red) phalloidin (Molecular Probes) were used to stain the nucleus and actin, respectively, of macrophages.

**Western blot analysis**

Bacterial cultures were grown in 3 ml of HIB at 28°C and 37°C overnight with shaking. The bacteria were harvested, dissolved by boiling in 2x SDS-PAGE sample buffer, and then checked for sterility. Western blot analysis was performed with specific primary anti-Lpp or anti-Pla antibodies (1:1,000 dilution), followed by horseradish peroxidase-labeled goat anti-mouse secondary antibodies (1:5,000 dilution). The blots were developed using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). The antibodies to Lpp and Pla were available in the laboratory.

**Pla protease activity assay**

All tested cultures (WT CO92, its mutants, and the complemented strains) were plated on HIB agar plates from -80°C glycerol stocks and incubated at 28°C for 36 h. Single colonies of each culture were then re-plated on fresh HIB agar plates and incubated at 28°C or 37°C for 20-22 h. Bacteria from each plate were suspended and diluted in PBS to obtain absorbance values ($OD_{600}$) of 0.1 and 0.5 in a spectrophotometer (SmartSpec$^{Tm}$ 300, Bio-Rad). For each strain, 50 µl suspensions were added to wells of a black microtiter plate (Costar Corning Inc., Corning, NY) in triplicate. The hexapeptide substrate (5.0 µg/µl) in dimethyl sulfoxide (DMSO) (Agarkov *et al.*, 2008), was diluted to a final concentration of 2.5 µg/50 µl, and added to each well containing bacterial cells. Pla activity was then

measured in a fluorometric assay (Excitation/Emission 360/460nm) at 37°C on BioTek Synergy HT spectrophotometer (BioTek Instruments Inc., Winooski, VT). The substrate (DABCYL-Arg-Arg-Ile-Asn-Arg-Glu (EDANS)-$NH_2$) was synthesized on Sieber amide resin (Agarkov *et al.*, 2008). The kinetics of substrate cleavage by Pla was measured every 15 min for 2 h. The increase in fluorescence as a function of time for WT CO92, its mutants, and the complemented strains was recorded and $V_{max}$ calculated using Prism (GraphPad, La Jolla, CA).

**Measurement of cytokines in the supernatants of WT *Y. pestis* CO92 and the Δ*lpp* Δ*pla* double mutant- infected macrophages**

The supernatants from infected macrophages were collected for each cell line at 100-105 min (post-infection and gentamicin treatment), and filtered using 0.1 μm centrifuge tube filters from Costar (Corning Inc.). The cytokine levels in undiluted supernatant samples were analyzed by 6-plex Bio-Rad Bioplex, as we previously described (Sha *et al.*, 2013; van Lier *et al.*, 2014).

**Measurement of nitric oxide levels in the supernatants of macrophages infected with WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant**

Nitric oxide (NO) levels in WT CO92 and its Δ*lpp* Δ*pla* double mutant stimulated macrophages was determined by measuring the accumulation of nitrite, a stable metabolite of the reaction of NO with oxygen, using the Griess reaction assay kit (Molecular Probes). The supernatants were collected 12 h after infection, and an aliquot (130 μl) of each supernatant was mixed with 20 μl of Griess reagent (0.5% sulfanilamide; 0.05% *N*-[1-

naphthyl] ethylenediamine in 2.5% acetic acid), and 150 µl deionized water. The accumulation of nitrite was then measured spectrophotometrically (SpectraMax M5$^{e}$, Molecular Devices, Sunnyvale, CA) at 548 nm. The concentration of nitrite was calculated by using a standard curve prepared with sodium nitrite.

**Phagocytosis of Zymosan particles by macrophages infected with WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant**

Macrophages (RAW 264.7, MH-S, and HMDMs) were infected with WT CO92 and the Δ*lpp* Δ*pla* double mutant as described above. After 1 h of gentamicin treatment, infected host cells were incubated with serum (mouse or human depending upon the cell type used)-opsonized synthetic latex beads (1 µm size) at an MOI of 1 per well (Life Technologies, Carlsbad, CA). The plates were incubated for 1.5 h at 37°C with slow agitation (80 rpm). After incubation with the beads, the macrophages were washed with PBS, also collecting all of the supernatants for floating cells, and spun to collect a pellet at 1,200 rpm for 10 min. Macrophages were then re-suspended in Trypan blue, fixed with 10% formalin, and checked for sterility. The samples were read on the LSRII Fortessa (BD Biosciences, San Jose, CA) (Excitation/Emission 580/605nm) and analyzed with FlowJo (Ashland, OR).

**Statistical analyses for *in vitro* macrophage studies**

Whenever appropriate, ANOVA with the Bonferroni correction or Tukey's *post hoc* test with one-way ANOVA was employed for data analysis. The $p$ values of $\leq 0.05$ were considered significant. All of the experiments were performed in triplicate.

# Chapter 3: Bubonic plague in a murine model

## *In vitro* characterization of Δ*lpp* and Δ*pla* single- and Δ*lpp* Δ*pla* double- mutants of *Y. pestis* CO92

An in-frame deletion of the *pla* gene from WT bacteria and the Δ*lpp* mutant of CO92 resulting in Δ*pla* single- and Δ*lpp* Δ*pla* double- mutant was ascertained by DNA sequencing of the flanking regions to the target gene. The growth rate of the mutants compared to WT CO92 did not show any defects (data not shown). The deletion of the *pla* gene from multiple copies of the pPCP-1 plasmid as well as of the *lpp* gene from the chromosome in the mutants was confirmed by PCR and Western blot analysis (data not shown).

The secretion and translocation of Yops through the T3SS represents a key virulence mechanism of *Y. pestis*, which are tightly regulated. Under high calcium environment (e.g., HIB medium), secretion and translocation of Yops is restricted but triggered rapidly when bacteria come in contact with the eukaryotic cells or when calcium is chelated by the addition of EGTA (Rosenzweig *et al.*, 2005; Lawal *et al.*, 2013; Sha *et al.*, 2013). To determine whether deletion of the above-mentioned genes affected any other phenotype(s) in the mutant which could compromise bacterial virulence, we evaluated the functionality of the T3SS, specifically by measuring the secretion of T3SS effectors (YopE and YopH) via Western blot analysis. As shown in **Fig. 1A**, similar levels of these effectors were noted in the bacterial pellet and the supernatant across all tested strains upon addition of EGTA. We also examined cell membrane integrity by performing transmission electron

microscopy and found no indication of membrane blebbing when comparing WT CO92 with its Δ*lpp* Δ*pla* double mutant.

However, in some bacterial mutant cells, the outer membrane appeared to be vesiculated along their surfaces (**Fig. 1B**). Likewise, it was noted that some Δ*lpp* Δ*pla* double mutant cells had a tighter periplasmic space which seemed to be filled with electron-dense material when compared to the WT CO92 cells.

To examine survivability of *Y. pestis* CO92 mutants in macrophages, RAW 264.7 cells were used. As depicted in **Fig. 2**, WT CO92 could survive in macrophages 4 h p.i. in a gentamicin protection assay, but the single (Δ*lpp*- and Δ*pla*-) and the double (Δ*lpp* Δ*pla*) mutants could not efficiently resist the hostile environment of the macrophages. Similar survival data were noted at later time points of 8 and 24 h (data not shown). These effects were not related to the differential sensitivity to gentamicin as the MIC of this antimicrobial for both the WT CO92 and the Δ*lpp* Δ*pla* double mutant was 0.5 μg/ml at 37°C and 0.75 μg/ml at 28°C.

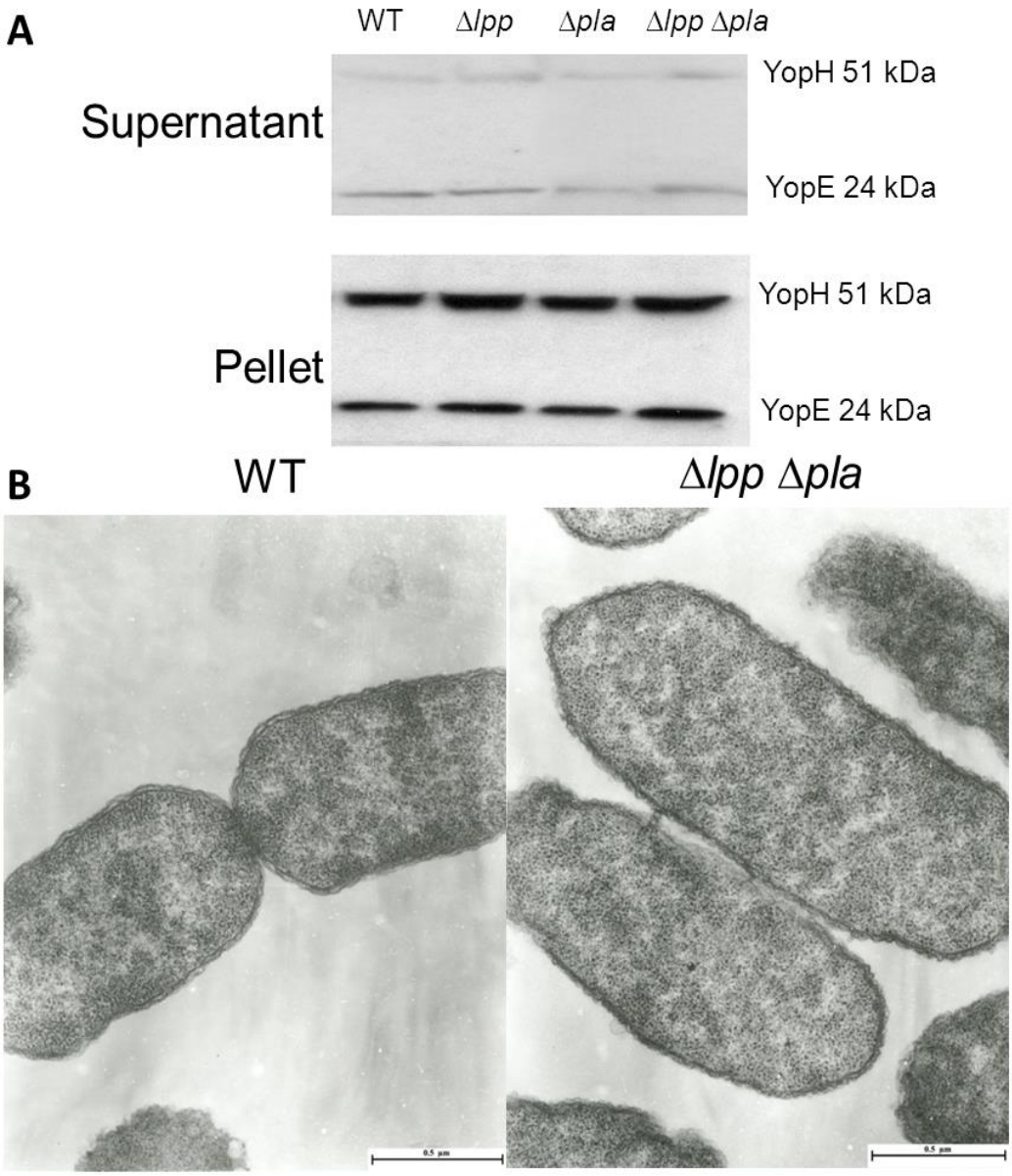


**Fig. 1: T3SS function and transmission electron microscopy.**

WT CO92, Δ*lpp*, Δ*pla*, and Δ*lpp* Δ*pla* mutants were grown overnight and then diluted 1:20 with fresh HIB. The cultures were grown for an additional 3 h at 28°C and then shifted to 37°C for 2 h. The secretion of YopE and YopH was induced by the addition of 5 mM EGTA. Culture supernatants and pellets were collected 5 min after the EGTA addition. The presence of YopE and YopH in the samples was analyzed by antibodies to YopE and YopH **(A)**. To evaluate membrane integrity, both the WT CO92 and the Δ*lpp* Δ*pla* double mutant were grown to exponential phase at 28°C ($OD_{600}$ of 0.8). The cells were washed, pelleted, fixed, and subjected to transmission electron microscopy **(B)**.

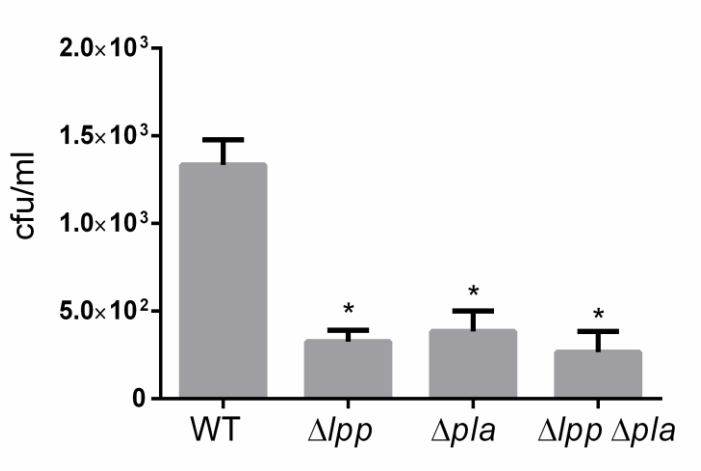


**Fig. 2: Intracellular survival of WT *Y. pestis* CO92 and its mutant strains in murine macrophages.**

RAW 264.7 macrophages were infected at an MOI of 1 with WT and its tested mutants for 30 min. Monolayers were treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. At 4 h (post-gentamicin treatment), macrophages were lysed and cultured on SBA plates. Three independent experiments were performed, and the data analyzed using one-way ANOVA with Bonferroni correction and a $p$ value of $\leq 0.05$ was considered significant. Asterisks denote comparison of the mutants with WT CO92.

**Attenuation of the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 in a bubonic plague mouse model and generation of a protective immune response**

The virulence potential of the Δ*lpp* Δ*pla* double mutant in a bubonic plague model was determined by infecting animals via the s.c. route with 5 x $10^5$ cfu dose (representing 10,000 $LD_{50}$, where 1 $LD_{50}$= 50 cfu of the WT CO92) (Liu *et al.*, 2010). As depicted in **Fig. 3**, mice started dying between days 4-6 p.i., and all of the animals infected with the WT CO92 succumbed to infection by day 6. However, an increased survival rate was observed at 80%, 90%, and 100%, respectively, for the Δ*lpp* and Δ*pla* single-, and the Δ*lpp* Δ*pla* double- mutant infected animals. Importantly, no or extremely mild signs of discomfort were observed in surviving mice infected with either the Δ*lpp* Δ*pla* double- or the Δ*lpp* or Δ*pla* single- mutant, respectively. In contrast, mice infected with the WT CO92 exhibited ruffled fur, hunched back, lethargy, buboes, and inability of animals to groom. Although the temperature of infected animals was not monitored, no significant difference was observed regarding their food consumption and body weight in the mutant-infected groups. By using telemetry (Lawrence *et al.*, 2009), we will be able to measure physiological functions of the animals (e.g., respiration rate, cardiac function) in our future studies.

To evaluate specific immunity that could have been developed in mice after infection with the mutants to WT CO92, we challenged survivors of the Δ*pla* single and Δ*lpp* Δ*pla* double mutant-immunized groups with a 10 $LD_{50}$ (1 $LD_{50}$= 500 cfu) of WT CO92 via the i.n route 30 days following the initial infection. The i.n. route was chosen as it has the highest CFR in humans. In **Fig. 3,** 90% of mice that were initially infected with

the Δ*lpp* Δ*pla* double mutant survived i.n. challenge with the WT CO92. All of the age-matched naïve animals infected with WT CO92 died by day 5 p.i.

The highly attenuated phenotype of the Δ*lpp* Δ*pla* double mutant in mice prompted us to evaluate the mutant's resistance to serum killing. Our data showed that both the WT CO92 and its Δ*lpp* Δ*pla* double mutant had a 100% survival rate in either untreated or heat-inactivated sera and/or PBS at 37°C. These data indicated that deletion of the *lpp* and *pla* genes did not affect bacterial resistance to serum (Lathem *et al.*, 2007; Sha *et al.*, 2008).

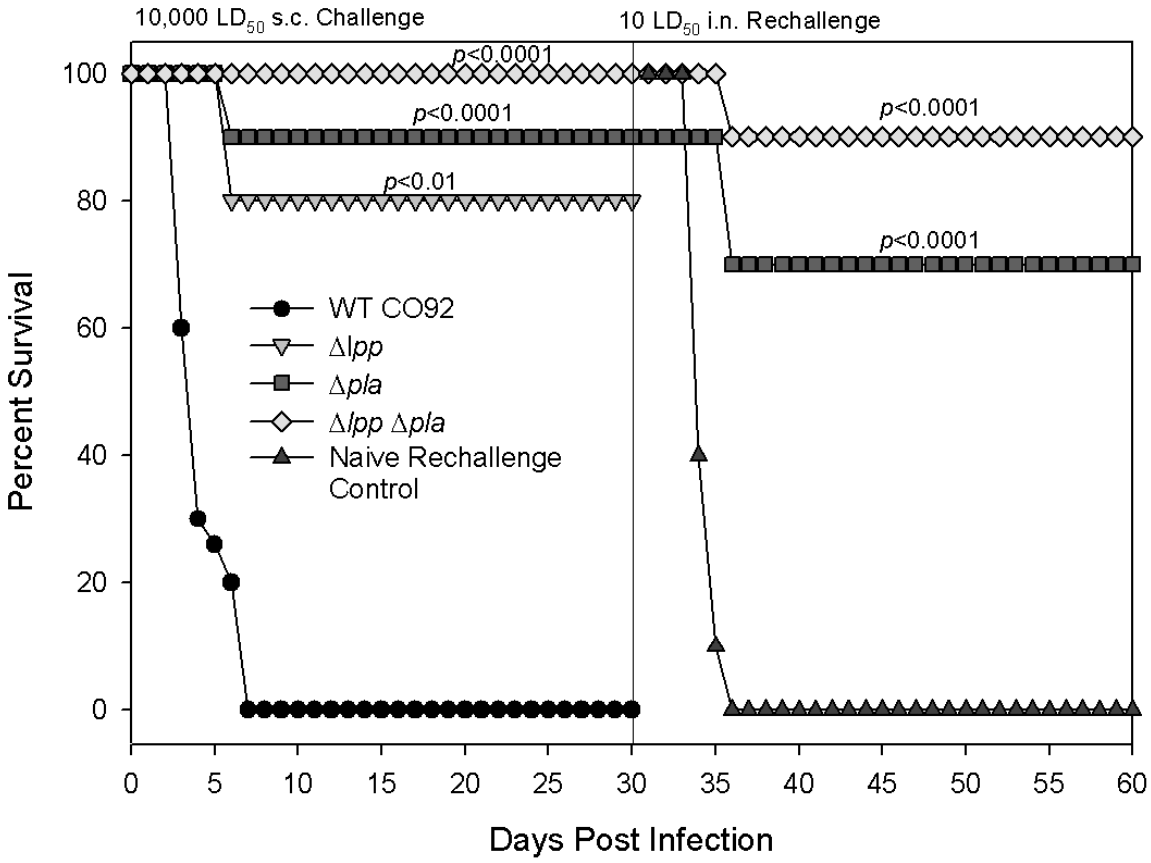


**Fig. 3: Survival analysis of mice infected with WT *Y. pestis* CO92 and its mutant strains by the bubonic plague route.**

Mice were challenged by the s.c. route with 5 x $10^5$ cfu (representing 10,000 $LD_{50}$ of WT CO92) of WT *Y. pestis* CO92 and its various mutants at day 0 and observed for mortality. Thirty days p.i., mice that survived the initial infection were challenged with 10 $LD_{50}$ of WT CO92 by the i.n. route. We found a 90% survival rate for the Δ*lpp* Δ*pla* double mutant immunized animals and 77% for the Δ*pla* single mutant-immunized mice following challenge with WT CO92. The data were analyzed by using Kaplan Meier's survival estimates, and *p* values of ≤0.05 were considered significant.

