Early Host Response to Infection is Influenced by the *Listeria monocytogenes* Broad-Range Phospholipase C

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ABSTRACT

Listeria monocytogenes is a facultative intracellular bacterial pathogen that causes disease in humans and animals. A mutant strain that has lost the ability to control the activity of a phospholipase C is attenuated in mice. This attenuation is not due to a lack of bacterial fitness, but appears to result from a modified immune response to infection. In a competitive assay, the mutant strain compromises the ability of wild-type (wt) *L. monocytogenes* to multiply in the liver of infected animals. To assess how the mutant modifies the hepatic immune response to infection, we monitored the kinetics of immune cell recruitment in the liver of infected mice and levels of serum inflammatory cytokines up to three days post-infection. At day 3, an increase in neutrophils was only detected in wild-type infected mice, whereas an increase in dendritic cells was only detected in mutant infected mice. Other innate immune cell types were recruited to the same levels in mice infected with either wt or mutant bacteria. Interferon gamma was detected in the serum of mice infected with wt bacteria at day 1-3 post-infection, but only at day 2 post-infection in mice infected with the mutant strain. Interleukin 12 was only detected in mice infected with wt bacteria. Also, transcriptional profiling of macrophages indicated that immune related genes were similarly up-regulated within both mutant and wt infections, while varying regulation of pathways revealed possible differences in host response to infection. At 60 minutes post infection mutant bacteria demonstrated increased efficiency at vacuolar escape and decreased mitochondrial damage at 90 minutes versus wt. Together, these results suggested that PC-PLC potentiates the host ability to clear *L. monocytogenes* in a way that does not involve an increase in the inflammatory response to infection.

BIOGRAPHICAL SKETCH

Bryant Blank was born in Santa Monica, California and raised in Kansas. He received a Bachelor of Science in 2005 from Kansas State University following completion of Biology major and Animal Sciences and Industry minor. He received a Doctorate of Veterinary Medicine from Kansas State University in 2009. Following veterinary school, he completed a Laboratory Animal Medicine Residency at Cornell University within the Cornell Center for Animal Resources and Education. During his residency he was accepted into the Cornell Graduate School in the Field of Comparative Biomedical Sciences from which he will be receiving a Master of Science in January 2013.

This is dedicated to Ashley and Ella for all their love, support & patience.

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TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION	1
CHAPTER TWO: MATERIALS AND METHODS	6
CHAPTER THREE: RESULTS	13
CHAPTER FOUR: DISCUSSION	31
BIBLIOGRAPHY	37

LIST OF FIGURES

Figure 1: Kinetics of *L. monocytogenes* growth in tissues of infected BALB/c mice 15

Figure 2: 17
The *L. monocytogenes plcB*Δpro mutant interferes with growth of wild-type bacteria in the liver of infected BALB/c mice

Figure 3: 18
Total number of immune cells types recruited to the liver of infected BALB/c mice

Figure 4: 19
The *plcB*Δpro mutant does not generate a pro-inflammatory response in infected mice

Figure 5: 24
Host transcriptional response of immune related genes during *L. monocytogenes* infection

Figure 6: 26
Ratio of *L. monocytogenes plcB*Δpro vacuolar escape versus wild-type *L. monocytogenes*

Figure 7: 28
Percentage of HeLa cells with fragmented mitochondria after infection with *L. monocytogenes plcB*Δpro or wild-type *L. monocytogenes*

Figure 8: Ubiquitination of *L. monocytogenes* within macrophage host cells 30

LIST OF TABLES

Table 1: 21
Expression of immune related genes in *L. monocytogenes* infected macrophages

CHAPTER ONE:

INTRODUCTION

Listeria monocytogenes is a facultative intracellular, gram-positive bacterium [1,2]. Human infection commonly occurs through ingestion of contaminated foods, such as raw vegetables, hot dogs, deli meats, and dairy products [3,4]. *L. monocytogenes* will proliferate *ex vivo* at a large range of pH values, low temperatures, and high salinity, making it a common pathogen found in ready-to-eat foods [2,5-7]. A portion of the population is at higher risk for bacteremia and the associated sequelae, including neonates, older individuals, and pregnant women [8,9]. Patients on immunomodulatory treatments or with underlying disease, such as certain cancers, human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), renal disease, and liver cirrhosis, are also considered to be at a greater risk [10].

L. monocytogenes uses unique methods for infection and proliferation. Once ingested by the host, *L. monocytogenes* enters intestinal epithelial cells and translocates through host lymphatics and vasculature to the liver and spleen, ultimately proliferating in parenchymal cells of the liver and spleen [11]. Initial infection may be asymptomatic or cause febrile enteritis [12]. Generally, immunocompetent people are able to clear the infection without septicemia and associated morbidity; but if bacteria invade the feto-placental and blood brain barriers then abortions or meningitis/meningoencephalitis may occur, respectively [2]. A similar pathophysiology occurs in ruminants used in agricultural production, often presenting with neurologic dysfunction [1,2], and is historically linked to contaminated silage, although this connection is debated within the literature [1,13].

The host immune response to *L. monocytogenes* makes it a model pathogen for immunological research. In order to clear *L. monocytogenes* infections the host innate and adaptive immune responses are triggered [14,15]. The strong cell mediated immunity trigger in the host makes it an ideal vaccine vector for targeting both infections and cancerous cells [14]. Due to the intracellular nature of *L. monocytogenes* residing in both phagosomal and cytosolic regions of the host cell, degraded bacterial proteins are loaded onto both MHC class I and MHC class II molecules resulting in activation of both CD4+ and CD8+ T lymphocytes [14]. Thus, due to *Listeria*'s natural ability to up-regulate the immune response and the easy manipulation of the bacterial genome, modulation of host's immunity can be readily developed with *L. monocytogenes* infection, leading to further immunological studies and vaccine development.

L. monocytogenes is one of the few bacterial species which invades host cells and multiplies intracellularly. The bacterium uses internalin A /E-cadherin interaction, in a species specific manner to enter into epithelial host cells and transverse natural host barriers, such as the intestinal barrier and feto-placental barrier [16,17]. The bacterium is phagocytized by the host, resulting in the formation of a membrane-bound vacuole. *L. monocytogenes* will escape from the vacuole using a combination of bacterial secreted proteins, including the pore-forming hemolysin, known as lysteriolysin O (LLO) and phodphatidylinositol-specific phosholipase C (PI-PLC) [18-20]. Upon escape from the vacuole *L. monocytogenes* replicates within the host cytosol and the ActA protein mediates polymerization of host actin filaments into actin comet tails [21]. This actin motility is essential for the spread of *L. monocytogenes*, which uses the actin to propel itself from one cell into an adjoining cell forming a double membrane-bound vacuole [20]. LLO and the broad range phospholipase C (PC-PLC) are utilized for dissolution and escape from the secondary vacuole, followed by further replication and infection [22]. This

method of cell-to-cell spread allows *L. monocytogenes* to colonize adjoining host cells without ever leaving the intracellular milieu, resulting in evasion of host humoral immunity [20].