**Dissemination of *Y. pestis* CO92 mutants to peripheral organs in a bubonic plague mouse model**

We challenged 20-25 mice per group by the s.c. route with 5 x $10^5$ cfu of WT CO92, Δ*lpp*-, Δ*pla*-, or Δ*lpp* Δ*pla* mutants. Five-seven mice per time point (2, 4, 8, and 16 days) were sacrificed, and the cfu per organ or ml was determined. As depicted in **Fig. 4**, WT CO92 could be detected in the lungs of some mice 2 days after infection, reaching an average number of ~8 x $10^7$ cfu/organ (5/7 mice were positive) by day 4 prior to death. Likewise, an increase in cfu of single mutants (Δ*lpp*- and Δ*pla*-) in the lungs of some mice (2-3/6) was noted over a period of 4 days, albeit these bacterial numbers were lower than those from animals infected with the WT CO92 at the corresponding time point of 4 days. Importantly, the Δ*lpp* Δ*pla* double mutant was below the limit of detection in the lungs of mice (4/5) until day 4. On day 8, while the Δ*lpp* single mutant was minimally detected (1/5 mice), the average numbers of the Δ*pla* single- and the Δ*lpp* Δ*pla* double- mutant in the lungs of animals were ~8 x $10^4$ (4/5 mice were positive) and 2 x $10^4$ (2/5 mice were positive) cfu/organ, respectively. By day 16, no mutant bacteria (Δ*lpp*, Δ*pla*, Δ*lpp* Δ*pla*) were detected in the lungs (data not shown).

A similar bacterial dissemination pattern for WT CO92 and single mutants (Δ*lpp*- and Δ*pla*-) emerged in the spleen and liver of mice up to day 4. By day 8, all mutants were cleared from the spleen **(Fig. 4)**. The Δ*lpp* Δ*pla* double mutant was not cultivated from the spleen at any time point between 2-16 days p.i. In the liver, while the WT CO92 (7/7 mice were positive) and its Δ*lpp* single mutant (1/5 were positive) could be detected on day 4, both Δ*pla*- and Δ*lpp* Δ*pla*- mutants were either observed at much decreased levels or were

undetectable between 2-16 days p.i. Finally in the blood of mice, only WT CO92 was detectable on day 4 (4/5 mice were positive) and in one mouse infected with an Δ*pla* single mutant on day 8. Neither the Δ*lpp* single- nor the Δ*lpp* Δ*pla* double- mutant was cultivated from the blood at any time point between days 2-16 **(Fig. 4;** day 16 data not shown**)**.

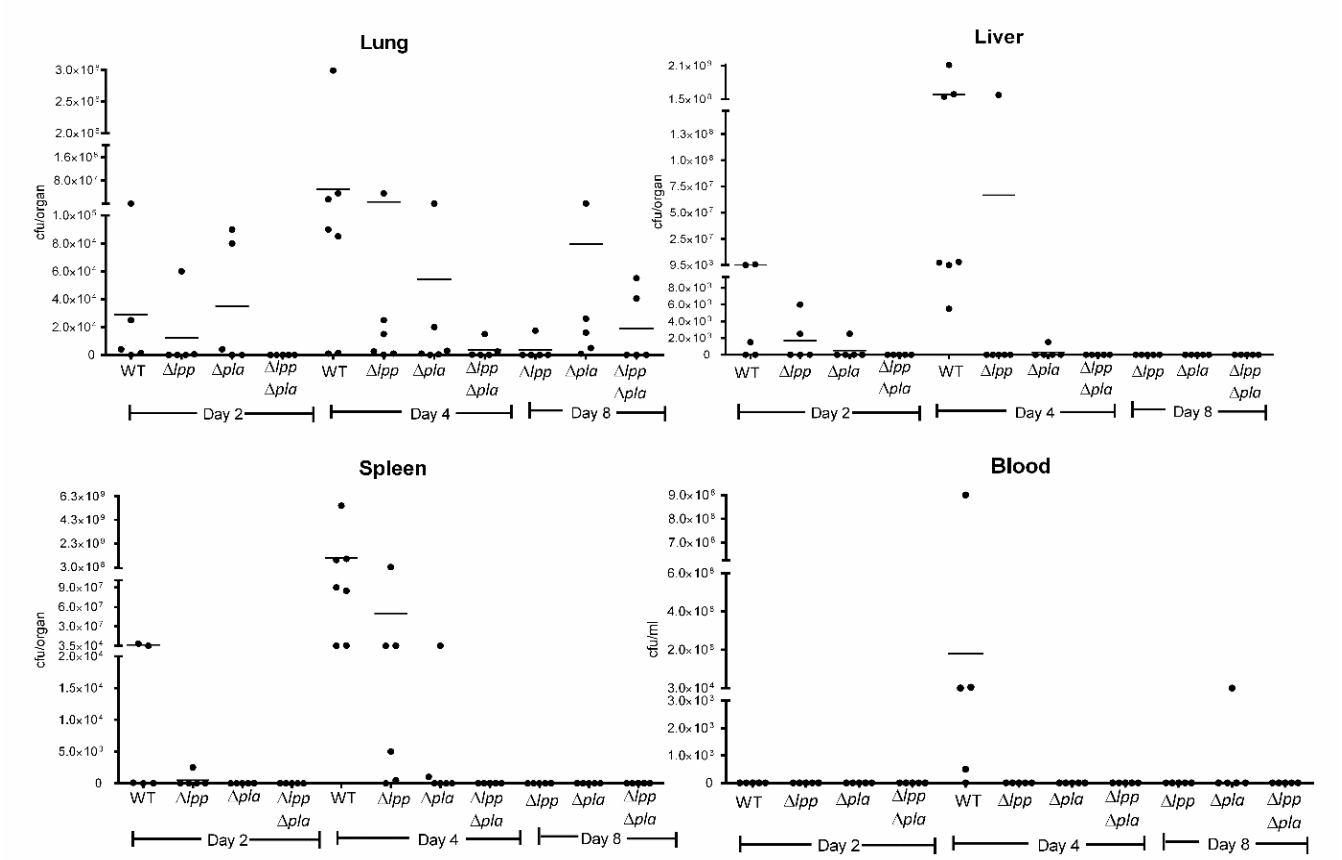


**Fig. 4: Dissemination of WT *Y. pestis* CO92 and its mutant strains to the peripheral organs of mice infected by the bubonic plague route.**

Mice (20-25 per group) were challenged by the s.c. route with 5 x $10^5$ cfu of WT *Y. pestis* CO92 and its various mutants at day 0. On days 2, 4, 8, and 16, five to seven animals per group were sacrificed and the spleen, liver, lungs, and the blood collected. Each organ was homogenized (except for the blood) and plated to determine bacterial load. The data were analyzed by one-way ANOVA and Tukey's *post-hoc* test and a *p* value of ≤0.05 was considered significant. Because of the terminal nature of some animals, blood could not be drawn from them.

**Cytokine/chemokine levels in sera of mice infected with WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant in a bubonic plague model**

There was a general decrease in the cytokine/chemokine levels in sera of mice infected with the Δ*lpp* Δ*pla* double mutant when compared to levels in the WT CO92-infected animals (**Table 3**). We have shown data for day 4, as animals infected with the WT CO92 were terminal at this time point. Of the 31 cytokines/chemokines, G-CSF, IL-1β, IL-6, IP-10, MCP-1, and RANTES were significantly reduced in the sera of mice infected with the Δ*lpp* Δ*pla* double mutant when compared to the sera of animals infected with the WT CO92. This general trend of lower cytokine/chemokine response in the mutant-infected mice correlated with the observation that there was a rapid clearance of the Δ*lpp* Δ*pla* double mutant from mouse organs and the blood **(Fig. 4)**.

**Table 3: Changes in cytokine and chemokine levels in mouse sera comparing WT CO92 to Δ*lpp* Δ*pla* double mutant**

| Cytokine/ Chemokine | Day 4 (s.c.) | | Day 2 (i.n.) | |
|---|---|---|---|---|
| | WT (pg/mL) | Δ*lpp* Δ*pla* (pg/mL) | WT (pg/mL) | Δ*lpp* Δ*pla* (pg/mL) |
| TNF-α | 310±507 | ND | 69±45 | 37±12* |
| IFN-γ | 2260±4330 | ND | 274±217 | 380±417 |
| G-CSF | 10918±5975 | 1708±987*** | 26495±12979 | 14299±8604 |
| GM-CSF | 248±159 | ND | 274±79 | 185±71 |
| M-CSF | 62±38 | 20±20 | 89±51 | 47±9 |
| IL-1α | 321±113 | 279±179 | 876±177 | 808±184 |
| IL-1β | 103±23 | 23±26* | 148±98 | 54±41 |
| IL-2 | 21±25 | 9±5 | 11±7 | 4±4 |
| IL-3 | 307±480 | ND | 28±7 | 16±12 |
| IL-4 | 90±173 | 11±2 | 1±3 | ND |
| IL-5 | ND | 9±20 | 56±43 | 19±13 |
| IL-6 | 3858±3587 | 309±248** | 7458±3643 | 5465±4315 |
| IL-7 | 33±40 | ND | 23±7 | 14±5 |
| IL-9 | 46±104 | ND | 1054±165 | 1054±153 |
| IL-10 | 77±67 | 22±22 | 139±78 | 62±27* |
| IL-12(p40) | ND | ND | 24±10 | 25±18 |
| IL-12 (p70) | 62±89 | ND | 290±166 | 213±144 |
| IL-13 | 1190±1837 | ND | 265±100 | 240±112 |
| IL-15 | 124±204 | ND | 77±22 | 55±34 |
| IL-17 | 230±516 | ND | 376±366 | 101±116*** |
| IP-10 | 1740±828 | 348±97*** | 1654±742 | 1643±429 |
| Eotaxin | 1469±1020 | 1070±199 | 2024±413 | 1289±342*** |
| KC | 3528±5069 | 213±114 | 5588±4282 | 814±401*** |
| LIF | 70±123 | ND | 98±46 | 42±40 |
| LIX | 7822±2819 | 8111±4454 | 15891±508 | 8389±2100 |
| MCP-1 | 3411±4627 | 102±39* | 2323±2376 | 94±38 |
| MIG | 7403±3517 | 798±363 | 52532±10237 | 8079±4499 |
| MIP-1α | 515±276 | 271±79 | 316±122 | 316±232 |
| MIP-2 | 874±1272 | 201±128 | 385±109 | 262±42 |
| RANTES | 78±57 | 22±6*** | 81±40 | 27±10 |
| VEGF | 11±7 | 5±1 | 33±34 | 16±7 |

*$p$<0.05, **$p$<0.01, ***$p$<0.001

ND: not detected

**Antibodies specific to *Y. pestis* WT CO92 and its antigens (F1-LcrV or V) were detected in mice challenged with the Δ*pla* single- and Δ*lpp* Δ*pla* double- mutant in the bubonic plague model**

Surviving mice challenged with 5 x $10^5$ cfu of Δ*pla* single- or Δ*lpp* Δ*pla* double-mutant by the s.c. route **(Fig. 3)** were bled on day 16 for performing ELISA. Animals challenged with the WT CO92 did not survive this two-week time frame.

As noted in **Fig. 5A**, animals infected with either the Δ*pla* single- or the Δ*lpp* Δ*pla* double- mutant exhibited significant total IgG titers to the whole CO92 cells, when compared to naïve mice. Likewise, when the ELISA plates were coated with the F1-V antigen, significant IgG antibody titers were detected in the sera of both groups of mutant-infected mice compared to naïve sera (**Fig. 5B**). However, the Δ*pla* single mutant seemed to generate better IgG1 **(Fig. 5C)** and IgG2a **(Fig. 5D)** antibody responses compared to that of the Δ*lpp* Δ*pla* double mutant. These data indicated a trend towards a $T_H2$-based immune response in a mouse model of bubonic plague.

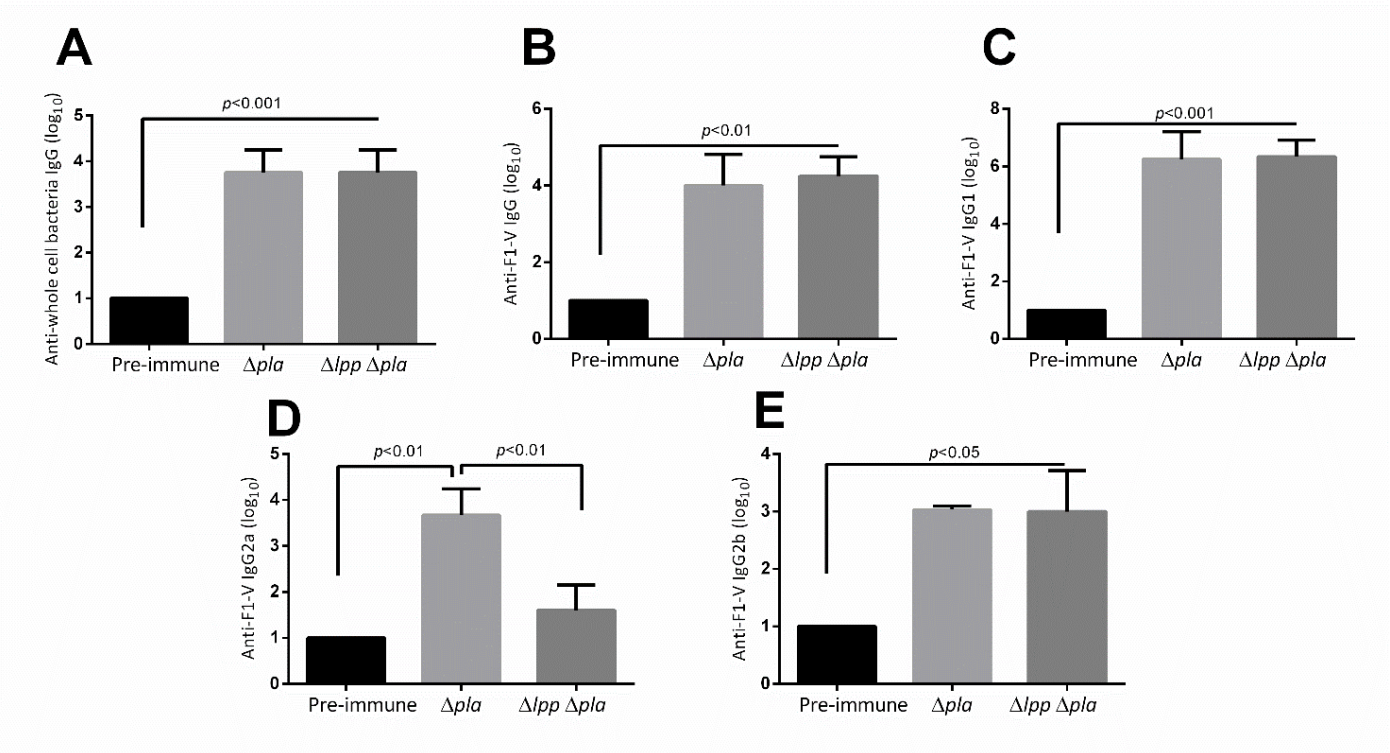


**Fig. 5: Antibody responses of mice challenged with the mutant strains of *Y. pestis* CO92 by the bubonic plague route.**

Mice were challenged by the s.c. route with 5 x $10^5$ cfu of Δ*pla* single- or the Δ*lpp* Δ*pla* double mutant at day 0 and survivors bled 16 days later to determine antibody titers. To observe general antibody responses (IgG) to *Y. pestis*, we grew WT *Y. pestis* CO92 at 28°C overnight and then shifted temperature to 37°C for 4 h and used to coat the plates for ELISA **(A)**. An ELISA was also performed on plates coated with F1-V antigen to measure overall antigen specific IgG response **(B)** and its isotypes IgG1**(C)**, IgG2a **(D)** and IgG2b **(E)**. The geometric mean of each sample (n=5) was used for data plotting. The data were analyzed statistically by one-way ANOVA with Tukey's *post-hoc*, and *p* values of ≤0.05 were considered significant.

# Chapter 4: Pneumonic plague in a murine model

### Attenuation of the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 in a pneumonic plague mouse model and generation of a protective immune response

The virulence potential of the Δ*lpp* Δ*pla* double mutant in comparison to that of WT CO92, and its single mutants Δ*lpp*- *and* Δ*pla*-, in a pneumonic plague model was determined by infecting animals by the i.n. route with 5 x $10^5$ cfu (representing 1,000 $LD_{50}$ for the WT CO92 where 1 $LD_{50}$=500 cfu). In **Fig. 6**, mice started dying between days 3-5 p.i., and an increased survival rate for animals infected with the Δ*pla*- and Δ*lpp* Δ*pla*- mutants was observed. Mice infected with the WT CO92 and its Δ*lpp* single mutant died by day 5, with a slight increase in mean time to death of the latter compared to WT-challenged animals, while only 40% of the mice infected with the Δ*pla* single mutant survived. It was expected that animals infected with the Δ*lpp* single mutant at such a high challenge dose would not survive (Sha *et al.*, 2008; Agar *et al.*, 2009; Liu *et al.*, 2010; Sha, J. *et al.*, 2013). On the contrary, the Δ*lpp* Δ*pla* double mutant-infected mice showed 100% survival. Similar to the bubonic plague model, the Δ*lpp* Δ*pla* double mutant-infected animals were clinically healthy while mice infected with the WT CO92 as well as with Δ*lpp* or Δ*pla* single- mutants exhibited marked clinical signs. These data indicated that although individual mutations alone were not sufficient to protect animals from high bacterial challenge doses, deletion of both the *lpp* and *pla* genes led to synergistic attenuation of the double mutant in a mouse model of pneumonic plague.