Upon infection with *L. monocytogenes*, nonspecific innate immunity is quickly triggered to control bacterial proliferation and prevent sepsis during the initial 1-2 days post infection [23]. Sublethal *L. monocytogenes* i.v. injection results in rapid uptake by the liver and spleen, approximately 90% of bacteria within 10 minutes, and bacteria continue to proliferate exponentially for approximately 3-4 days [15,24]. Macrophages and neutrophils are the predominant leukocytes at foci of infection within hepatic and splenic tissue during this initial period of infection [15]. Resident macrophages, such as Kupffer cells, within the liver play a predominant role in the initial trapping of many bacteria, either by phagocytosis or extracellular binding [23,25,26]; but a large proportion, 38% reported by Gregory et al., are found associated with parenchymal hepatocyes at the 10 minute post infection time point [27]. Neutrophil infiltration also plays a critical role in nonspecific early host defense against *L. monocytogenes* [28,29]. Neutrophils are able to engulf and kill extracellular bacteria outside of liver parenchymal cells [27] and have demonstrated the ability to lyse infected hepatocytes in order to control bacterial numbers within the liver [30]. DCs serve as the crucial link between innate immunity and adaptive immunity, by priming the T cell response through antigen presentation [31]. CD4 and CD8 T cells are then responsible for the majority of *L. monocytogenes* induced adaptive immunity [14,26].

Along with early cellular infiltration, innate immune responses rely on production of cytokines, namely tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin 12 (IL-12), and their resultant interaction and effects upon inflammation to mediate infection. IL-12 produced by resident macrophages result in natural killer (NK) cells producing IFN-γ, which

then activates macrophages, resulting in increased cytokine synthesis and further NK stimulation [32]. Other cell types, such as γ/δ T cells, in peritoneal infection of *L. monocytogenes*, have also been shown to be a key producer of IFN- γ at early time points of infection [33] and IL-12 production can also be seen in activated neutrophils [34], further demonstrating the complex interaction of leukocytes and cytokines involved in innate immunity.

Activation of PC-PLC is under tight control within the vacuole bound bacteria. PC-PLC is produced as a proenzyme which is inactive until proteolytic cleavage of a 24-amino-acid N-terminal propeptide [35,36]. The proform of PC-PLC does not efficiently translocate across the bacterial cell wall, leading to a pool of PC-PLC at the membrane-cell-wall interface [37,38]. The PC-PLC propeptide is a checkpoint in enzymatic activity and promotes accumulation of bacterial enzymes at the membrane cell-wall interface [37,39,40]. Both maturation of the proenzyme and translocation of pooled protein are under the regulation of the *L. monocytogenes* metalloprotease [41] and dependent upon vacuolar acidification [40,42]. Upon a drop in vacuolar pH the active form of PC-PLC is released into the vacuole as a bolus to aid in vacuolar lysis and bacterial release into host cytosol [37-39].

In our current studies, we look to elucidate the processes by which host cells eliminate the *L. monocytogenes plcB*Δpro mutant more efficiently than wild-type bacteria. This mutant contains a deletion in the propeptide domain of PC-PLC, allowing constitutive secretion of active PC- PLC which effectively translocates across the bacterial cell wall at physiologic pH [40]. We have previously shown in J774 cells, a murine macrophage cell line, that the mutant behaves similarly to wild-type bacteria in regards to invasion, growth, and cell-to-cell spread; but the mutant is attenuated in murine models through both oral and i.v. infection [43]. In this study we aimed to determine the cause of the *plcB*Δpro *in vivo* attenuation. We find that co-infection of

*plcB*Δpro mutant and wild-type bacteria *in vivo* results in attenuation of both strains. The effect of constitutive secretion of PC-PLC on leukocyte migration to the liver was largely similar, with notable differences being a lack of significant neutrophil populations and significant increases in dendritic cells in mutant infected mice at day 3 post-infection. Serum cytokine production was drastically different in *plcB*Δpro mutant and wild-type murine infections, showing decreased production of IFN-γ and IL-12 at days 1, 2, and 3 post-infection. Transcriptional analysis demonstrated similar responses of immune related genes in macrophage infection with both strains *in vitro*. Also, the mutant showed increased efficiency at escape from the primary vacuole in macrophages and reduced ability to induce mitochondrial fragmentation in the host cell, when compared to wild-type *L. monocytogenes.* The mutant is able to establish hepatic infection; but appears to have an ultimately deleterious effect on proliferation. Further understanding and manipulation of host-pathogen interactions that result from constitutive secretion of PC-PLC could allow for immune modulation and possible therapies in the future.

CHAPTER TWO:

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Listeria monocytogenes 10403S or the murinized derivative *inlA*^{m} (NF-L1556/HEL-921) [44] were used as wild-type strains. The *plcB*Δpro mutant in 10403S (HEL-335) [40] and in 10403S *inlA*^{m} (HEL-923) were used as mutant strains. HEL-923 was generated by allelic exchange using plasmid pHEL-913 [44] and HEL-335 [40]. Bacteria were grown overnight in BHI at 30°C, passaged 1/10 or 1/100 in BHI and incubated at 30°C, 200 rpm until reaching an OD600 of 0.6-0.8. Bacteria were washed once in PBS, diluted, and suspended in appropriate volume for *in vitro* or *in vivo* infection.

Animals and housing conditions.

Female mice of the BALB/cAnNHsd inbred strain (Harlan, Indianapolis, IN) were used for infection experiments and tissue collection. All mice were murine pathogen free by supplier standards. Mice were held in single-sex groups of 3-5 animals per cage based on the parameters of the experiment and maintained at Animal Biosafety Level 2. Mice were housed in individually ventilated, positively pressured cages (Allentown Inc., Allentown, PA) at Cornell University, an AAALAC accredited institution. All experiments were approved by the Cornell University Institutional Animal Care and Use Committee.