To evaluate *Y. pestis*-specific immunity developed in the surviving mice, they were challenged with 10 $LD_{50}$ of WT CO92 (5,000 cfu) via the i.n route 30 days following the initial infection. As shown in **Fig. 6,** 70% of the animals that were initially infected with the Δ*lpp* Δ*pla* double mutant survived challenge with the WT CO92 strain. Although 100% of mice initially infected with the Δ*pla* single mutant survived re-challenge, there was a significant mortality rate (60%) during the initial phase of infection. Mice that were age- and sex- matched served as controls and succumbed to infection. Overall, our results indicated that the double mutant was not lethal to mice but provided significant protection to animals from subsequent WT CO92 infection, irrespective of the route of initial challenge, s.c. or i.n. **(Figs. 3 and 6)**.

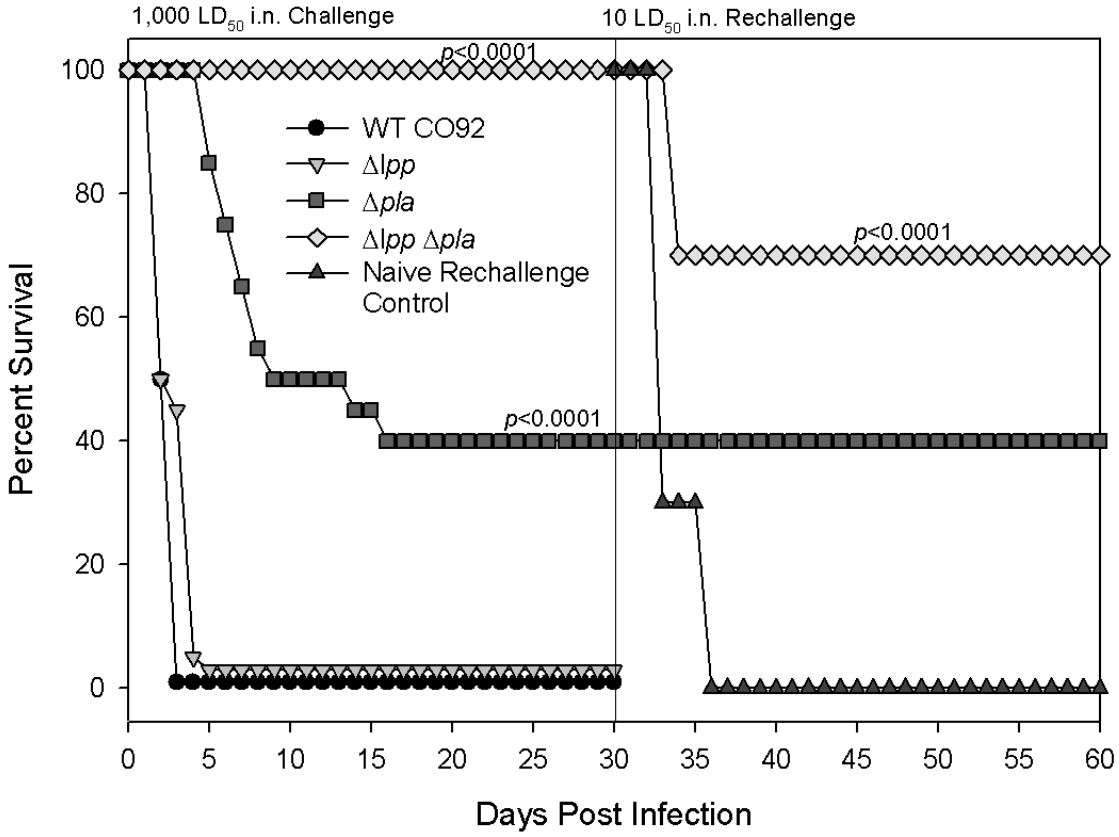


**Fig. 6: Survival analysis of mice infected with WT *Y. pestis* CO92 and its mutant strains by the pneumonic plague route.**

Mice (20-30 per group) were challenged by the i.n. route with 5 x $10^5$ cfu (representing 1,000 $LD_{50}$ of WT CO92) of WT *Y. pestis* CO92 and its various mutants at day 0 and observed for mortality. Thirty days p.i., survivors were re-challenged with 10 $LD_{50}$ of WT CO92 strain by the i.n. route and observed for mortality. The data were statistically analyzed by using Kaplan-Meier's survival estimates, a *p* value of $\leq$0.05 was considered significant.

**Dissemination of *Y. pestis* CO92 mutants to peripheral organs in a pneumonic plague mouse model**

First, 20 to 30 mice per group were challenged by the i.n. route with 5 x $10^5$ cfu of WT CO92, Δ*lpp*-, Δ*pla*-, or the Δ*lpp* Δ*pla* mutant. Then 5-10 mice per time point (2, 6, 8, and 14 days) were sacrificed and the cfu determined in various organs. By day 2, significant multiplication of WT CO92 (10/10 mice) and its Δ*lpp* single mutant (10/10 mice) was seen in lungs, liver, and the spleen **(Fig. 7)**. In the blood, 4/5 animals infected with either the WT CO92 or its Δ*lpp* single mutant had high bacterial counts, while 2/5 mice were positive for the Δ*pla* mutant although at low numbers. The Δ*pla* single mutant was generally detected in lower numbers (close to the challenge dose) in all of these organs on day 2 **(Fig. 7)**, with the Δ*lpp* Δ*pla* double mutant was found at low-to-undetectable numbers in the peripheral organs. In the lungs, all of the mutants were at a statistically lower level compared to animals that were infected with the WT CO92. By day 6, all of the animals infected with the WT CO92 and its Δ*lpp* single mutant died, and, hence there were no data for those groups. Although the Δ*pla* single mutant was detectable in the lungs up to day 8 (2/5 mice was positive), the spleen was negative on day 8 **(Fig. 7)**. The double mutant was only minimally detected, if at all, in all of the organs between days 6-14 (day 14 data not shown). The Δ*lpp* Δ*pla* double mutant was undetectable in the blood at all the time points.

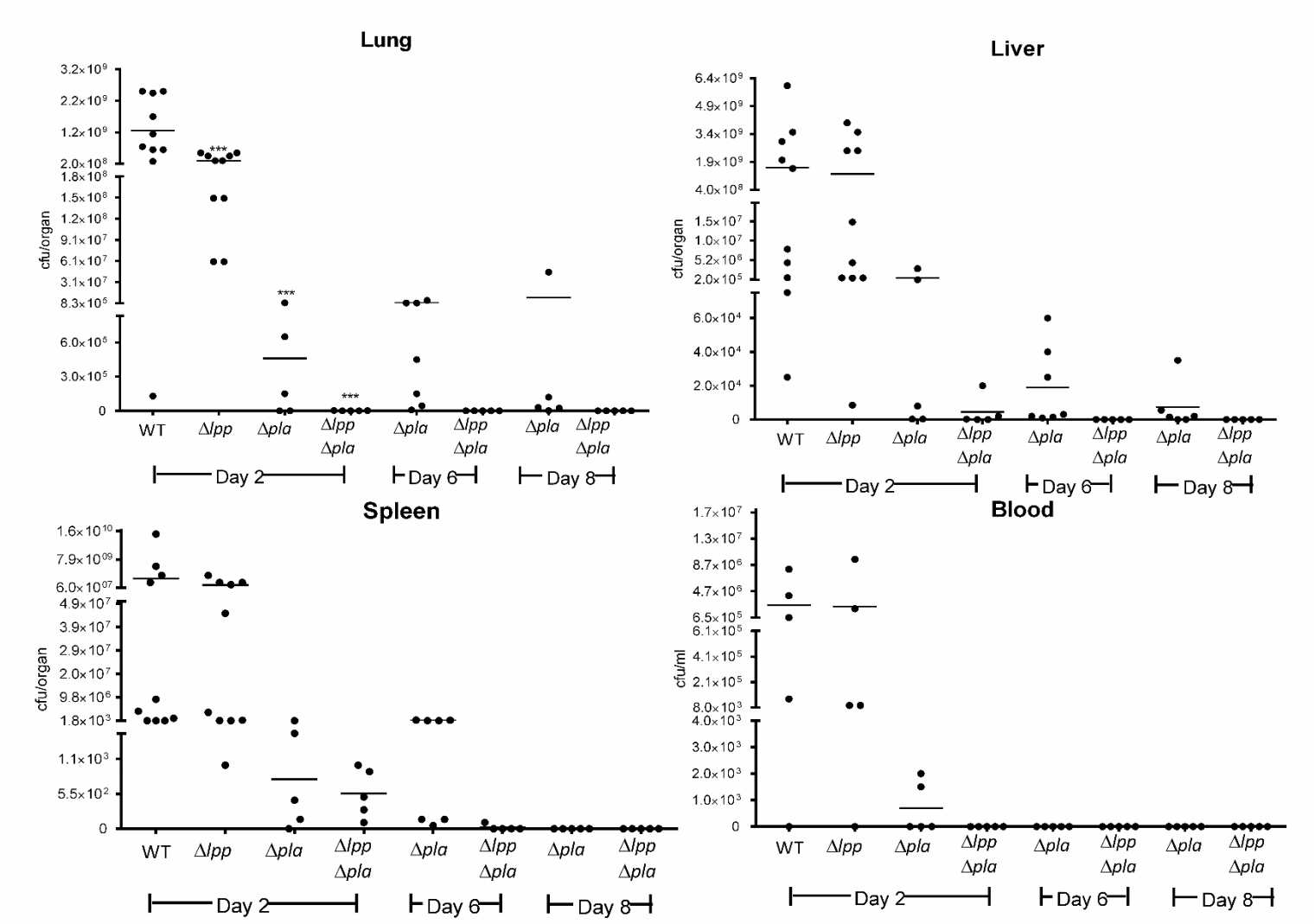


**Fig. 7: Dissemination of WT *Y. pestis* CO92 and its mutant to peripheral organs of mice infected by the pneumonic plague route.**

Mice were challenged by the i.n. route with 5 x $10^5$ cfu of WT *Y. pestis* CO92 and its various mutants at day 0. On days 2, 6, 8 and 14, five to ten animals per group were sacrificed and the spleen, liver, lungs, and the blood collected. Each organ was homogenized (except for the blood) and plated to determine bacterial load. The data were analyzed by one-way ANOVA and Tukey's *post-hoc* test. Statistically significant values are denoted by asterisks (***$p$<0.001) and these data were compared to animals infected with WT CO92. By day 2 all of the WT bacteria- and Δ*lpp* mutant-infected had died. Because of the terminal nature of some animals, blood could not be drawn from them.

**Cytokine/chemokine levels in sera of mice infected with WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant in a pneumonic plague model**

As noted for the bubonic plague mouse model, there was a general decrease in the cytokine/chemokine levels in the sera of mice infected with the Δ*lpp* Δ*pla* double mutant when compared to those of WT CO92-infected animals (**Table 3**). Specifically, levels of TNF-α, IL-10, IL-17, and keratinocyte-derived chemokine (KC) were significantly down regulated when compared to their levels in WT bacteria-infected mice 2 days after infection. This general trend of a lower cytokine/chemokine response in the mutant-infected mice correlated with rapid clearance of the Δ*lpp* Δ*pla* double mutant from organs and the blood **(Fig. 7)**.

**Antibodies specific to WT *Y. pestis* CO92 and its antigens F1-V were detected in mice challenged with the Δ*pla* single- and Δ*lpp* Δ*pla* double- mutant in the pneumonic plague model**

Mice challenged by the i.n. route with the Δ*pla* single- and Δ*lpp* Δ*pla* double-mutant exhibited significantly higher total IgG titers (1:10,000) compared to those in pre-immune sera when ELISA plates were coated with the WT CO92 on day 14 **(Fig. 8)**. Likewise, high IgG titers of 1:3,125 for both the mutants were observed when ELISA plates were coated with F1-V. Titers of 1:3,125 were noted for IgG1 and IgG2a, indicating that a balanced $T_H1$ and $T_H2$ response was generated by both the Δ*pla* single and Δ*lpp* Δ*pla* double mutant-challenged mice (**Fig. 8**). Our data indicated that the $T_H1$ response generated by the double mutant might be stronger than that generated by the Δ*pla* single mutant (see IgG2a antibody titers). The IgG2b titers against F1 and LcrV antigens were similar for both the single and the double mutants (**Fig. 8**). The relative ratio of IgG1:IgG2a was 1:1 for

both Δ*pla* single and Δ*lpp* Δ*pla* double mutant, thereby indicating a trend towards a balanced $T_H1$-$T_H2$ immune response.

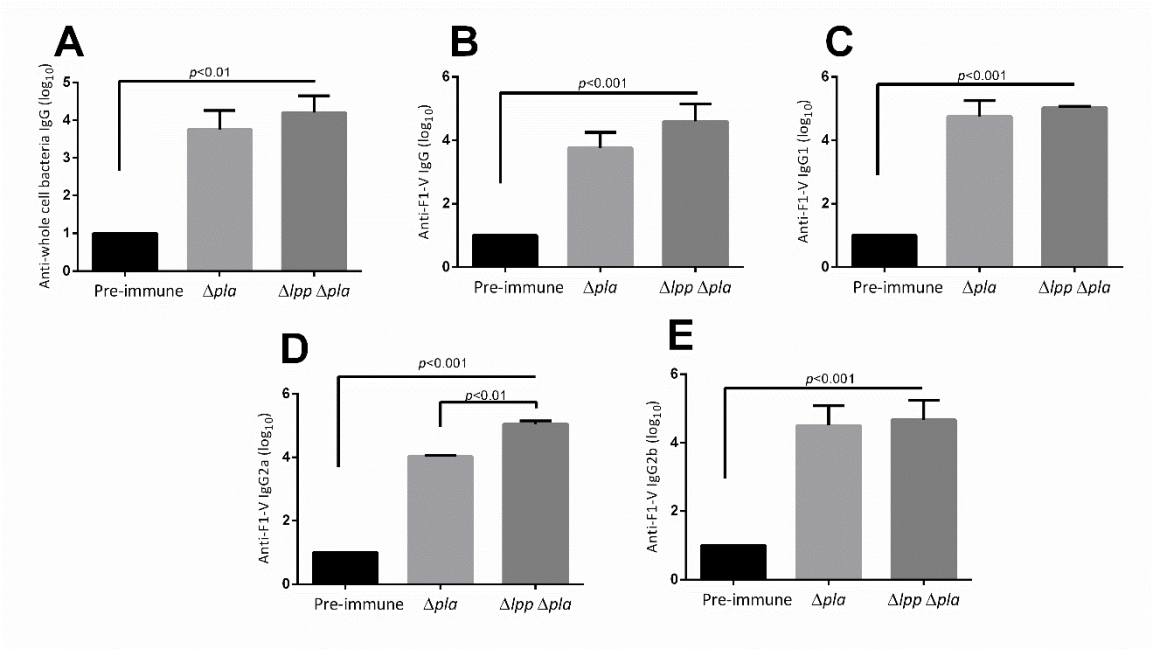


**Fig. 8: Antibody responses of mice challenged with the mutant strains of *Y. pestis* CO92 by the pneumonic plague route.**

Mice (n=10) were challenged by the i.n. route with 5 x $10^5$ cfu of Δ*pla* single- or the Δ*lpp* Δ*pla* double mutant on day 0 and bled 14 days later to determine antibody titers in the sera. WT *Y. pestis* CO92 was grown to coat the plates for ELISA to observe total IgG antibody response to *Y. pestis* **(A)**. An ELISA was also performed to examine the total antigen specific IgG **(B)**, IgG1**(C)**, IgG2a **(D)**, and IgG2b **(E)** responses when the plates were coated with the F1-V antigen of *Y. pestis*. The geometric mean of each sample (n=5) was used for data plotting. The data were analyzed statistically by one- way ANOVA with Tukey's *post-hoc*, and *p* values of ≤0.05 were considered significant.

**Complementation of the single (Δ*pla*-) and Δ*lpp* Δ*pla*- double mutants of *Y. pestis* CO92 in a mouse model of pneumonic plague**

To test whether we could demonstrate the restoration of Δ*pla*- and Δ*lpp* Δ*pla* double- mutants' virulence, we complemented them with the *pla* gene in *trans* and tested the mutants in a mouse model of pneumonic plague **(Fig. 9)**. All of the animals infected with the WT CO92 [pBR322] **(Table 1)** by the i.n. route at a dose of 1,000 $LD_{50}$ died by day 3. Likewise, all of the animals infected with the Δ*lpp* [pBR322] single mutant died at the WT CO92 dose equivalent of 1,000 $LD_{50}$ by day 5. However, an attenuation of this Δ*lpp* mutant was observed, as the mean time to death of mice was increased when compared to that of the WT CO92-infected animals (day 3 versus day 5). While both Δ*pla* [pBR322] single- (60% survival) and Δ*lpp* Δ*pla* [pBR322] double- (100% survival) mutants were attenuated at the same challenge dose of WT CO92, the death rates of mice increased to 100% (0% survival by day 3) when the above-mentioned mutants were complemented with the *pla* gene. We also observed a statistically significant increase in survival when comparing Δ*pla* [pBR322] single- and Δ*lpp* Δ*pla* [pBR322] double-mutants.