***In vivo* proliferation.**

Six- to 8-week-old female BALB/c mice were infected by gavage with log-phase bacterial cells in 200 µl of PBS. Mice were sacrificed at specific time points post-infection. The intestines were isolated and flushed with cold PBS to remove all feces. The intestines were cut longitudinally and homogenized in 0.02% NP-40/0.2 mM EDTA. Serial dilutions were plated on LB plates supplemented with streptomycin (200 µg/ml) to determine bacterial counts. Alternatively, mice were infected i.v. by retro-orbital injection. When indicated, the mice were perfused post-mortem with PBS to remove hematogenous bacteria before collecting spleen and liver. The organs were homogenized in 2 ml of 0.02% NP40 Alternative (CalBiochem, La Jolla, CA) and serial dilutions were plated on LB agar with streptomycin to quantify bacterial load. In order to differentiate bacterial strains, 100-200 colonies were picked from each co-infection sample and streaked on egg-yolk agar containing activated charcoal (0.2%)-treated LB, 25mM glucose-1-phosphate, and 1.25% egg yolk. The constitutive secretion of PC-PLC allows differentiation of mutant strain after 24 hours of incubation at 37°C on egg-yolk agar plates [40].

Detection of leukocytes.

Six- to 8-week-old female BALB/c mice (Taconic, Hudson, NY) were infected i.v. as described elsewhere in the manuscript. At indicated times post-infection mice were euthanized and perfused with PBS in order to remove hematogenous leukocytes. Liver leukocytes were purified. Briefly, livers were ground through coarse mesh strainers and cells were then washed in PBS, and resuspended in Dulbecco's Modified Eagle Medium (DMEM). Homogenates were digested with 50 mg/ml Type IV collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C, pelleted, and resuspended in PBS. A single cell suspension was

made by straining through 40 μm nylon Cell Strainer (BD Biosciences, Durham, NC). Leukocytes were separated from red blood cells and hepatocytes through a discontinuous Percoll (GE Healthcare, Piscataway, NJ) gradient. The leukocytes were then pelleted, resuspended in PBS, and counted. Cells were stained with different combinations of antibodies from eBioscience (San Diego, CA): PE anti-CD45 (30-F11), PE-Cy7 anti-CD11b (M1/70), Alexa Fluor 647 anti-CD11c (N418), eFluor 660 anti-CD3e (17A2), FITC anti-CD49b (DX5), and FITC anti-Ly-6G (1A8). Stained cells were differentiated using a BD FACSCanto II (BD Biosciences, Durham, NC) and data were analyzed with FlowJo (Tree Star Inc, Ashland, OR).

Bone marrow-derived macrophage infections.

Bone marrow cells were collected from BALB/c mice and macrophages were differentiated in medium containing 50% DMEM, 20% Fetal Bovine Serum (FBS), 30% L-cell conditioned medium, 100 U/ml Penicillin G,100 μg/ml Streptomycin, 1mM Sodium pyruvate, and 2mM L-glutamine. Differentiated macrophages were seeded at a concentration of 3.6×10^6 cells in 100 mm petri dishes containing four 12 mm glass cover slips, and incubated overnight at 37°C, 5% CO_2. Macrophages were infected with either wild-type *L. monocytogenes* or *L. monocytogenes plcB*Δpro at an MOI of 1. Cells were washed in PBS at 30 min post infection and tissue culture medium was added to dishes. At one hour post-infection, gentamicin was added at a final concentration of 50 μg/ml. At three hours post-infection, the medium was replaced with antibiotic-free medium to prevent intracellular bacterial killing as the mutant affects membrane permeability [43]. Samples were collected at 3, 6, and 9 hr p.i. for RNA purification. Cover slips were collected at the same time points to determine bacteria counts as previously described.

Microarray.

Total RNA from the BMMΦ infections was isolated and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) per manufacturer's instructions. RNA sample purity was assessed using Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA). All samples were >9.1 RIN (RNA Integrity Number). The samples were processed as follows by the Cornell University Life Sciences Core Laboratories Center: A total of 825 ng per sample of Cy3-labelled cRNA was fragmented, blocked, hybridized to Agilent Whole Mouse Genome Microarray 4x44K (G4122F), and washed according to manufacturer's instructions. The slides were then scanned using the one-color scan setting for 4x44 array slides (scan area 61 x 21.6 mm, scan resolution 5um, dye channel set to red and green, and PMT 100%) using the Agilent DNA Microarray Scanner (G2505B). Feature Extraction Software 9.1 (Agilent) analyzed the scanned images for background, subtracted and spatially detrended processed signal intensities, and excluded non-uniform outliers flagged in feature extraction (protocol GE1-105_DEC8 and Grid: 012391_D_20060331). Data was analyzed using GeneSpring11.5 (Agilent Technologies, Santa Clara, CA), threshold to raw signal was set to 1.0, shifted to 75.0 percentile to remove lower percentile intensity values (background noise from probes where gene is not expressed), and baseline set to median of all samples. Experimental groupings were created by dividing the samples into conditions (a) wild-type infected (triplicate),(b) *plcB*Δpro mutant (triplicate), and (c) uninfected (duplicate). Interpretations were created using the average replicates in condition, non-numeric, continuous parameters, and including detected, not detected, and compromised flags. Statistical analysis was then performed between conditions (a)/(c) & (b)/(c), unpaired T-test, no correction, p-value <0.05. We then performed functional annotation using our significant gene lists through the Database for Annotation, Visualization

and Integrated Discovery (DAVID 6.7, National Institute of Allergy and Infectious Diseases – National Institutes of Health) to find pathways significantly affected by up- or down-regulated genes [45].

Cytokine assays.

Whole blood was collected from mice by cardiac puncture immediately following euthanasia with CO_2. Cytokine serum levels were determined using ELISA Ready-SET-Go! Kits from eBioscience (San Diego, CA) for Mouse IL-1ß, TNF-α, IL-12 p70, and IFNγ. Corning 96 Well High Bind Microplates were used (Corning Inc., Corning, NY) for the assays and the results were read using a BioTek ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT).

Vacuolar escape assays.

Bone marrow derived macrophages from BALB/c mice were used for this assay. Differentiated macrophages were seeded on cover slips in 35 mm tissue culture dishes at a concentration of approximately 6 x 10^5 cells/dish and infected the following day with wild-type *L. monocytogenes* or *L. monocytogenes plcB*Δpro at a MOI of 5 [46]. After 30 min incubation at 37°C, 5% CO_2, the cells were washed three times with PBS and either fixed in formalin or incubated for 30 or 60 more minutes in tissue culture medium supplemented with gentamicin (10 µg/ml), before being washed and fixed. Bacteria and actin were stained for fluorescence microscopy as previously described [47]. Samples were examined using a Zeiss Axio Imager M1 and AxioCam MRm (Carl Zeiss Microscopy, Thornwood, NY). Intracellular bacteria were scored as being decorated with actin (cytosolic) or not (vacuolar).

Mitochondrial fragmentation assays.

HELA cells were trypsinized and seeded onto coverslips in 35 mm tissue culture dishes at a concentration of 1x 10^6 cells per dish in RPMI 1640, 10% FBS. After an overnight incubation, cells were infected with wild-type *L. monocytogenes* or *L. monocytogenes plcB*Δpro at a MOI of 50 and incubated for 90 min at 37°C, 5% CO_2. Pre-warmed MitoTracker Deep Red FM (Invitrogen, Eugene, OR) was then added to media at a concentration of 300 nM for 15 minutes followed by 3 washes in pre-warmed medium. Cells were fixed in pre-warmed formalin for 15 minutes at 37°C, 5% CO_2, then with acetone on ice for 5 minutes. Bacteria were stained for immunofluorescence as per manufacturer's instructions (Invitrogen). Coverslips were mounted on glass slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen, Eugene, OR). Samples were examined using Zeiss Axio Imager M1 and AxioCam MRm (Carl Zeiss Microscopy, Thornwood, NY).

Ubiquitination assays.

BMMΦ from BALB/c mice were used for this assay. Differentiated macrophages were seeded on 18 x 18 mm glass cover slips in 6-well tissue culture plates at a concentration of approximately 1 x 10^6 cells/well in BMMΦ media and infected the following day with wild-type *L. monocytogenes* or *L. monocytogenes plcB*Δpro at a MOI of 0.5. After 30 min incubation at 37°C, 5% CO_2, the cells were washed three times with PBS. After 60 min incubation 1 μg/ml gentamicin was added and cells were incubated for a total of 4 hrs. Samples were fixed in formalin and blocked/permeablized. Coverslips were stained with biotin-conjugated FK2 (Enzo Life Sciences, Farmingdale, NY) and then mounted on glass slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen, Eugene, OR). Samples were examined using a Zeiss Axio

Imager M1 and AxioCam MRm (Carl Zeiss Microscopy, Thornwood, NY). Intracellular bacteria were scored as being ubiquitin decorated or not.

CHAPTER THREE:

RESULTS

Attenuation of the *plcB*Δpro mutant is unlikely due to a lack of bacterial fitness.

Previously, we showed using a competitive infection assay that *L. monocytogenes plcB*Δpro is outcompeted by wild-type strain in the intestines, liver, and spleen of BALB/c mice at days 2, 3, and 4 following infection by gavage [43]. To assess whether this attenuation was due to a defect in initial colonization of the intestines, we infected mice by gavage with wild-type strain or the *plcB*Δpro mutant and monitored intestinal colonization at early time points post-infection. The results indicated that *L. monocytogenes plcB*Δpro is as efficient as wild-type strain at colonizing the intestines of BALB/c mice within the first 28 hours post-infection (Fig. 1a). However, a statistically significant difference in bacterial numbers was observed by 40 hours post-infection, with the mutant strain being less abundant than wild-type.

Previously, we also showed that, following an i.v. infection, the mutant competed equally with wild-type strain in the initial stages of infection [43]. Using the competitive indexes and total bacterial numbers reported in the Yeung paper for the i.v. infection assay [43], we plotted the kinetics of bacterial growth in the liver and spleen. The results indicated that mutant and wild-type strains were found in equivalent numbers by 7 hours post-infection, and increased by 1-2 logs in numbers between 7 and 24 hours post-infection (Fig. 1b). However, mutant attenuation became apparent with time as mutant bacterial numbers were 34 and 66 fold lower than wild-type by 72 hours post-infection in spleen and liver respectively. This attenuation in virulence was further emphasized by determining that the mutant LD_{50} was increased a hundred

fold in BALB/c mice: 2 x 10^6 for the mutant vs. 2 x 10^4 for wild-type (data not shown). Overall, these results suggested that virulence attenuation of *L. monocytogenes plcB*Δpro is unlikely due to an initial lack of bacterial fitness, since the mutant is as efficient as wild-type strain at colonizing the intestines, spleen, and liver in the initial stage of infection and that it is also capable of multiplying in tissues.