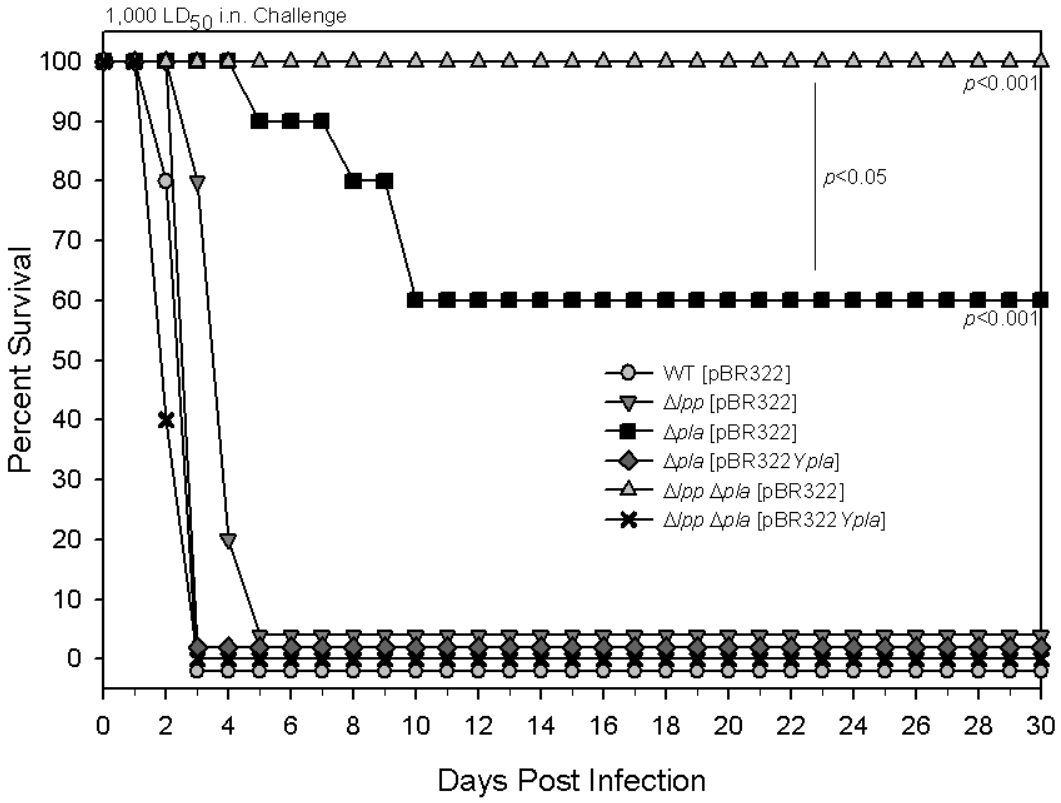


**Fig. 9: Survival analysis of mice infected with WT *Y. pestis* CO92 and its complemented mutant strains by the pneumonic plague route.**

Mice (10 per group) were challenged by the i.n. route with 5 x $10^5$ cfu (representing 1,000 $LD_{50}$ of WT CO92) of WT *Y. pestis* CO92, its various mutants, and the complemented Δ*pla* single- or the Δ*lpp* Δ*pla* double mutant (at day 0) and observed for mortality. The data were analyzed by using the Kaplan Meier's survival estimates, and a *p* value of ≤0.05 was considered significant. The data were compared between WT CO92 and its various mutants as well as between Δ*pla* single- and Δ*lpp* Δ*pla*- double mutants

**Histopathology**

Portions of the mouse tissues were stained with H&E to assess the level of tissue damage after infection (pneumonic plague) with the WT CO92 and its single (Δ*lpp*- and Δ*pla*-)- and Δ*lpp* Δ*pla* double- mutant at a dose of 5 x $10^5$ cfu. Uninfected control mice exhibited no significant lesions and tissues appeared normal (data not shown). All of the WT-, Δ*lpp*-, Δ*pla*-, and Δ*lpp* Δ*pla*- mutant infected mice had lung lesions 2 days p.i., but the severity and prevalence of the lesions were notably decreased in the Δ*lpp* Δ*pla* double mutant- challenged animals **(Fig. 10)**. At 2 days p.i., mild-to-moderate tissue lesions (20-50% tissue involvement) were noted in the lungs of animals infected with WT CO92, Δ*lpp*-, and Δ*pla*- single mutants. Lung lesions included edema, hemorrhage, fibrinous infiltrates, acute inflammation, and leukocytosis. Additionally, bacteria were observed in WT- and the Δ*lpp* mutant-challenged mice. The enlarged inset on the WT CO92 image shows the presence of bacteria in the lungs (**Fig. 10**).

Further, in the lungs of mice infected with WT CO92, Δ*lpp*- and Δ*pla*- single mutants, there was a noticeable collapse in the structure of the alveoli accompanied by congestion and disintegration of the alveolar sacs with an increase in the neutrophil recruitment, which led to an acute inflammatory response. The Δ*lpp* Δ*pla* double mutant-infected mice, on the other hand, exhibited only mild lesions (10-20% tissue involvement) in their lungs, indicative of a decrease in tissue damage consistent with a decline in virulence of this mutant (**Fig. 6**). Although some inflammation was noted in the lungs of mice infected with the Δ*lpp* Δ*pla* double mutant **(Fig. 10)**, recruitment of neutrophils was at a much reduced level, which also correlated with our observed decrease in pro-inflammatory cytokine levels in sera of mice 2 days p.i. (**Table 3**).

In the liver, WT CO92-infected mice exhibited mild, acute inflammation (3/3 mice) and the presence of bacteria (1/3 mice). Two of 3 Δ*lpp*-, and 3 of 3 Δ*pla*- and Δ*lpp* Δ*pla*-infected mice had no liver lesions, and no mutant-infected animals had bacteria in the liver; indicative of a decreased ability of mutants to disseminate (**Fig. 7**). All WT CO92-infected animals had splenic lesions, which included acute inflammation, myeloid hyperplasia, and decreased cellularity in the red pulp, and mild-to-moderate (20-50% of tissue involvement) depletion in the marginal zones of the white pulp.

All Δ*lpp*-, Δ*pla*-, and 2 of 3 Δ*lpp* Δ*pla* mutant-infected mice had splenic lesions that were similar to those infected with WT CO92, which was expected, as we observed a cell-based immune response. All mice tended to show some alterations in the red pulp, indicative of antigens processing from the lymph and bloodstream in addition to the recruitment of lymphocytes from the spleen (**Fig. 10**). Subsequently at days 6 and 8 p.i., a general trend towards clearing and containing of the infection was observed with the mutants, notably the double Δ*lpp* Δ*pla* mutant-infected mice never showed bacteria in any of the examined organs, and inflammatory tissue involvement did not exceed 20% (data not shown).

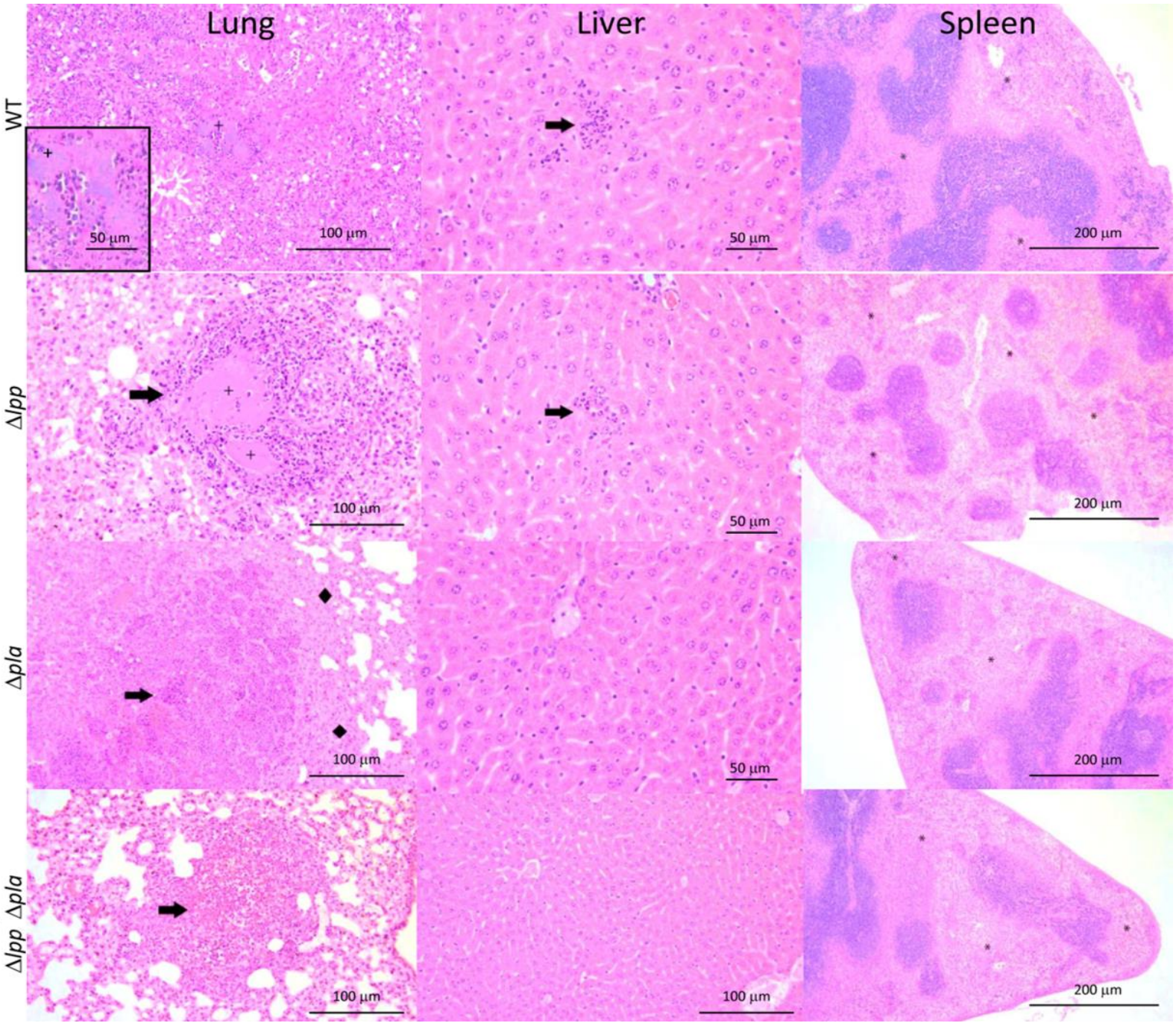


**Fig. 10: Histopathology of mouse tissues following infection with WT *Y. pestis* CO92 and mutant strains.**

Mice were challenged by the i.n. route with 5 x $10^5$ cfu of either WT CO92 or its mutants. Two days p.i., a portion of the lungs, liver, and the spleen was stained with H&E and evaluated. Organs from 3 animals were examined, and representative data are shown here. The arrows in the lungs and liver indicate inflammation, plus signs in the lungs indicate observable bacterial presence, and the diamonds indicate edematous areas. The asterisks in the spleen represent areas of decreased cellularity in the red and marginal zones of the white pulp.

**T cell activation by the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 in a mouse model of pneumonic plague**

To investigate the specific T cell responses in mice infected with the Δ*lpp* Δ*pla* double mutant, T cells were isolated from the spleens of animals challenged with a sub-lethal dose of either WT CO92 or its Δ*lpp* Δ*pla* mutant at three time points: 14, 21, and 60 days. Although some mice infected with the WT CO92 died during the course of infection, we had enough survivors (at least n=5) for each time point to obtain statistically significant data. We chose these time points to examine early and recall (memory) immune responses. The isolated T cells were then stimulated for 3 days with APCs that had been incubated with heat-killed WT CO92 and then γ-irradiated. As shown in **Fig. 11**, T cells from both the WT CO92 and its Δ*lpp* Δ*pla* mutant-infected mice proliferated strongly, specifically on days 21 and 60. Importantly, T cells from the mutant-infected mice proliferated more when compared to T cells from the WT CO92-infected animals at all 3 time points tested (**Fig. 11**). Together, these data indicated that the Δ*lpp* Δ*pla*-mutant immunized mice had a better recall response to WT CO92 antigens than the WT bacteria-immunized animals.

The supernatants from APC-stimulated T cells from WT CO92 and Δ*lpp* Δ*pla* double mutant-challenged mice were harvested at 48 h for 6-Plex analysis. We found that T cells from animals immunized with the Δ*lpp* Δ*pla* double mutant had higher levels of IFN-γ and interleukin (IL)-4 on day 14; although, the data did not reach statistical significance **(Fig. 12)**. The levels of other cytokines (TNF-α, IL-5, and IL-6) were similar across the three time points in the Δ*lpp* Δ*pla* double mutant versus the WT bacteria-immunized mice (**Fig. 12**). The presence of both TNF-α and IFN-γ indicated a $T_H1$-based response, which is critical in clearing an intracellular infection. Overall, our data provided

evidence that the Δ*lpp* Δ*pla* double mutant was capable of stimulating a protective, cell-based immune response, while highly attenuated in causing an infection in animals. We did not observe any IL-17 response from T cells of WT- or the double mutant-immunized mice (data not shown). These cytokine data also indicated a balanced $T_H1/T_H2$ immune response was generated by the Δ*lpp* Δ*pla* double mutant.

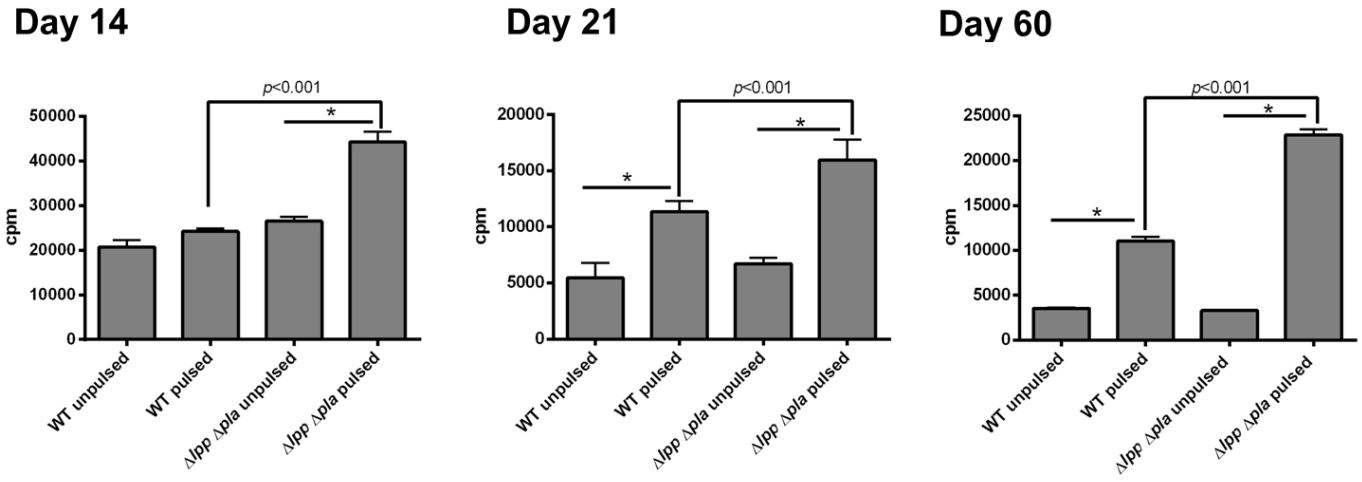


**Fig. 11: Proliferation of T cells isolated from WT *Y. pestis* CO92 and Δ*lpp* Δ*pla* mutant-infected mice.** A total of 60 mice were i.n. challenged with sub-lethal dose (250 cfu) of WT CO92 or Δ*lpp* Δ*pla* double mutant (30 mice per group). At 14, 21, and 60 days p.i., T cells were isolated separately from spleens of 5 mice in each infected group and stimulated for 3 days with APCs that had been pulsed with heat-killed WT CO92 and then γ-irradiated. Data were analyzed by using Bonferroni correction with a one-way ANOVA, and a *p* value of ≤0.05 was considered significant. Asterisks indicate a statistical significance in comparing un-pulsed to pulsed T cells ($p<0.001$) for both WT- and the mutant- infected mice. The results represent average from 2 independent experiments.

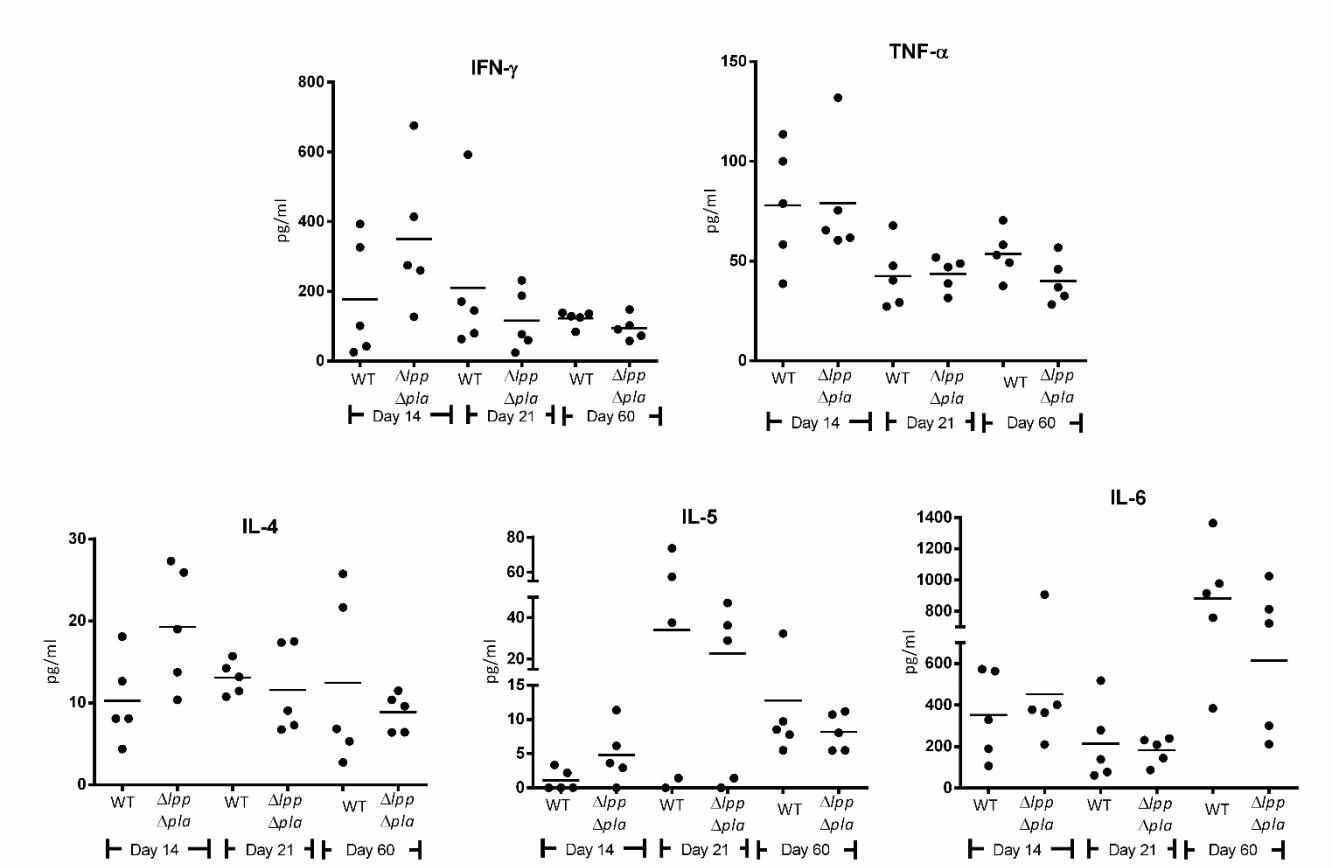


**Fig. 12: Cytokine production by T cells isolated from WT *Y. pestis* CO92 and its mutant-immunized mice after *ex vivo* pulsing with plague bacterium antigens.**

A total of 60 mice were i.n. challenged with sub-lethal dose (250 cfu) of WT or Δ*lpp* Δ*pla* mutant (30 mice per group). At 14, 21, and 60 days p.i., spleens from 5 mice in each infected group (at each time point) were harvested, and T cells isolated from each animal separately (including uninfected controls) and incubated with pulsed or un-pulsed APCs. After 48 h of incubation, supernatants were harvested for analysis by Milliplex. Data were analyzed by Bonferroni correction with one- way ANOVA, and a *p* value of ≤0.05 was considered significant.