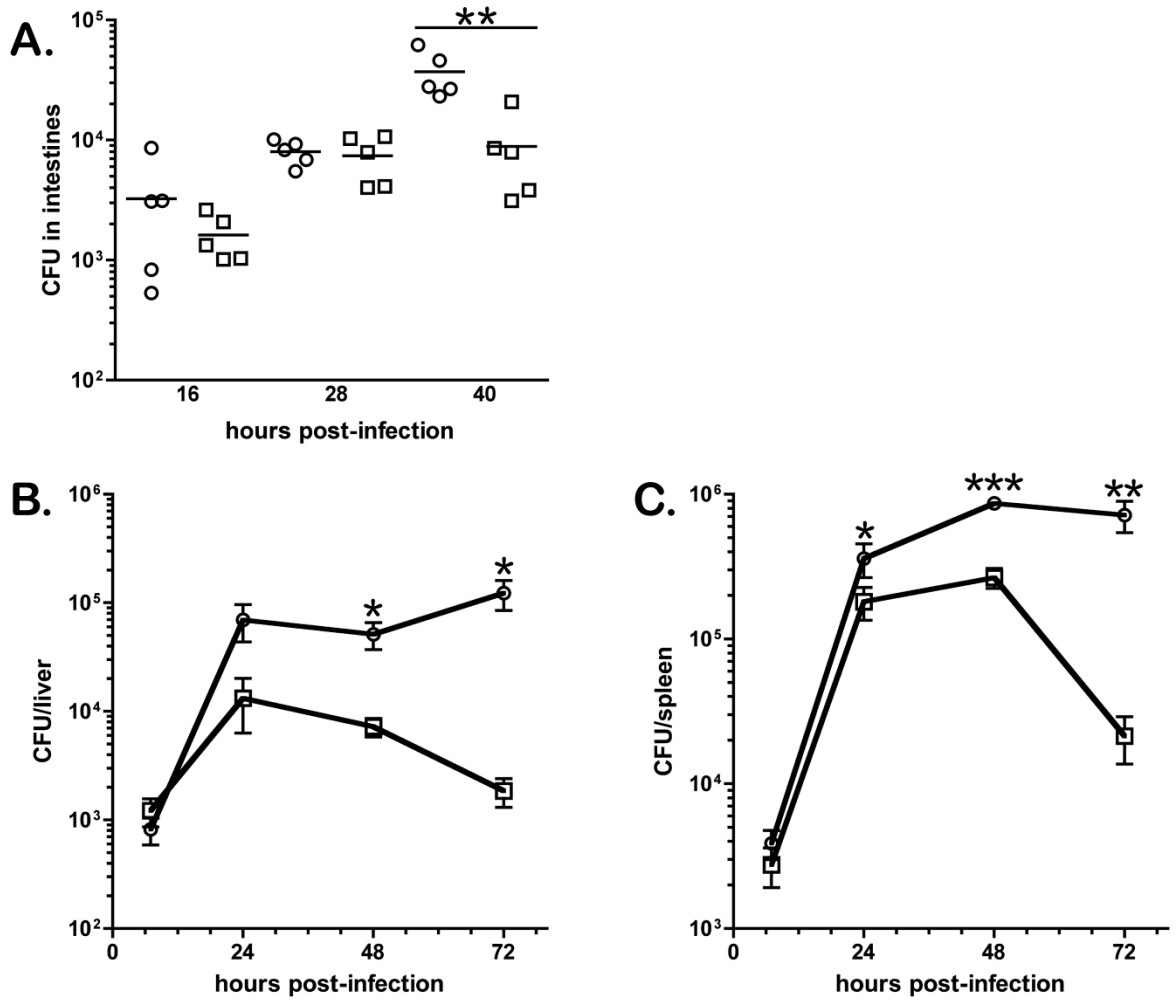

Figure 1. Kinetics of *L. monocytogenes* growth in tissues of infected BALB/c mice. A. Total CFU recovered from intestinal tissues of mice infected by gavage with 6 x 10^9 *L. monocytogenes* *inlA*^{m} (round symbols) or *inlA*^{m} *plcB*Δpro (squares symbols). Each data point represents counts from one mouse and horizontal bars represent the mean. The difference in CFU between the two bacterial strains is statistically significant only at 40 hours post-gavage: Student's t test unpaired two-tailed p value <0.01 (**). Total CFU recovered from the liver (B.) and spleen (C.) of mice co-infected i.v. with 10^4 wild-type *L. monocytogenes* (round symbols) and 10^4 of the *plcB*Δpro mutant (square symbols). Bacterial numbers were calculated from data reported in Yeung et al [43]. Each time point represents the mean ± SEM of four to seven mice. Data were analyzed by the Student's t test and paired two-tailed p values are: * <0.05, ** < 0.01, *** <0.001. Experiment in A performed by Hélène Marquis.

The *plcB*Δpro mutant interferes with the growth of wild-type strain.

The above results indicated that the loss of regulation of PC-PLC activity compromises the ability of *L. monocytogenes* to expand the infection, and we speculated that attenuation of the *plcB*Δpro mutant results from a change in how the host perceives signals received from the infected cells. To test this possibility, we assessed whether the mutant strain would affect the growth of wild-type bacteria during a mixed infection. Three groups of mice were infected i.v.: the 1st group received ~10^4 *L. monocytogenes inlA*^{m}, the 2nd group received twice as many of the mutant strain (*inlA*^{m} *plcB*Δpro), and the third group received the same inoculum as groups 1 and 2 together. Bacterial counts in spleen and liver were determined at 72 hours post-infection and the wild-type and mutant strains were differentiated by plating isolated colonies on egg yolk agar to detect phospholipase activity. No significant changes in numbers of wild-type and *plcB*Δpro bacteria were seen in the spleen of mice when comparing single strain infections to mixed infections (Fig. 2a). Also, no significant changes in numbers of *plcB*Δpro bacteria were seen in the liver of mice when comparing single strain infections to mixed infections (Fig. 2b). However, there was a reproducible statistically significant difference in the numbers of wild-type bacteria recovered from the liver of mice when comparing single strain infections to mixed infections. Overall, these results show that wild-type *L. monocytogenes* does not affect the growth of the *plcB*Δpro mutant, but the *plcB*Δpro mutant has a negative effect on the growth or survivability of the wild-type strain in the liver of co-infected mice.

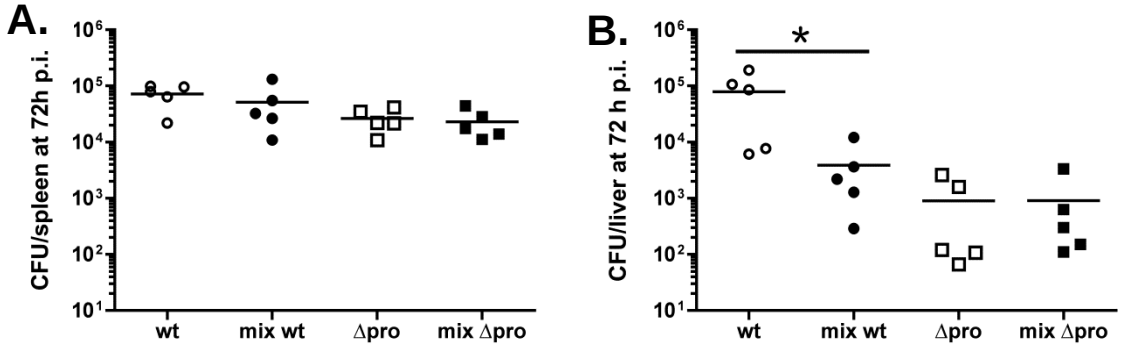

Figure 2. The *L. monocytogenes plcB*Δpro mutant interferes with growth of wild-type bacteria in the liver of infected BALB/c mice. BALB/c mice were infected i.v. with 7.0-8.5 x 10^3 CFU of *L. monocytogenes inlA*^{m} (wt) or 1.7 x 10^4 CFU of *inlA*^{m} *plcB*Δpro (Δpro), or with both strain at the same concentrations (mix). Bacterial counts in spleen (A) and in liver (B) were determined at 72h post-infection. Each data point represents counts from one mouse and horizontal bars represent the mean. For the mix infection, specific numbers of wt (mix wt) and mutant (mix Δpro) recovered were determined. The difference in CFU between the single and mix infections was statistically significant for wild-type strain recovered from the liver: Student's t test unpaired one-tailed p value <0.05 (*). Experiment performed in collaboration with Lauren Griggs.

The *plcB*Δpro mutant does not enhance the innate immune response to infection in vivo.

Due to the significant effect of *L. monocytogenes plcB*Δpro on the virulence of wild-type *L. monocytogenes*, we hypothesized that the mutant enhances or hastens the host innate immune response to infection. To test this hypothesis, the kinetics of leukocyte recruitment to the liver of infected mice was examined. BALB/c mice were infected intravenously with 10^4 of either the wild-type strain or the *L. monocytogenes plcB*Δpro mutant. At 1, 2, and 3 days post infection mice were sacrificed and liver leukocytes were purified and analyzed by FACS. Total numbers of T lymphocytes, macrophages, neutrophils, natural killer (NK) cells, NK-T cells, and dendritic cells were determined. No changes were observed between control and infected mice at day 1 and 2 post-infection, except for a 4-fold increase in macrophages ($p = 0.008$) at day 2 post-

infection in mutant-infected mice. At day 3 post-infection, a variety of innate immune cells were significantly increased in the liver of mice infected with either the wild-type or mutant strains (Fig. 3). Interestingly, an increase in neutrophils was only detected in wild-type infected mice, whereas an increase in dendritic cells was only detected in mutant infected mice.

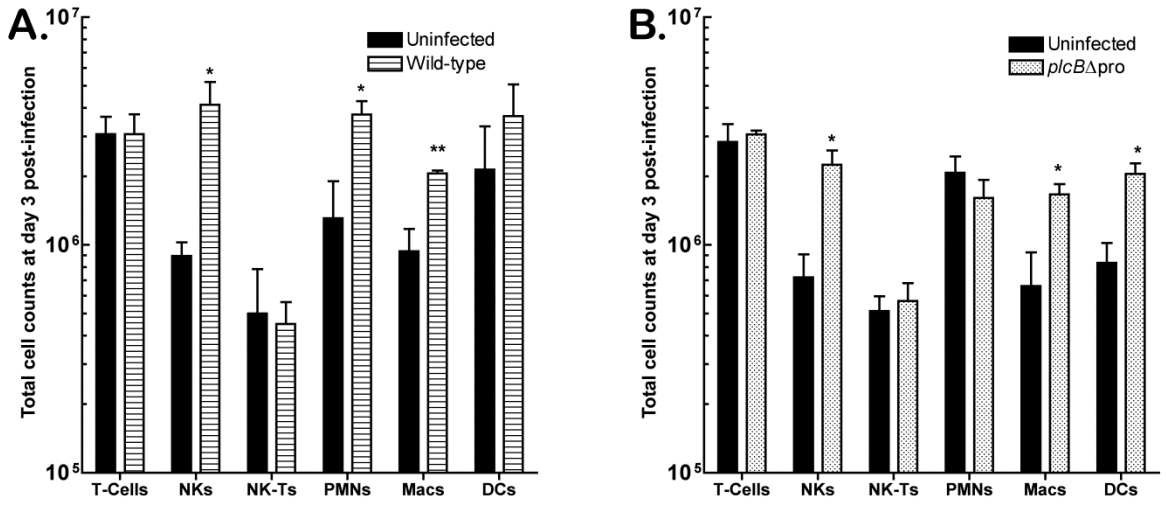

Figure 3. Total number of immune cells types recruited to the liver of infected BALB/c mice. Mice were injected with 10^4 *L. monocytogenes* $inlA^m$ (wild-type), 10^4 $inlA^m$ *plcB*Δpro (*plcB*Δpro), or sterile PBS (uninfected). Mice were sacrificed at 72 hours post-infection, livers were perfused with PBS, and leukocytes were purified. Purified leukocytes were stained with specific antibodies and analyzed by FACS to differentiate the various types of cells. T cells ($CD45^+$, $CD3^+$, $CD49b^-$), NK cells ($CD45^+$, $CD3^-$, $CD49b^+$), NK-T cells ($CD45^+$, $CD3^+$, $CD49b^+$), neutrophils ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^+$), macrophages ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$), and dendritic cells ($CD45^+$, $CD11b^{+/-}$, $CD11c^+$, $Ly6G^-$). Data were analyzed by the Student's t test comparing uninfected to infected groups of mice within each experiment. P values are: * <0.05, ** < 0.01. n=3 per group within each experiment. Experiment performed in collaboration with Alan Pavinski Bitar.

To further elucidate the innate immune response to infection, serum levels of pro-inflammatory cytokines were measured. No differences between wild-type and mutant infected mice were detected in serum levels of interleukin-1β (IL-1ß), interleukin 10 (IL-10), and tumor necrosis factor alpha (TNF-α) (data not shown). However, IL-12 and interferon gamma (IFN-γ) were significantly elevated in wild-type infected mice at day 2 and 3 post-infection, respectively, but not in mutant infected mice (Fig. 4). Together, the data suggest that *L. monocytogenes plcB*Δpro does not enhance the innate immune response to infection.

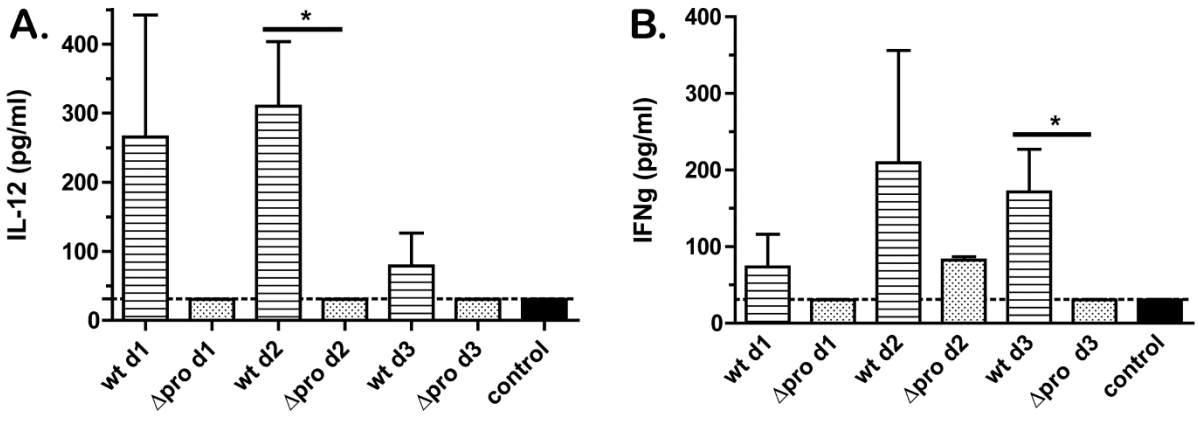

Figure 4. The *plcB*Δpro mutant does not generate a pro-inflammatory response in infected mice. BALB/c mice were injected with 10^4 *L. monocytogenes inlA*^{m} (wt), 10^4 *inlA*^{m} *plcB*Δpro (Δpro), or sterile PBS (control). Mice were sacrificed at 24 (d1), 48 (d2) or 72 (d3) hours post-infection and blood was immediately collected. Serum levels of IL-12 (A) and IFN-γ (B) were determined by ELISA. Data were analyzed by ANOVA using a Tukey's multiple comparison test. *: p value of <0.05. n=3 per group. ELISA performed by Alan Pavinski Bitar.

Transcriptional profiling does not reveal differences in the expression of immune related genes in wild-type and mutant infected macrophages.

To further assess host cell response to infection, we examined the transcriptional response of mouse bone marrow derived macrophages infected with wild-type *L. monocytogenes* or the *plcB*Δpro mutant. As previously reported in murine macrophages, genes coding for IFNβ, IL-6, TNF-α, IL-1α, and IL-1β were among the most abundant transcripts in infected cells, validating our microarray data [48,49]. Moreover, our results indicate that there is a similar pattern of expression with most immune response genes at 3, 6, and 9 hours post-infection in cells infected with wild-type strain or the *plcB*Δpro mutant (Table 1). Heat maps illustrate the similarities between genes expressed by wild-type strain or the *plcB*Δpro mutant, while both are increased over the uninfected controls, and further demonstrate the changes in overall immune related expression between the 3, 6, and 9 hour post-infection time points (Fig. 5). These data suggest that the *L. monocytogenes plcB*Δpro mutant does not modulate the macrophage innate immune response differently than wild-type bacteria.