**Activation of T cell subsets by the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 in a mouse model of pneumonic plague**

T cells from animals infected with the WT CO92, Δ*lpp* Δ*pla* double mutant, or uninfected mice were cultured with pulsed APCs for 5 days. As shown in **Fig. 13,** we noted a significant increase in $CD4^+$ TNF-α secreting T cells in the Δ*lpp* Δ*pla* mutant-immunized mice on day 14 compared to those in WT bacteria-infected animals **(Panel A)**. On day 60, however, a similar percentage of $CD4^+$ TNF-α-producing T cells from WT CO92 and Δ*lpp* Δ*pla* double mutant-immunized animals were observed **(Panel B)**. Likewise, the percentage of $CD8^+$ T cells producing TNF-α also increased on day 14 in the mutant-immunized mice **(Panel C)**, while both WT- and mutant-immunized animals had similar percentages of $CD8^+$ T cells producing this cytokine on day 60 **(Panel D)**. These data indicated that the Δ*lpp* Δ*pla* double mutant was capable of inducing at least as much or better $CD4^+$ and $CD8^+$ T cell-specific responses in terms of TNF-α production when compared to that in the WT bacteria-immunized mice. Such a cellular immune response is important in protecting mice against pneumonic plague.

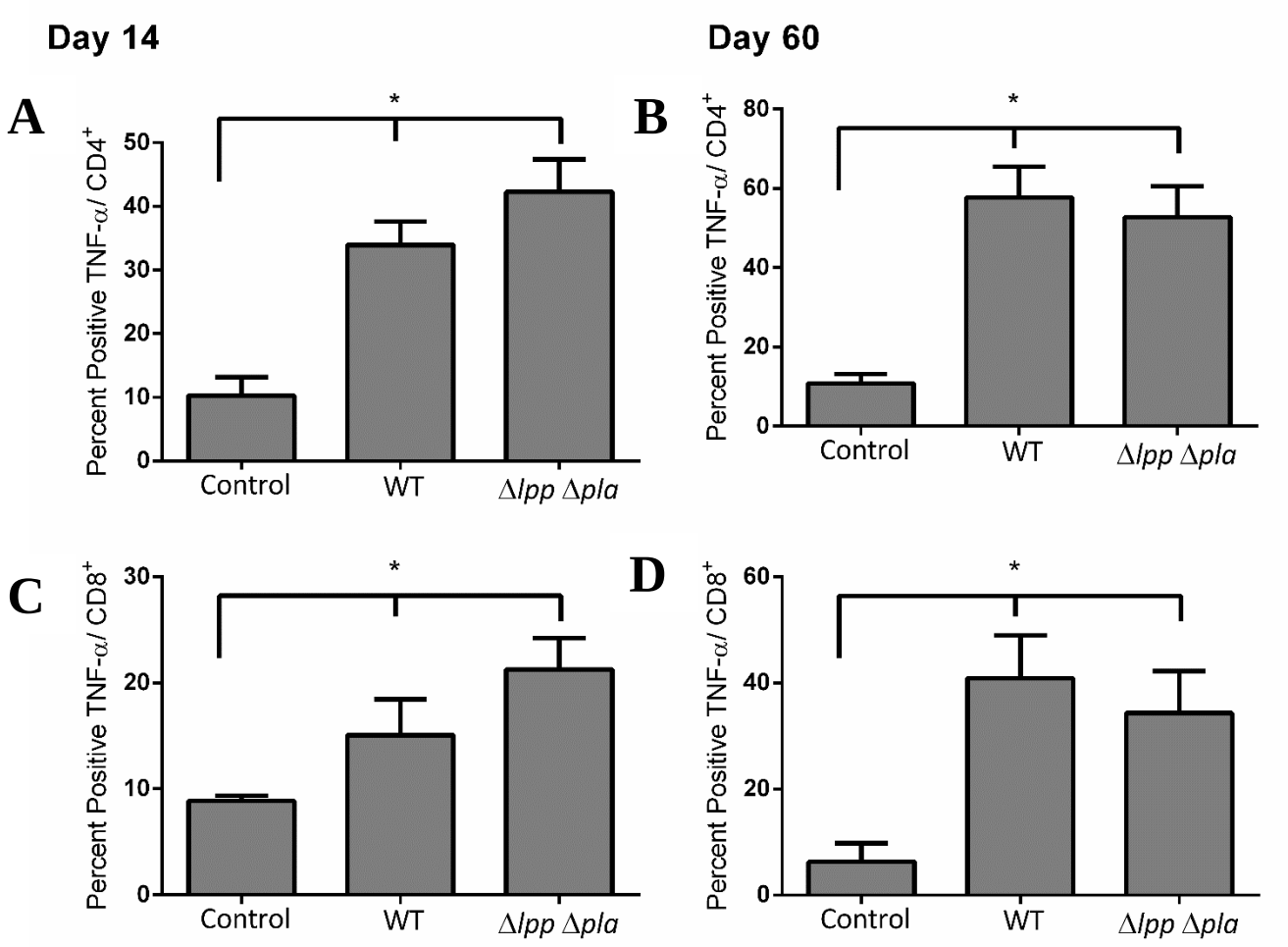


**Fig. 13: Flow cytometry of T cells isolated from WT *Y. pestis* CO92 and its mutant-immunized mice after *ex vivo* pulsing with plague bacterium antigens.**

T cells from animals infected with WT CO92, Δ*lpp* Δ*pla* double mutant, or uninfected mice (see legend to Fig. 12) were cultured with pulsed APCs for 5 days. Cells were then stained, read on the flow cytometer (LSRII Fortessa), and the data analyzed by using FACS diva software. T cells were stained on days 14 **($CD4^+$, panel A and $CD8^+$, panel C)** and 60 **($CD4^+$, panel B and $CD8^+$, panel D)**. These data represent an average from two independent experiments. Data were analyzed by Tukey's *post-hoc* test with one- way ANOVA, and a *p* value of ≤0.05 was considered significant. Asterisks represents statistical significance ($p<0.05$) between control versus WT CO92 or Δ*lpp* Δ*pla* double mutant.

# Chapter 4: Discussion of murine *in vivo* results

Our study has potentially developed a platform that could lead to the development of a legitimate and well-defined live-attenuated plague vaccine, as the generated Δ*lpp* Δ*pla* double isogenic mutant was highly attenuated and effective at establishing long- term immunity in the host against plague. Although the subunit plague vaccines composed of F1 and LcrV antigens are currently in clinical trials, these vaccines only evoke strong humoral immune response, which may not be adequate in protecting humans (Titball and Williamson, 2001; Kummer, L. W. *et al.*, 2008; Smiley, 2008; a). The lack of FDA approved vaccine and the need for both humoral and cell-mediated immune responses to effectively combat plague infections warrant continue searching of new live-attenuated plague vaccine candidates. The Δ*lpp* Δ*pla* double mutant possibly could provide the background strain, which after some additional manipulations to reduce reactogenicity, might become an excellent vaccine candidate.

In an earlier study, we demonstrated that when the pPCP-1 plasmid harboring the *pla* gene was cured from the Δ*lpp* single mutant, the resulting strain was highly attenuated in a mouse model of pneumonic plague (Agar *et al.*, 2009). However, since the pPCP-1 plasmid encodes *pst* and *pim* genes among others, it was essential to create an isogenic Δ*pla* mutant for vaccine development purposes.

Based on both *in vitro* and *in vivo* characterization (Bartra *et al.*, 2008; Agar *et al.*, 2009), it was apparent that there was no pleiotropic effects associated with the deletion of these two genes, *lpp* and *pla*, from WT CO92 (Figs. 1, 3, 6 & 9). To be an attractive live-attenuated vaccine strain, it should be cleared quickly from the host after triggering both arms of the immune response. Therefore, we examined the survivability of the double

mutant (Δ*lpp* Δ*pla*) in macrophages, the primary cell type that is inflicted during pneumonic plague. Indeed, we noted that the Δ*lpp* Δ*pla* double mutant was unable to survive in macrophages beyond 4 h *in vitro* (Fig. 2) and that it largely cleared from all organs of mice by day 2 in bubonic and pneumonic plague mouse models (Figs. 4 & 7).

In our earlier study, we have shown the Δ*lpp* single mutant was also unable to survive within macrophages due to the down-regulation of a gene encoding the global stress response protein A (GsrA) (Agar *et al.*, 2009; Galindo *et al.*, 2010). However, it is unclear how Pla directly or indirectly contributes to bacterial survival in the host cell, but the literature indicates that Pla can enhance bacterial invasion of epithelial cells and mediate the delivery of T3SS effectors into the host (Caulfield and Lathem, 2012).

We were intrigued by our results that the Δ*pla* single mutant was unable to survive in macrophages (Fig. 2). These data were in contrast to our earlier study in which we demonstrated that curing of the pPCP-1 plasmid from WT CO92 was initially (4-8 h) sensitive to the macrophage environment but recovered and survived similar to that of WT CO92 by 24 h (Agar *et al.*, 2009). Therefore, it is tempting to speculate that other genes present on the pPCP-1 plasmid (e.g., *pst* and *pim*) might modulate survival of the plasmid-cured CO92 strain in macrophages, when compared to the Δ*pla* single mutant, and needs further investigation. Finally, although we used murine RAW 264.7 macrophages, we have shown that macrophages derived from the human HL-60 cells behaved very similarly (Sha *et al.*, 2013).

Our data corroborated with the earlier findings that the ability of Δ*pla* single mutant to disseminate to peripheral organs was impeded in a bubonic plague mouse model (Sodeinde *et al.*, 1992; Sebbane, Jarrett, *et al.*, 2006). However, in a pneumonic plague

mouse model, our data indicated that the Δ*pla* single mutant retained its capability to disseminate to liver and the spleen up to day 6 p.i., although the number of bacteria and animals that were positive for infection was greatly reduced when compared to WT CO92-infected mice (Fig. 7). Importantly, on day 2 p.i., there was a statistically significant decrease of the Δ*pla* single mutant at the initial infection site (lungs) compared to WT CO92 (Fig. 7). These data indicated failure of the Δ*pla* mutant to colonize the lung tissue, as reported earlier by Lathem *et al*. (Lathem *et al.*, 2007). The difference in the pattern of Δ*pla* mutant dissemination in bubonic and pneumonic mouse models could be related to the highly vascularized nature of the lungs, allowing escape of some bacteria through alveolar capillaries, and, thus initiating systemic infection (Luo *et al.*, 2013).

Pla facilitates digestion of fibrin matrices at peripheral sites of infection, thereby disrupting physical barriers that impede bacterial dissemination (Caulfield and Lathem, 2012). The inability of the Δ*lpp* Δ*pla* double mutant to dissolve fibrin deposits due to the absence of Pla, would enable innate and adaptive host effector cells to quarantine and destroy the invading bacteria. A recent study has also implicated the role of fibrin in innate and T cell-mediated protection of mice against pneumonic plague (Luo *et al.*, 2013), as fibrin-dependent signals provide a cue for the survival of neutrophils to combat *Y. pestis* infection. However, in the above-mentioned study (Luo *et al.*, 2013), it was also noted that the fibrinogen-deficient mice which had antibodies to F1 and LcrV were protected from developing pneumonic plague. These data suggested that fibrinogen did not contribute to antibody-mediated protection of animals. Taken together, the Δ*lpp* Δ*pla* double mutant would avoid mitigation of the innate and cell-mediated signals associated with fibrin deposits.

In a pneumonic plague mouse model, four cytokines/chemokines showed statistically significant decreases when compared to their levels in the WT CO92-infected animals (Table 3). One of them was KC which attracts neutrophils to the site of an infection, and this may contribute to the impaired neutrophil infiltration observed in the tissues of mice infected with the Δ*lpp* Δ*pla* double mutant when compared to mice infected with the WT CO92 (Fig. 10).

The levels of IL-17 and TNF-α were also significantly reduced in the Δ*lpp* Δ*pla* double mutant-infected mice. IL-17 is known to increase chemokine production in tissues to recruit neutrophils and monocyte to the inflammation site, and, in conjunction with IL-23, is responsible for severe tissue damage (Lin *et al.*, 2011). In addition, IL-17 synergizes with TNF-α to produce damaging effects in the host (Kuby, 2007; Lin *et al.*, 2011). Finally, the double mutant produced significantly less anti-inflammatory cytokine IL-10 (Table 3) when compared to WT bacteria-infected animals. This could, possibly in part, be related to deletion of the *lpp* gene from the double mutant, as the absence of Lpp would result in a more greatly reduced activation of TLR-2, latter of which LcrV also uses to promote production of IL-10 (Lui *et al.*, 2010). Our future study will investigate this possibility in detail.

Production of both IgG1 and IgG2a antibodies in a mouse model of pneumonic plague infected with the Δ*lpp* Δ*pla* double mutant indicated a balanced $T_H1/T_H2$ $CD4^+$-based immune response (Fig. 8). The IgG1 isotype corresponds to an increased stimulation of $T_H2$ $CD4^+$ T cell subset, principally producing IL-4 and thereby stimulating an antibody-mediated immune response. Indeed T cells from our double mutant-immunized mice had higher IL-4 levels when compared to WT CO92-infected animals at the sub-lethal dose

(Fig. 12). The IgG2a isotype, on the other hand, corresponds with an increase in a $T_H1$ $CD4^+$ T cell subset which mainly secretes TNF-α and IFN-γ, required for combating an intracellular infection with activated cytotoxic lymphocytes (Dubois *et al.*, 2007). T cells from the Δ*lpp* Δ*pla* double mutant-immunized mice similarly had higher levels of IFN-γ and TNF-α (Figs. 12 & 13). The current notion in plague field is that cellular immunity augments antibody-mediated defense (Smiley, 2008; Lin *et al.*, 2011). In other words, antibodies alone provide unreliable protection, while a cellular immune response, such as the one elicited by a live-attenuated vaccine, exhibits dependable protection. Consistent with this hypothesis, our data depicted both a cell-based (Figs. 11-13) and humoral immunity (Fig. 8) that were elicited by the Δ*lpp* Δ*pla* double mutant, providing comprehensive protection against pneumonic plague.

Route of infection seems to play a role in dictating which type of antibodies is produced during plague by the Δ*lpp* Δ*pla* double mutant. For example in a bubonic plague model, the Δ*pla* single mutant produced both IgG1 and IgG2a, while the Δ*lpp* Δ*pla* double mutant predominately produced IgG1 (Fig. 5). However, animals infected with the above-mentioned single or the double mutant in a pneumonic plague model elicited similar levels of IgG1 and IgG2a (Fig. 8). An earlier study also indicated a predominant $T_H2$ response in mice by immunization with F1-V by the s.c. route and a balanced $T_H1/T_H2$ response when immunized by the i.n. route (Anderson *et al.*, 1997).

No detectable IgA was measured in the sera of mice infected with the Δ*pla* single or the Δ*lpp* Δ*pla* double mutant by any route (data not shown). However, IgA antibodies are rarely seen in the sera during pneumonic plague, as the hallmark of disease is not the colonization of the lungs by the bacteria, but rather its rapid dissemination from the lungs.

Thus the lack of an IgA response may not be a necessity to contain *Y. pestis* infection (Lukaszewski *et al.*, 2005).

The T cell proliferative responses specific to *Y. pestis* antigens were significantly more robust in the Δ*lpp* Δ*pla* double mutant-immunized mice compared to those in animals infected with the WT CO92 (Fig. 11). Live-attenuated vaccine strains tend to exhibit reduced T cell responses compared to those with WT bacteria due to over attenuation (Meyer, 1970; Smiley, 2008; Feodorova and Motin, 2012), thus making our findings significant. In our early study in which mice were infected with another double mutant, Δ*lpp* Δ*msbB* of *Y. pestis*, we showed T cell proliferative responses comparable to that of the WT CO92-infected animals (Sha *et al.*, 2013). Here, we provided evidence that a majority of the T cells that were proliferating after immunization of mice with either the Δ*lpp* Δ*pla* double mutant or the WT CO92 were TNF-α producing $CD4^+$ and $CD8^+$ cells (Fig. 13).

We preferred to measure cytokine responses both in the T cell supernatants by Multiplex as well in the $CD4^+$ and $CD8^+$ subsets of T cells by flow cytometry in WT- and double mutant-immunized mice. Our rationale was not to miss their detection due to low levels or degradation. Indeed, we could detect IFN-γ production in the T cell supernatants by Multiplex but not in T cell subsets. Conversely, TNF-α was seen in both T cell subsets as well as in the supernatants of T cells. These data could be explained by the fact that the detection of cytokines in the supernatant (such as in Multiplex) is dependent on the total number of T cells used and their ability to produce these mediators. Further, some discrepancy in the levels of cytokines detected could also be related to the sensitivity of the two methods used. Mechanistically, both TNF-α and IFN-γ combat *Y. pestis* infection

by up-regulating the production of iNOS (inducible nitric oxide synthase-2) in macrophages (Miossec *et al.*, 2009). In addition, both of these cytokines have the potential to augment oxidative mechanisms in neutrophils (Miossec *et al.*, 2009).

The protective role of these two cytokines during plague has further been established, as neutralizing antibodies against these cytokines negatively impact antibody-mediated (to F1 and LcrV) protection against pneumonic plague (Smiley, 2008; Lin *et al.*, 2011). Conversely, endogenous TNF-α and IFN-γ could protect mice against *Y. pestis* infection without any protective antibodies to F1 and LcrV (Smiley, 2008).