Table 1. Immune-related gene expression.

Expression levels of 43 immune-related genes of macrophages after infection with either *L. monocytogenes* inlAm or inlAm plcBΔpro at 3, 6, and 9 hours. Results are shown as mean of fold change compared to uninfected controls in three replicates for each infection at the given time point. Significant genes (bolded) have a P-value < 0.05 based on Student's t test analysis.

Table 1. Immune-related gene expression.

	3 hrs		6 hrs		9 hrs		
GeneSymbol	*plcB*Δpro	*wt*	*plcB*Δpro	*wt*	*plcB*Δpro	*wt*	Description
Cytokines							
Ifna2	3.14	2.48	**7.48**	**7.21**	**6.76**	**6.47**	Interferon alpha 2
Ifnab	-1.05	-1.21	-1.10	**1.32**	1.14	1.13	Interferon alpha B
Ifnab1	**44.19**	**44.83**	**352.79**	**232.92**	**79.92**	**70.90**	Interferon beta 1
Il1a	1.93	1.77	**5.95**	**5.74**	**28.61**	**24.58**	Interleukin 1 alpha
Il1b	**2.67**	**2.23**	**16.78**	**30.05**	**55.08**	**51.86**	Interleukin 1 beta
Il6	**3.68**	**3.28**	**489.26**	**513.41**	**62.68**	**67.24**	Interleukin 6
Il7	1.59	1.63	2.14	**3.08**	1.59	1.72	Interleukin 7
Il10	1.10	1.06	4.77	**2.34**	**5.12**	**5.14**	Interleukin 10
Il12b	**2.60**	**2.14**	**7.27**	**8.92**	**21.90**	**19.70**	Interleukin 12b (p40)
Il15	**-1.17**	**-1.29**	**3.08**	**4.46**	**2.58**	**2.69**	Interleukin 15
Il18	1.00	-1.00	2.13	**2.13**	**3.73**	**4.06**	Interleukin 18
Tnf	**5.71**	**6.13**	**57.39**	**41.07**	**37.25**	**31.70**	Tumor necrosis factor
Cytokine receptors							
Tnfrsf1b	1.00	-1.01	-1.22	**1.15**	1.26	1.22	Tumor necrosis factor receptor superfamily, member 1b
Chemokines							
Ccl2	**1.78**	**1.75**	**33.77**	**33.29**	**30.33**	**32.81**	Chemokine (C-C motif) ligand 2
Ccl3	**4.29**	**3.87**	**13.84**	**19.92**	**24.59**	**25.38**	Chemokine (C-C motif) ligand 3
Ccl5	**1.61**	1.64	**61.72**	**81.38**	**25.38**	**31.25**	Chemokine (C-C motif) ligand 5
Ccl7	**2.49**	**2.54**	**80.05**	**73.61**	**60.70**	**65.27**	Chemokine (C-C motif) ligand 7
Ccl8	1.03	-1.03	1.32	**1.85**	1.50	1.46	Chemokine (C-C motif) ligand 8
Ccl12	1.26	**1.26**	**95.12**	**83.39**	**60.19**	**69.67**	Chemokine (C-C motif) ligand 12
Cxcl1	**10.17**	**10.56**	**26.30**	**36.03**	**77.43**	**69.54**	Chemokine(C-X-C motif) ligand 1
Cxcl2	**7.91**	**7.31**	**32.78**	**42.08**	**77.71**	**79.52**	Chemokine(C-X-C motif) ligand 2
Cxcl3	**3.53**	**3.04**	**6.31**	**9.61**	**14.69**	**17.32**	Chemokine(C-X-C motif) ligand 3
Cxcl9	1.07	-1.30	**19.36**	**28.69**	**19.37**	**20.88**	Chemokine(C-X-C motif) ligand 9
Cxcl10	**3.93**	**3.88**	**134.57**	**132.24**	**124.36**	**128.29**	Chemokine(C-X-C motif) ligand 10
Cxcl11	1.22	**1.10**	**111.70**	**112.36**	**148.52**	**163.77**	Chemokine(C-X-C motif) ligand 11
Cxcl16	**1.32**	**1.27**	**1.69**	**1.66**	**2.20**	**2.03**	Chemokine(C-X-C motif) ligand 16
Cx3cl1	1.32	1.26	1.87	**2.10**	**3.13**	**2.32**	Chemokine(C-X3-C motif) ligand 1

Table 1 (continued). Immune-related gene expression.

	3 hrs		6 hrs		9 hrs		
GeneSymbol	*plcB*Δpro	*wt*	*plcB*Δpro	*wt*	*plcB*Δpro	*wt*	Description
Chemokine receptors							
Ccr2	-1.23	-1.22	-3.51	**-1.69**	**-2.47**	**-2.24**	Chemokine (C-C motif) receptor 2
Ccr7	1.48	1.17	**1.59**	**2.29**	**4.25**	**3.82**	Chemokine (C-C motif) receptor 7
Transcription factors							
Irf7	-1.13	-1.02	**11.55**	**8.63**	**4.76**	**4.54**	Interferon regulatory factor 7
Nfkb1	**1.36**	**1.28**	1.91	**1.95**	**2.35**	**2.34**	NF-kappa-B DNA binding subunit
Innate immune receptors							
Myd88	**1.19**	**1.20**	**2.79**	**2.17**	**2.56**	**2.57**	Myeloid differentiation primary response gene 88
Tlr2	**1.67**	**1.62**	2.21	**3.55**	**6.15**	**6.12**	Toll-like receptor 2
Tlr3	-1.10	-1.17	**6.04**	**8.48**	**4.68**	**5.09**	Toll-like receptor 3
Nod1	1.00	-1.02	6.05	**5.75**	**3.88**	**4.26**	Nucleotide-binding oligomerization domain containing 1
Nod2	**2.13**	**2.04**	14.70	**7.73**	**5.88**	**6.51**	Nucleotide-binding oligomerization domain containing 2
Other immune related molecules							
Icam1	**1.71**	**1.86**	**2.08**	**2.45**	**3.97**	**3.64**	Intercellular adhesion molecule 1
Ifit1	**1.91**	**1.50**	**94.81**	**126.99**	**49.83**	**53.48**	Interferon-induced protein with tetratricopeptide repeats 1
Ifit2	1.06	1.02	**65.33**	**122.11**	**53.07**	**61.86**	Interferon-induced protein with tetratricopeptide repeats 2
Ifit3	1.00	-1.06	**84.87**	**110.22**	**57.30**	**61.78**	Interferon-induced protein with tetratricopeptide repeats 3
Casp1	1.07	-1.04	1.83	**2.06**	**1.75**	**2.04**	Caspase 1
C3	1.10	1.01	**2.60**	**2.07**	**2.42**	**2.52**	Complement component 3
Peli1	1.15	1.16	**4.14**	**4.44**	**1.92**	**1.79**	Pellino 1