Taken together, our data tend to suggest that TNF-α and IFN-γ produced by T cells during the immunization of mice with the Δ*lpp* Δ*pla* double mutant would activate macrophages to kill intracellular plague bacterium during secondary infection (Fig. 6), while activation of neutrophils by the same cytokines would kill extracellular *Y. pestis*, possibly in a fibrin-dependent manner (Szaba and Smiley, 2002; Luo *et al.*, 2013). Finally, the role of IL-17 produced by T cells has been implicated in protection of the host against pneumonic plague (Lin *et al.*, 2011), although how it contributes to protection is unclear as it is not related to rapid bacterial clearance or neutrophil recruitment (Kuby , 2007; Kamei *et al.*, 2011; Lin *et al.*, 2011; Wilke *et al.*, 2011; Ali *et al.*, 2013). In our study, we were unable to detect IL-17 from T cells in WT CO92 or the double mutant-immunized mice. However, because the Δ*lpp* Δ*pla* double mutant rapidly clears from the host and production of TNF-α and IFN-γ by T cells would activate neutrophils, IL-17 might not be absolutely required for protection and this needs further investigation. In summary, our data indicated that the Δ*lpp* Δ*pla* double mutant stimulated a WT bacterium-like or better protective humoral and cellular immune responses in mice without lethality.

The 5 x $10^5$ cfu dose of Δ*lpp* Δ*pla* double mutant which was used to induce pneumonic plague in this study represents a lower dose than the EV76 strain ($5.8 \times 10^6$ cfu) that is used as a live-attenuated vaccine strain in the Former Soviet Union (Titball and Williamson, 2001). Since the Δ*lpp* Δ*pla* double mutant still possesses an intact LPS, it would have issues related to reactogenicity. Consequently, introduction of additional mutations in the current Δ*lpp* Δ*pla* double mutant, e.g., reducing the biological potency of LPS or deleting other virulence genes may present a logical step in the future. In our earlier study, we modified LPS of *Y. pestis* CO92 by deleting the *msbB* gene which encodes an acyltransferase that is responsible for the addition of lauric acid to the lipid A moiety of LPS (Sha, J. *et al.*, 2013). We have shown that the Δ*lpp* Δ*msbB* mutant was more attenuated than the single mutants (i.e., Δ*lpp* or Δ*msbB*) alone in a mouse model of pneumonic plague and it generated cell-mediated immune responses (Sha *et al.*, 2013). On the other hand, studies have also implicated opsonic antibodies to the antigenically variable LPS O antigens as a primary immune effector against *Pseudomonas aeruginosa* associated acute lung infection (Pier *et al.*, 1995; Kamei *et al.*, 2011). Therefore, fine tuning of attenuation and the immunogenicity is a key to the development of a successful live attenuated vaccine.

# Chapter 5: Further characterization of the Δ*lpp* Δ*pla* double mutant in macrophages

*Y. pestis* is a facultative intracellular pathogen, and during the early stages of an infection, the organism invades both macrophages and neutrophils (Lukaszewski *et al.*, 2005). While neutrophils typically kill *Y. pestis*, the organism survives, replicates, and acquires anti-phagocytic capabilities (e.g., by synthesizing capsule and activating T3SS) that enable survival of extra-cellular bacteria *in vivo* (Yamamoto *et al.*, 1996; Perry and Fetherston, 1997; Pujol and Bliska, 2003; Lukaszewski *et al.*, 2005). This virulence attribute of *Y. pestis* represents an important first step in subverting the innate immune response of the host, which aids in subsequent bacterial dissemination leading to progressive bubonic, septicemic, and pneumonic plague (Peters *et al.*, 2013).

*Y. pestis* hijacks macrophages from within by preventing phagosome acidification and nitric oxide production (Sebbane *et al.*, 2006; Amedei *et al.*, 2011). Macrophages are one of the key players in innate immunity and they also are central to bridging innate and adaptive immunities against microbial infections (Gennery A.R., 2001). During bubonic and pneumonic plague, macrophages either in the regional lymph nodes or the lungs are the primary targets for initial bacterial replication (Perry and Fetherston, 1997; Peters *et al.*, 2013), thus providing the microbe with a unique niche to avoid antigen presentation, and, therefore, delaying the development of specific immune responses in the host.

Macrophages have been classified into various subsets based on their location, surface markers, and function. Two populations of macrophages that reside in the lungs are interstitial and alveolar. Interstitial lung macrophages closely resemble monocytes and are

more involved in immune regulation than alveolar macrophages. Further, due to their location, these macrophages are in direct contact with epithelial and endothelial cells and contribute more to the pathology than alveolar macrophages (Laskin *et al.*2001). In contrast, alveolar macrophages have greater functional activities, such as their ability to undergo phagocytosis and to produce reactive oxygen species (Peters *et al.*, 2013). The alveolar macrophages reside in an environment of high lung surfactant that provides survival advantage to them due to the activation of pro-survival signaling molecules (Janssen *et al.*, 2011) when compared to the recruited macrophages (e.g., from bone marrow or those circulating in the blood), which have a shorter lifespan (Janssen *et al.*, 2011). In addition, macrophages possess a variety of receptors for lineage-determining growth factors, and, therefore, classified into two populations: M1 and M2 (Fernando *et al.*, 2014). The M1 refers to classically activated macrophages that are capable of sustaining an immune response to pathogens through the release of proinflammatory factors, as well as efficient antigen presentation and T-cell stimulation. The M2 refers to alternatively activated macrophages, a very heterogeneous group of cells contributing to resolution of inflammation, tissue repair, extracellular matrix remodeling, and pathogen scavenging (Bayer *et al.*, 2013).

In efforts to search for a novel live-attenuated plague vaccine, we recently generated a Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 (van Lier *et al.*, 2014). Braun lipoprotein (Lpp) is an outer bacterial membrane protein that activates toll-like receptor (TLR)-2 of the host to initiate an inflammatory cascade; its role in evoking bubonic and pneumonic plague has been well characterized in our laboratory (Sha *et al.*, 2008). Likewise, plasminogen activator (Pla) protease is a proven virulence factor of *Y. pestis*

which facilitates bacterial dissemination during bubonic and pneumonic plague (Lathem *et al.*, 2007; van Lier *et al.*, 2014). The generated Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 was highly attenuated at doses of 1,000 and 10,000 ($5 \times 10^5$ colony forming units [CFU]) equivalent LD50s of WT CO92 with 100% survival rates when delivered by the intranasal or the subcutaneous route in pneumonic and bubonic plague mouse models, respectively (van Lier *et al.*, 2014). The mutant bacteria were cleared from the mouse organs rapidly, but Δ*lpp* Δ*pla* double mutant still generated adequate humoral and cell-mediated immune responses to significantly protect animals from developing subsequent pneumonic plague caused by infection with WT CO92 (van Lier *et al.*, 2014).

Our recent data indicated that both Lpp and Pla contributed to the survival of *Y. pestis* CO92 in murine RAW 264.7 macrophages (van Lier *et al.*, 2014). We also showed that the role of Lpp in *Y. pestis* CO92 intracellular survival in these macrophages was linked to the decreased production of a heat shock protein GsrA in the microbe (Galindo *et al.*, 2009). GsrA is a periplasmic protease required for bacterial adaptation to stress stimuli, and it has been implicated in the survival of *Y. enterocolitica* and *E. coli* within macrophages (Yamamoto *et al.*, 1996). However, the contribution of Pla to intracellular survival of *Y. pestis* in macrophages represents a new paradigm; as previous studies from other groups have shown that *Y. pestis* strains lacking pPCP1 plasmid, which encodes Pla, were still able to replicate inside the macrophages (Straley and Harmon, 1984a; b; Pujol *et al.*, 2005). The mechanism(s) by which Pla contributes to *Y. pestis* intracellular survival is currently unknown.

Considering the curial role of macrophages during *Y. pestis* infection and the functional heterogeneity of macrophages, it is important to further evaluate interaction of the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 with different types of macrophages and gauge its potential as a live-attenuated vaccine candidate. Three different types of macrophages, namely murine RAW 264.7 and MH-S, and human primary macrophages derived from blood monocytes of healthy donors were used in this study. While the MH-S cell line represents resident lung macrophages, both RAW 264.7 and human primary macrophages closely mimic the recruited macrophages which are derived from the bloodstream monocytes (Laskin *et al.*, 2001; Liu *et al.*, 2006; Berghaus *et al.*, 2010). Consequently, due to some intrinsic differences between resident versus recruited macrophages (Laskin *et al.*, 2001; Liu *et al.*, 2006), the intracellular survival of *Y. pestis* mutants could differ in various types of macrophages and was the focus of this study.

Our results showed that the Δ*pla* single and the Δ*lpp* Δ*pla* double mutant were unable to survive efficiently in all tested macrophages compared to WT CO92 and that both Lpp and Pla contributed to intracellular survival by operating through different mechanisms. Importantly, the Δ*lpp* Δ*pla* double mutant retained the ability to elicit innate and subsequent acquired immune responses in the host similar to that of WT CO92. This is the first detailed study delineating the role of Lpp and Pla, as well as their interplay, in the survival of *Y. pestis* in professional macrophages.

**Survival of various mutant strains of *Y. pestis* CO92 in murine RAW 264.7 and MH-S macrophages**

The first step during *Y. pestis* infection is the phagocytosis of the pathogen by macrophages within which the organisms survive and replicate before being further disseminated to cause a progressive disease. Consequently, we examined intracellular survival of WT CO92 and its various mutants (Δ*lpp* and Δ*pla* single, and the Δ*lpp* Δ*pla* double mutant), as well as their respective complemented strains in murine RAW 264.7 macrophages when infected at an MOI of 1. We chose this cell line to confirm our previous findings (van Lier *et al.*, 2014) with additional control strains to authenticate the data.

As shown in **Fig. 14A**, WT CO92 had an intracellular survival rate of 55% at 4 h post-gentamicin treatment, and in comparison, the Δ*lpp* and Δ*pla* single mutants were below 10% survival. Complementation of the Δ*lpp* (Δ*lpp* Tn*7*-*lpp* [*in cis*]) and Δ*pla* (Δ*pla* pBR322*Ypla* [*in trans*]) **(Table 1)** single mutants resulted in restoration of their intracellular survival phenotype similar to that of WT CO92 (~50-60%) (**Fig. 14A**). Likewise, the Δ*lpp* Δ*pla* double mutant survived minimally in RAW 264.7 macrophages (less than 10%), while complementation of the double mutant with the *pla* gene (Δ*lpp* Δ*pla* pBR322*Ypla* [*in trans*]) exhibited 60% survival rate, similar to that of the WT CO92 strain. These results correlated with our previous study in RAW 264.7 cells which also showed decreased intracellular survival of Δ*lpp* and Δ*pla* single, and the Δ*lpp* Δ*pla* double mutant (van Lier *et al.*, 2014). Importantly, we have now shown that the decreased intracellular survival of the Δ*pla* single and the Δ*lpp* Δ*pla* double mutant could be fully complemented when the *pla* gene was supplied *in trans*, confirming the role of Pla in *Y. pestis* intracellular survival.

Our results that the Δ*pla* single mutant was unable to survive in macrophages (**Fig. 14A**) contrasted from our earlier study in which we demonstrated that WT CO92 cured for the pPCP-1 plasmid was initially (4 to 8 h) sensitive to the macrophage environment but recovered and had a survived rate similar to that of WT CO92 by 24 h (Agar *et al.*, 2009). Therefore, it is tempting to speculate that other genes present on the pPCP-1 plasmid (e.g., *pst* and *pim*) might modulate the survival of the plasmid-cured CO92 strain in macrophages compared to that of the Δ*pla* single isogenic mutant which still contained the pPCP-1 plasmid but devoid of the *pla* gene.

Since deletion of both the *lpp* and *pla* genes individually decreased intracellular survival of the mutants drastically, we were unable to discern any additive effect of the combined deletion of these two genes from the Δ*lpp* Δ*pla* double mutant (**Fig. 14A**). Supplying *pla* in *trans* compensated the deletion effect of *lpp* in terms of intracellular survival of the Δ*lpp* Δ*pla* double mutant in RAW 264.7 macrophages (**Fig. 14A**). This was surprising as we anticipated only partial restoration of the intracellular survival phenotype in the double mutant. To further confirm and pursue our findings, MH-S, a murine immortalized alveolar macrophage cell line, was tested, as lungs are the primary target during pneumonic plague infection.

The MH-S cells were infected with an MOI of 1 with WT CO92, its various mutants, and the complemented strains (**Fig. 14B**). As noted, 4 h post-gentamicin treatment, WT CO92 not only survived but also proliferated in these cells, as the percent survival exceeded 100%. In comparison, the survival rates of the Δ*lpp* and Δ*pla* single mutants were 30 and 45%, respectively. We could fully complement the Δ*lpp* and Δ*pla* single mutants in terms of their intracellular survival phenotype with the corresponding

genes (**Fig. 14B**). Importantly, the Δ*lpp* Δ*pla* double mutant survived minimally (less than 20%) in MH-S cells and exhibited an additive decrease in its ability to survive intracellularly when compared to the Δ*lpp* and Δ*pla* single mutants **(Fig. 14B),** a phenomenon not observed in RAW 264.7 macrophages **(Fig. 14A)**. Interestingly, complementation of the double mutant with the *pla* gene restored the intracellular survival phenotype comparable to that of WT CO92 (**Fig. 14B**), as also noted in the RAW 264.7 cells.

As alluded to earlier, the role of Lpp in *Y. pestis* intracellular survival was linked to the decreased production of a heat shock protein GsrA within the microbe (Galindo *et al.*, 2009). Since the decreased intracellular survivability of the Δ*lpp* Δ*pla* double mutant in both RAW 264.7 and MH-S cells was fully complemented with the *pla* gene when supplied *in trans* (**Fig. 14A&B**), these findings suggested that Lpp and Pla might have had a similar mechanism of action, i.e., decreasing the production of GsrA, and thus overproduction of Pla from the recombinant pBR322 plasmid could compensate the effect of Lpp in the complemented strain **(Fig. 14A&B)**. Alternatively, there might be an interplay between Pla and Lpp as the deletion of one gene could possibly affect the expression of the other gene, eventually leading to an alteration in the level of GsrA. As mentioned earlier for RAW 264.7 macrophages, we expected only partial restoration of the intracellular survival of the double mutant with the *pla* gene in MH-S cells if Pla had a different mechanism of action than Lpp, a possibility which was further investigated in this study.

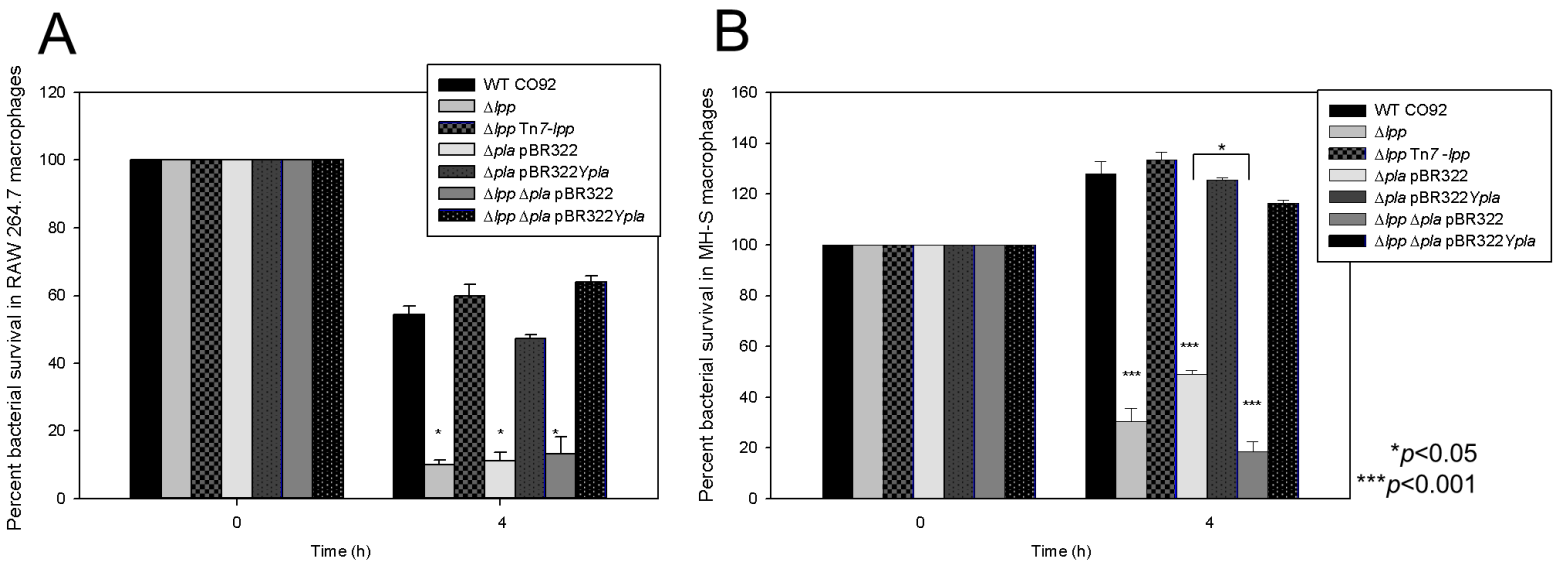


**Fig. 14: Intracellular survival of WT *Y. pestis* CO92 and its mutant strains in murine macrophages.**
RAW 264.7 macrophages **(A)** and MH-S alveolar macrophages **(B)** were infected with WT CO92, its single mutants (Δ*lpp* and Δ*pla*), and the Δ*lpp* Δ*pla* double mutant, as well as the complemented strains for 45 min at an MOI of 1. Monolayers were then treated with gentamicin for 1 h, and harvested at 0 and 4 h post-gentamicin treatment. Bacteria were enumerated and the percent survival rate was calculated based on the number of bacteria recovered at each specific time point over that at the 0 h time point. At least three independent experiments were performed. The data from both of the cell lines were analyzed using one-way ANOVA with Bonferroni correction and $p$ values $\leq 0.05$ were considered significant. Asterisks indicate statistically significant differences when compared to WT CO92-infected cell lines or between two groups as indicated by a bracket.

**Crosstalk between Lpp and Pla, and the role of GsrA in modulating *Y. pestis* CO92 survival in macrophages**

To further dissect the mechanism of Pla in *Y. pestis* intracellular survival and to test the possibility whether deletion of the *pla* or the *lpp* gene from WT CO92 would affect expression and production of these membrane proteins, we performed Western blot analysis and the Pla activity assay. As noted from **Fig. 15A**, while WT CO92 and the Δ*pla* single mutant had similar levels of Lpp, no Lpp production was noted in the Δ*lpp* single or the Δ*lpp* Δ*pla* double mutant. Likewise, similar levels of Pla were noted in the Δ*lpp* single mutant when compared to its level in the WT CO92 (**Fig. 15B&C**, **insets**). However, Pla was absent from the Δ*pla* single and the Δ*lpp* Δ*pla* double mutants. The production of Pla was restored in the above-mentioned two mutants after complementation with the *pla* gene *in trans* (**Fig. 15B&C**, **insets**).

It has been reported that lipopolysaccharide (LPS) present on the bacterial surface can affect the conformation of Pla, with the more biologically active form of Pla being displayed on the bacterial surface at 37$^{o}$C (Eddy *et al.*, 2014). Since both Lpp and Pla are also bacterial outer membrane components, we measured protease activity associated with Pla to ensure that the enzymatic activity of this protein remained unaffected by deletion of the *lpp* gene from WT CO92 by using a fluorometric assay and a hexapeptide substrate (Agarkov *et al.*, 2008). The Pla protease assay was performed at two different bacterial concentrations and temperatures to measure the kinetics of substrate cleavage (relative fluorescence units [RFU]). Subsequently, the maximal velocity ($V_{max}$) value, defined as the rate at which the substrate was cleaved by Pla was calculated. The use of two growth temperatures allowed us to measure Pla protease activity at both flea (28°C) and human

body temperatures (37°C). Further, as mentioned earlier, the bacterial surface structure changes at different temperatures. For examples, the F1 capsular antigen is primarily synthesized at 37°C, the lipid A of *Y. pestis* LPS shifts from a hexa-acylated form to a tetra-acylated form when the temperature shifts from 21-27$^{o}$C to 37$^{o}$C (Rebeil *et al.*, 2006). In addition, the production of Attachment Invasion Locus (Ail), another bacterial outer membrane component, is up regulated at 37$^{o}$C (Kolodziejek *et al.*, 2010). These surface alterations could affect the conformation of Pla and its associated enzymatic activity, and was tested.

As noted in **Fig. 15B-I**, when various bacterial strains were grown at 28°C and adjusted to an $OD_{600}$ of 0.1, WT CO92 and its Δ*lpp* single mutant had essentially similar kinetics of Pla substrate cleavage with $V_{max}$ values of 3719 and 4004, respectively. The Δ*pla* single and the Δ*lpp* Δ*pla* double mutant in comparison showed minimal background activity, with $V_{max}$ values of 210.6 and 207.3, respectively. Complete restoration of Pla protease activity was noted when the Δ*lpp* Δ*pla* double mutant was complemented with the *pla* gene in *trans* ($V_{max}$ of 4211) and was comparable to that of WT CO92 and its Δ*lpp* single mutant, with essentially similar Pla substrate cleavage kinetics curves over time. At an $OD_{600}$ of 0.5 at 28°C, WT CO92, Δ*lpp* single, and the Δ*lpp* Δ*pla* pBR322*Ypla* complemented strain behaved similarly with $V_{max}$ values of 4479, 4051, and 4451, respectively (**Fig. 15B-II**). The Δ*pla* single and the Δ*lpp* Δ*pla* double mutant had baseline $V_{max}$ values of 344.3 and 344.9, respectively.

As noted from **Fig. 15C-I**, at an $OD_{600}$ of 0.1 at 37°C, WT CO92 and its Δ*lpp* single mutant had essentially similar kinetics of substrate cleavage with $V_{max}$ values of 3266 and 3761, respectively. The Δ*pla* single and the Δ*lpp* Δ*pla* double mutant, respectively,

demonstrated minimal background activity with $V_{max}$ values of 248.5 and 219.4. Partial restoration of Pla protease activity was noted when the Δ*lpp* Δ*pla* double mutant was complemented with the *pla* gene in *trans* ($V_{max}$ of 1178), which was significantly higher when compared to the Δ*pla* single and the Δ*lpp* Δ*pla* double mutant ($p<0.001$). Overall, the kinetics of substrate cleavage over time by Pla was somewhat affected in the complemented strain, however, there was still notable restoration of Pla protease activity compared to the Δ*pla* single and the Δ*lpp* Δ*pla* double mutant.

When the WT CO92 and its Δ*lpp* single mutant were grown at 37°C and at an $OD_{600}$ of 0.5, both had essentially similar kinetics of substrate cleavage with $V_{max}$ values of 4278 and 4041, respectively (**Fig. 15C-II**). The Δ*pla* single and the Δ*lpp* Δ*pla* double mutant demonstrated minimal background level of protease activity. Restoration of Pla protease activity was noted when the Δ*lpp* Δ*pla* double mutant was complemented with the *pla* gene ($V_{max}$ of 4340), which was comparable to that of WT CO92. Although the kinetics of substrate cleavage by Pla was slower initially for the complemented strain, overall, it had similar $V_{max}$ over a period of 2 h, indicating that Pla protease activity almost reached saturation at an $OD_{600}$ of 0.5.

Except for the Δ*lpp* Δ*pla* double mutant complemented strain, similar kinetics of substrate cleavage by Pla and $V_{max}$ values were observed for WT CO92 and its Δ*lpp* and Δ*pla* single, and the Δ*lpp* Δ*pla* double mutant both at 28 and 37°C, indicating that a temperature shift did not impact Pla protease activity in these strains. However, misfolding of Pla could most likely account for the relatively lower Pla protease activity in the Δ*lpp* Δ*pla* double mutant complemented strain at 37°C **(Fig. 15C),** as the level of Pla production in this complemented strain was similar to that of WT CO92 level based on Western blot

analysis **(Fig. 15C-insets)**. Clearly, there did not seem to have a direct interaction between Pla and Lpp in terms of their production level and Pla protease activity (**Fig. 15**).

The data presented above were intriguing as supplying the *pla* gene *in trans* only partially restored Δ*lpp* Δ*pla* double mutant's protease activity at 37°C (**Fig. 15C**), but it could fully restore the intracellular survival of the Δ*lpp* Δ*pla* double mutant in RAW 264.7 and MH-S macrophages (**Fig. 14A&B**). This question led us to the second part of our hypothesis that Pla might have a different mechanism than Lpp (not through GsrA) in terms of bacterial intracellular survival. To address this, we complemented Δ*lpp* and Δ*pla* single, and the Δ*lpp* Δ*pla* double mutant with the *grsA* gene using the pBR322 vector system. Our results for the Δ*lpp* single mutant correlated with our earlier study (Galindo *et al.,* 2010) showing complete restoration of mutant's ability to grow within RAW264.7 macrophages when the Δ*lpp* single mutant was complemented with the *gsrA* gene (**Fig. 16**). However, when the Δ*pla* single mutant was complemented with the *gsrA* gene, there was no restoration of intracellular survival. Importantly, GsrA could only partially restore the intracellular survival of the Δ*lpp* Δ*pla* double mutant, suggesting that GsrA complemented the effect of Lpp deletion but not that of Pla (**Fig. 16**). These data confirmed that Lpp and Pla operated through separate mechanisms to control intracellular survival of *Y. pestis* CO92. However, it was still not clear why Pla could compensate the effect caused by Lpp deletion on the intracellular survival in macrophages of the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 **(Fig. 14A&B)**.

Bacterial heat shock proteins, collectively termed as stress proteins, play an important role in allowing organisms to successfully adapt to the hostile environment of the host phagosome (Mecsas *et al.*, 1993; Yamamoto *et al.*, 1996). HtrA, also known as

DegP (Strauch *et al.*, 1989) or GsrA, has serine protease activity (Lipinska *et al.*, 1990). GsrA degrades abnormal peptides generated after exposure of bacteria to stress before they accumulate to toxic levels in the periplasmic space (Lipinska *et al.*, 1990). The production of GsrA is controlled by the sigma factor E ($\sigma^E$), a global stress response regulator, and the activity of $\sigma^E$ is induced by either overproduction or misfolding of outer membrane proteins (OMPs), leading to the increased production of GsrA in *E. coli* and *Y. enterocolitica* (Mecsas *et al.*, 1993; Yamamoto *et al.*, 1997). Therefore, it is possible that the misfolded Pla in the complemented Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 **(Fig. 15C)** with low Pla protease activity could possibly activate the expression of the *gsrA* gene through $\sigma^E$ to exhibit full complementation of the intracellular survival defect **(Fig. 14A&B)**.

In addition to GsrA, other heat shock proteins such as DnaK, GroEL, and GroES are essential for bacterial growth and viability at all temperatures in bacteria (Bukau and Walker, 1989; Fayet *et al.*, 1989). It has also been shown that DnaK and GroEL bind to unfolded polypeptides and function as "molecular chaperones" in cells (Ellis, 1990). Although the role of Pla in *Y. pestis* intracellular survival is not through GsrA; however, as an OMP, it is still possible that deletion of the *pla* gene would lead to decreased production of other stress response proteins, and our future studies will investigate this possibility.

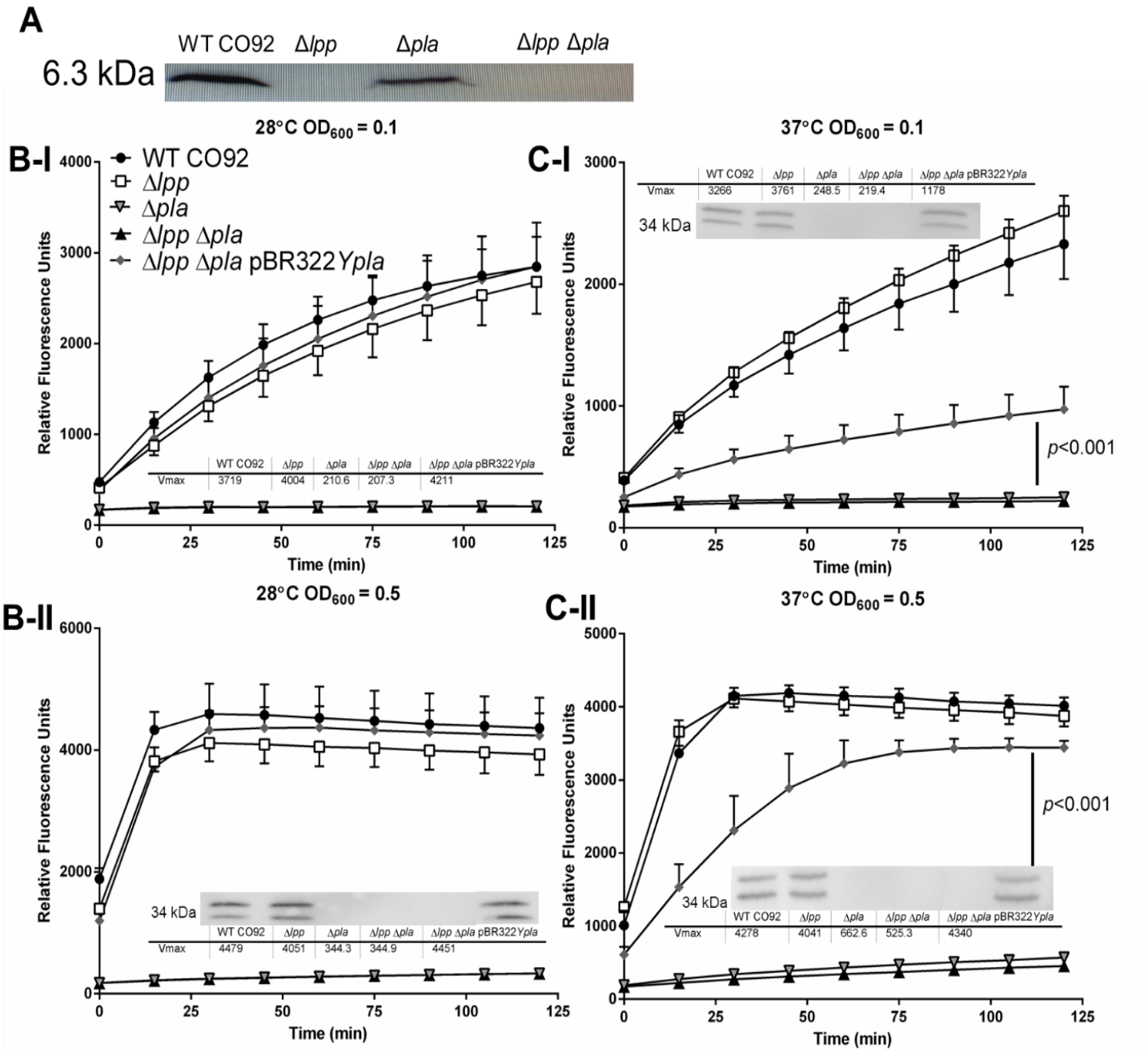


**Fig. 15: The expression of *lpp* and *pla* genes as well as Pla associated protease activity in various *Y. pestis* strains.** *Yersinia* cultures were grown overnight at 28°C. The expression of the *lpp* gene was evaluated by Western blot analysis with anti-Lpp monoclonal antibodies **(A)**. For Pla protease activity, *Yersinia* cultures were grown on the HIB agar plates either at 28°C **(B)** or 37°C (**C**) for 20-22 h and suspended in PBS to $OD_{600}$ of 0.1 (**B-I and C-I**) and 0.5 (**B-II and C-II**), respectively. Pla protease activity was measured in a fluorometric assay. The kinetics of substrate cleavage by Pla was plotted and the $V_{max}$ was calculated. The expression of the *pla* gene in the above-mentioned cultures was evaluated by Western blot analysis with anti-Pla polyclonal antibodies (**insets**). Statistical analysis was performed by one-way ANOVA with a Bonferroni *post-hoc* test. Statistically significant *p* values are shown between the groups by a vertical line. The upper band in the Western blots represented precursor form of Pla.

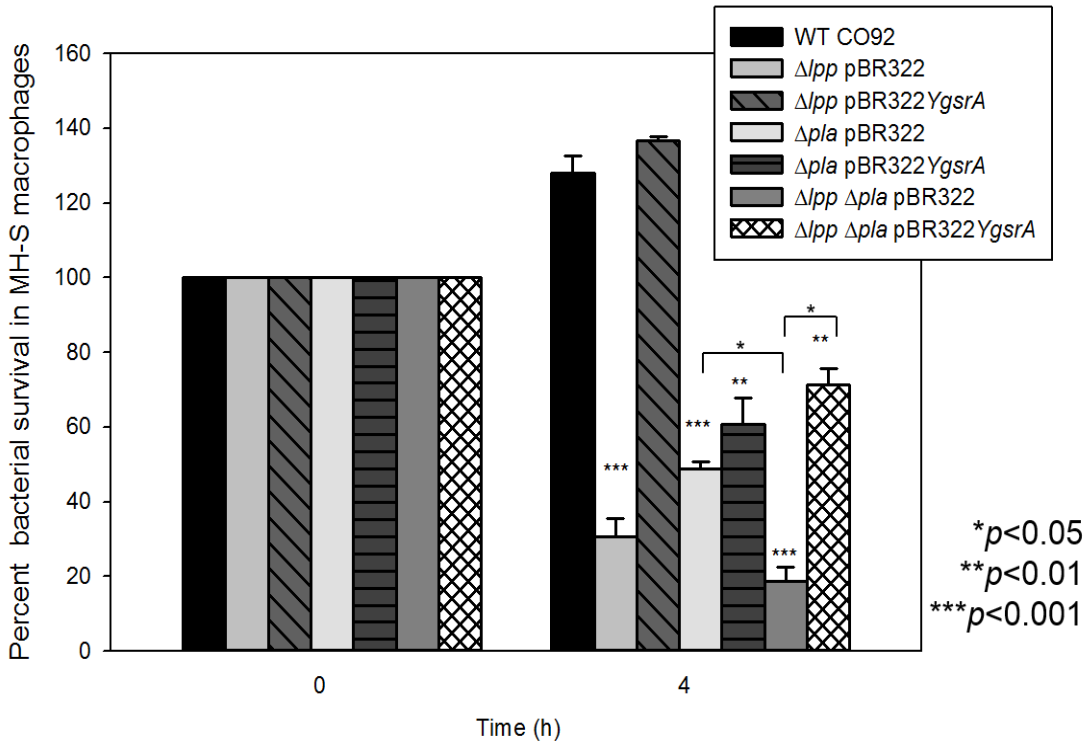


**Fig. 16: The role of GsrA in the intracellular survival of Δ*lpp* and Δ*pla* single, and the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92.**

MH-S macrophages were infected with WT CO92, its single mutants (Δ*lpp* and Δ*pla*), the Δ*lpp* Δ*pla* double mutant, as well as their *gsrA* complemented strains. Intracellular survival was assessed via a gentamicin protection assay as described in Fig. 1 legend. The experiments were performed in triplicate. The data were analyzed using One-way ANOVA with Bonferroni correction and $p$ values $\leq 0.05$ were considered significant. Asterisks indicate statistically significant differences when compared to WT CO92 infected MH-S cells or between the two groups indicated by brackets.

**Survival of WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant in human monocyte-derived macrophages (HMDMs)**

To be a vaccine candidate, it was crucial to evaluate the Δ*lpp* Δ*pla* double mutant of *Y. pestis* CO92 in primary human macrophages from healthy individuals. As shown in **Fig. 17A**, the WT CO92 exhibited survival rates of 100% and 50% at 1 and 2 h, respectively, post- gentamicin treatment, the double mutant survival rates were 80% and 25%, respectively, at the designated times. We focused on the Δ*lpp* Δ*pla* double mutant in HMDMs, as we showed earlier its potential as a viable plague vaccine candidate (van Lier *et al.*, 2014).

In addition to CFU determination, the HMDMs infected with either the WT CO92 or the Δ*lpp* Δ*pla* double mutant (1 h post gentamicin treatment) was stained for visualization of bacteria by confocal microscopy **(Fig. 17B)**. We chose to use antibodies specific to the F1 antigen to detect *Y. pestis* as we had previously shown that the production of F1 in the Δ*lpp* Δ*pla* double mutant was not altered compared to the WT CO92 (van Lier *et al.*, 2014). Following the addition of fluorescently labeled secondary antibodies, we detected much reduced level of the Δ*lpp* Δ*pla* double mutant (green) in infected macrophages when compared to WT CO92, thereby corroborating our *in vitro* results that the Δ*lpp* Δ*pla* double mutant was unable to survive as effectively as WT CO92 in macrophages **(Fig. 14A&B and 17A)**.

The inability of the Δ*lpp* Δ*pla* double mutant to survive within different types of macrophages (i.e., RAW 264.7, MH-S, and HMDMs) correlated with the mutant's inability to disseminate to the peripheral organs of mice during initial 2-8 days post infection in both bubonic and pneumonic plague models (van Lier *et al.*, 2014). Interestingly, WT *Y. pestis*

and its various mutant strains survived and replicated to higher numbers in the murine alveolar macrophages (MH-S) compared to RAW 264.7 and HMDMs, indicating intrinsic differences between resident versus recruited macrophages and signifies the role of alveolar macrophages during pneumonic plague. Previous studies showed that the dissemination of Pla-deficient *Y. pestis* was more severely affected in bubonic plague mouse model than in the pneumonic plague model (Sebbane *et al.*, 2006; Lathem *et al.*, 2007; van Lier *et al.*, 2014). Consequently, better survival and replication of the plague bacilli in alveolar macrophages might contribute to this phenomenon in addition to the highly vascularized nature of the lungs (Lathem *et al.*, 2007).

The role of Pla in promoting bacterial dissemination during bubonic and pneumonic plague had been previously attributed to its fibrinolytic activity (Lathem *et al.*, 2007; Korhonen *et al.*, 2013). However, considering the crucial role of macrophages in disseminating *Y. pestis in vivo*, the role of Pla in aiding bacterial intracellular survival may also contribute significantly to this feature. To our knowledge, this is the first report describing in detail the role of Pla in *Y. pestis* CO92 survival in macrophages.

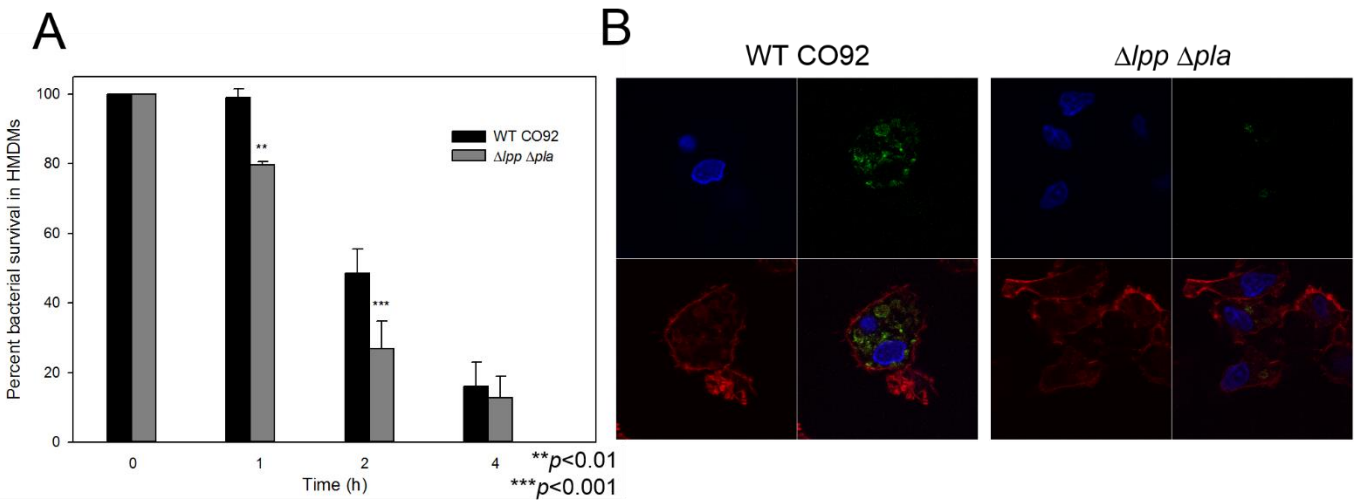


**Fig. 17: Intracellular survival of WT *Y. pestis* CO92 and its mutant strains in human monocyte-derived macrophages (HMDMs).**

Macrophages were infected at an MOI of 0.5 with either the WT CO92 or Δ*lpp* Δ*pla* double mutant for 40 min following 1 h gentamicin treatment (10 μg/ml). At 0, 1, 2, and 4 h (post-gentamicin treatment), macrophages were harvested and the number of bacteria inside macrophages was counted. The percent survival rate was calculated based on the number of bacteria recovered at each specific time point over that of the 0 h time point **(A)**. The data were analyzed using one-way ANOVA with Bonferroni correction and $p$ values $\leq 0.05$ were considered significant. Asterisks indicate statistically significant differences compared to WT CO92-infected HMDMs. At the 1 h time point, the infected HMDMs were also fixed and the bacteria inside the macrophages were detected by specific anti-F1 (capsular antigen) antibodies follow by secondary Alexa Fluor 488 antibodies (green). The actin filaments were stained with Alexa Fluor 568 phalloidin (red), and the cell nucleus was visualized with DAPI (blue). The images were taken by confocal microscopy with the magnification of 1000 **(B)**.

**Cytokine secretion levels are comparable in different types of macrophages when infected with WT *Y. pestis* CO92 and its Δ*lpp* Δ*pla* double mutant**

Three different types of macrophages were used to analyze whether their infection with WT CO92 or the Δ*lpp* Δ*pla* double mutant elicited similar levels of cytokine production. Supernatants from the infected macrophages were harvested immediately after an hour of gentamicin treatment (100-105 min post infection) and analyzed by 6-Bioplex from Bio-Rad. As shown in **Fig. 18A**, interleukin (IL-6) and tumor necrosis factor (TNF)-α were the only two cytokines among the six analyzed, which were detected at the highest concentrations across the three macrophage types used. Importantly, similar levels of these cytokines were detected irrespective of whether the macrophages were infected with the WT CO92 or the Δ*lpp* Δ*pla* double mutant. These data indicated that despite rapid clearance of the Δ*lpp* Δ*pla* double mutant from macrophages, the innate immune effector cells were still responding to activation evoked by this highly attenuated strain.

IL-6 is a notable pro-inflammatory cytokine secreted by various cell types, including the activated macrophages (Martin and Dorf, 1991). It has been shown that recombinant plant-derived *Y. pestis* F1, LcrV (also termed V) and F1-V, the key components of the next generation plague subunit vaccine, are TLR-2 agonists, and, importantly, they significantly increase IL-6 production in human monocytes (Amedei *et al.*, 2011). In addition, IL-6 switches the differentiation of monocytes from dendritic cells to macrophages, and, therefore, is essential in molecularly controlling antigen presentation and cell development (Chomarat *et al.*, 2000). IL-6 has also been reported to enhance polarization of alternatively activated macrophages, and, thus would promote resolution of inflammation and wound healing (Fernando *et al.*, 2014). On the other hand, TNF-α is one

of the most important pro-inflammatory cytokines which limits severity of bacterial infection (Amedei *et al.*, 2011). Interestingly, both IL-6 and TNF-α could act synergistically in prolonging plasma cell survival, leading to better antibody responses (Cassese *et al.*, 2003), which correlates with our previously published data indicating a robust humoral immune response generated by the Δ*lpp* Δ*pla* double mutant in mice (van Lier *et al.*, 2014). Thus, it is clear that both IL-6 and TNF-α play an important role in the host against *Y. pestis* infections, and the ability of Δ*lpp* Δ*pla* double mutant in eliciting high levels of these cytokines signifies the mutant's potential as a vaccine candidate.

**Nitric oxide production is similar when macrophages are stimulated with WT *Y. pestis* CO92 or the Δ*lpp* Δ*pla* double mutant**

Nitric oxide (NO) production is an essential bacterial killing mechanism employed by the professional macrophages and neutrophils, the two major primary cells of the host innate immune system. NO is produced by the inducible NO synthase (iNOS), which catalyzes the oxidation of L-arginine into NO and L-citrulline. NO has been shown to be important for killing or controlling the proliferation of many intracellular pathogens, such as *Leishmania major* (Liew *et al.*, 1990), *Toxoplasma gondii* (Scharton-Kersten *et al.*, 1997), *Mycobacterium tuberculosis* (Macmicking *et al.*, 1997) and *Y. pestis* (Pujol *et al.*, 2005).

The Griess assay was used to determine if the inability of the Δ*lpp* Δ*pla* double mutant to survive within macrophages was due to its increased killing by NO. In this assay, nitrite was the readout as opposed to NO, which is an unstable free radical. As shown in **Fig. 18B**, MH-S macrophages stimulated by infection with either the WT CO92 or its Δ*lpp* Δ*pla* double mutant produced 10 times higher levels of nitrite compared to RAW 264.7 and HMDMs. Surprisingly, all three types of macrophages (RAW 264.7, MH-S, and HMDMs) infected with either the WT CO92 or the Δ*lpp* Δ*pla* double mutant produced similar levels of nitrite in the supernatants after 12 h of infection (**Fig. 18B**).

In contrast, an earlier study indicated that the pigmentation (pgm) locus deletion mutant strain of *Y. pestis* stimulated robust production of NO, and, hence, was unable to survive within macrophages pre-activated with interferon-γ (Pujol *et al.*, 2005). This study further showed that the *ripA* gene encoded on the *pgm* locus was responsible allowing *Y. pestis* to combat NO killing (Pujol *et al.*, 2005). However, the Δ*lpp* Δ*pla* double mutant we used had an intact *pgm* locus and we also did not pre-activate the macrophages before

infection. Since the Δ*lpp* Δ*pla* double mutant was cleared from macrophages quickly **(Fig. 14& 17)** but it still induced similar levels of nitrite as the WT CO92, these data suggested that other mechanisms operating in macrophages might also play a major role in Δ*lpp* Δ*pla* double mutant's clearance.

**Zymosan particle uptake is increased in the WT *Y. pestis* CO92 and the Δ*lpp* Δ*pla* double mutant-infected macrophages versus uninfected cells**

Zymosan (synthetic particle) uptake was examined to delineate whether phagocytic ability of macrophages was altered after infection with either WT CO92 or the Δ*lpp* Δ*pla* double mutant. HMDMs in general had the lowest uptake of the zymosan beads even when the cells were uninfected, however, only approximately 8-9% of the macrophages were positive for zymosan uptake after infection with *Y. pestis* (**Fig. 18C**). In comparison, MH-S and RAW 264.7 murine macrophages both exhibited greater zymosan bead uptake (25 to 60%), and this difference was most likely related to the source of macrophages: human verse murine. A similar uptake rate of zymosan beads (~23.6%) was reported for the murine peritoneal macrophages (Carvalho *et al.*, 2000).

As expected, all three types of macrophages exhibited increase uptake of beads after infection compared to the uninfected ones. However, the uptake of zymosan particles was statistically significant only for the RAW 264.7 cells when comparing infected versus non-infected controls ($p<0.01$). Most importantly, there was essentially no difference in zymosan bead uptake across all three types of macrophages when they were infected with either the WT CO92 or its Δ*lpp* Δ*pla* double mutant **(Fig. 18C)**. We could recover similar numbers of WT CO92 and the Δ*lpp* Δ*pla* double mutant bacteria from these infected macrophages after the initial gentamicin treatment (data not shown), indicating that the T3SS was not altered in the Δ*lpp* Δ*pla* double mutant, which was in agreement with our previous report (van Lier *et al.*, 2014).

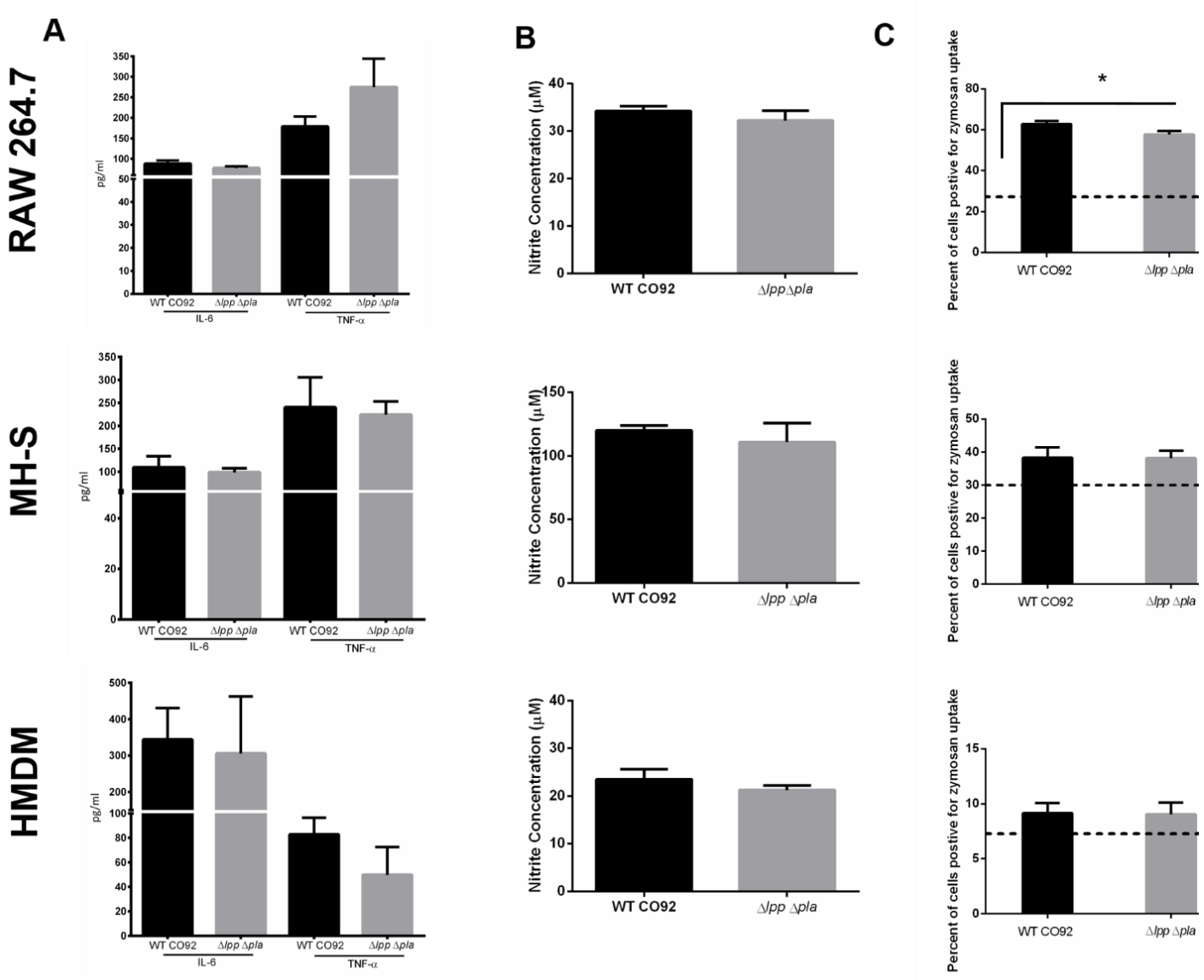


**Fig. 18: The cytokine and nitrite production as well as the zymosan uptake in macrophages elicited by infection with WT CO92 and its Δ*lpp* Δ*pla* double mutant.**

Macrophages were infected with WT CO92 and its Δ*lpp* Δ*pla* double mutant at MOI of 1 followed by gentamicin treatment. Culture supernatants were collected immediately after gentamicin treatment for analyzing cytokine production by using 6-plex Bio-Rad multiplex assay (**A**) or at 12 post infection for the production of nitrite by using the Griess assay (**B**). For the zymosan uptake (**C**), the above infected macrophages were incubated with 1 x $10^6$ (1 μm) of Texas-Red zymosan particles after the gentamicin treatment. The number of macrophages positive for beads was enumerated by flow cytometry. The dotted line indicates zymosan uptake by uninfected control macrophages. Statistical analysis was performed by one-way ANOVA with Tukey's *post-hoc* test, and the statistical significant difference is indicated by an asterisk with $p<0.05$ when compared to the uninfected control macrophages.

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**Conclusions and Future Directions**

The Δ*lpp* Δ*pla* double mutant represents an excellent candidate to be further developed into a viable live-attenuated plague vaccine. The double mutant is highly attenuated in evoking bubonic and pneumonic plague while retaining its ability to generate strong humoral and cell-mediated immune responses, and to maintain innate immune responses similar to that of the WT CO92. In future studies, we aim to determine the optimal dose of the Δ*lpp* Δ*pla* double mutant as well as number of boosters needed which would elicit a long-lasting protective humoral and cell-mediated immune responses when administrated by different routes in mice (e.g., intramuscular versus subcutaneous). Additional future studies would involve testing this double mutant in a rat model of pneumonic plague, and subsequently, in NHPs.

Most importantly, such vaccines as described in this study possibly could represent attractive options for immuno-competent individuals (military personnel and others who have been exposed to the pathogen); the suitability of vaccines in immuno-compromised people is always a concern. For example, cancer patients undergoing treatment and transplant and autoimmune patients treated with immunosuppressive agents represent a growing segment of the population. We would also investigate the possibility of attenuating this strain further by other gene deletions, to make it safer for use in a diverse human population. Consequently, the Δ*lpp* Δ*pla* double mutant could serve as a starting point or an excellent platform from which other virulence genes could be deleted to develop a safe and efficacious new generation vaccine for mass immunization.

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1. **van Lier CJ**, Sha J, Chauhan S, Vladimir ML, Fitts EC, Huante M, Endsley JJ, Chopra AK. 2014. Further characterization of a highly attenuated *Yersinia pestis* CO92 mutant deleted for the genes encoding Braun lipoprotein and plasminogen-activating protease in primary human macrophages. *Microb. Path.* Submitted.
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The *in vitro* macrophage research was supported by the NIH/NIAID grant AI064389 awarded to A.K.C. We also acknowledge a UC7 grant (AI070083) which facilitated our research in the Galveston National Laboratory, Galveston, TX. CJ van Lier was supported in part by the T32 Biodefense Training Grant (AI060549). Duraisamy Ponnusamy was supported by the James W. McLaughlin postdoctoral fellowship, UTMB.

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# Vita

Christina Josefa van Lier was born in Cleveland, Ohio on January 13, 1988 to Frank and Marianne van Lier and elder sister to Peter. She attended Hathaway Brown School, followed by Miami University of Ohio where she received a B.A. in Microbiology. After all that time in the snow, she then decided it was time for a change and went to study at The University of Texas Medical Branch in the Department of Microbiology and Immunology. During her PhD student tenure, she presented at six international and national meetings, was elected President of the Graduate Student Organization, and represented the students on the Microbiology and Immunology admissions committee. She was also an active member of the local community sitting on the Galveston Science and Engineering Fair Board and was a well-respected member of the Junior League of Galveston County.

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