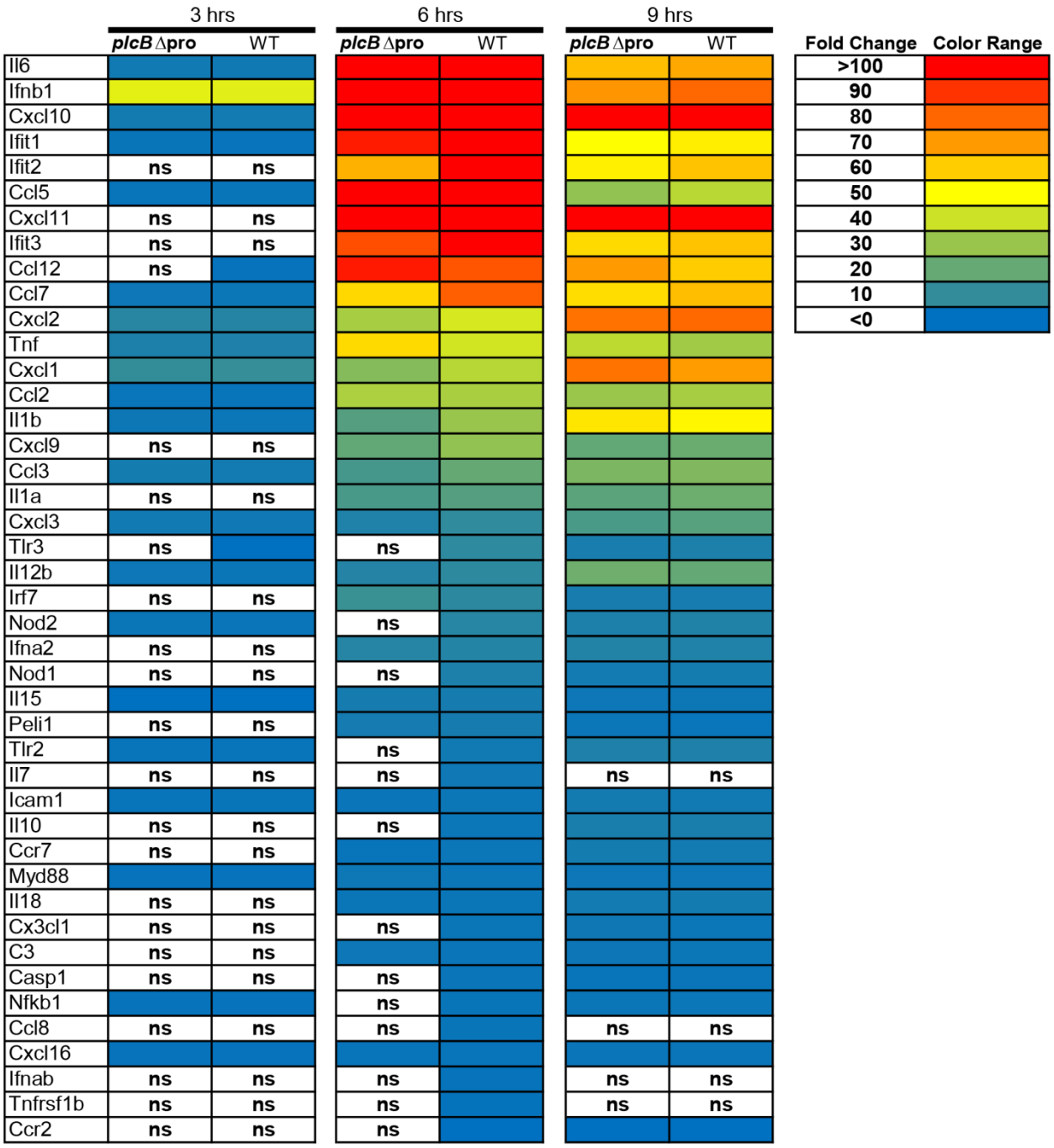

Figure 5. Host transcriptional response of immune related genes during *L. monocytogenes* infection. Expression levels of 43 immune-related genes of macrophages after infection with either *L. monocytogenes inlA*^{m} or *inlA*^{m} *plcB*Δpro at 3, 6, and 9 hours. The color scale represents a 3-color fold change range of 0 (blue), 50 (yellow), and 100 (red) with fold changes between 0-100 resulting in a blend of the relative colors and fold changes below 0 being blue and above 100 being red. Empty slots represent non-significant (ns) genes for infection and time-point. Results are shown as mean of fold change compared to uninfected controls in three replicates for each infection.

The *plcB*Δpro mutant is more efficient than wild-type at vacuolar escape.

Further examination of the microarray data revealed that the caspase-1 gene, involved in the inflammasome pathway [50], was significantly up regulated by 2.06 fold in wild-type, but not mutant infected mouse BMMΦ cells at the 6 hour time point. The ability of *L. monocytogenes* to activate the inflammasome is dependent on listeriolysin O (LLO), which enables bacterial escape from vacuoles [51]. Additionally, the mannose-6-phosphate receptor was down regulated by 1.10 fold in mutant infected cells and up regulated in wild-type infected cells by 1.08 fold at the 3 hour time point. Although small, these differences were statistically significant. This was an interesting finding as LLO pore forming activity is dependent on the host thiol-reductase GILT (gamma interferon inducible thiol-reductase) that requires the mannose-6-phosphate receptor to reach the *L. monocytogenes* containing vacuole [52]. Given that LLO is essential for escape from vacuoles in *L. monocytogenes* [53], and we noted significant differences in genes associated with this pathway, we compared the efficacy of wild-type and mutant bacteria to escape vacuoles in murine bone marrow-derived macrophages. To quantify bacterial escape at given time points we co-stained infected cells for bacteria and actin, as actin decorates exclusively cytosolic bacteria [47,54]. The data indicates that *L. monocytogenes plcB*Δpro is more efficient than wild-type bacteria at escaping vacuoles early during infection, as a statistically significant difference was detected at 60 min post-infection, but absent by 90 min post-infection (Fig. 6). These results indicate that the observed changes in transcriptional activity of genes involved in inflammasome and vacuolar transport are unlikely due to changes in the functionality of LLO. The increased efficiency of escape could be caused by active PC-PLC initiating vacuolar lysis at an earlier time point in the mutant strain.

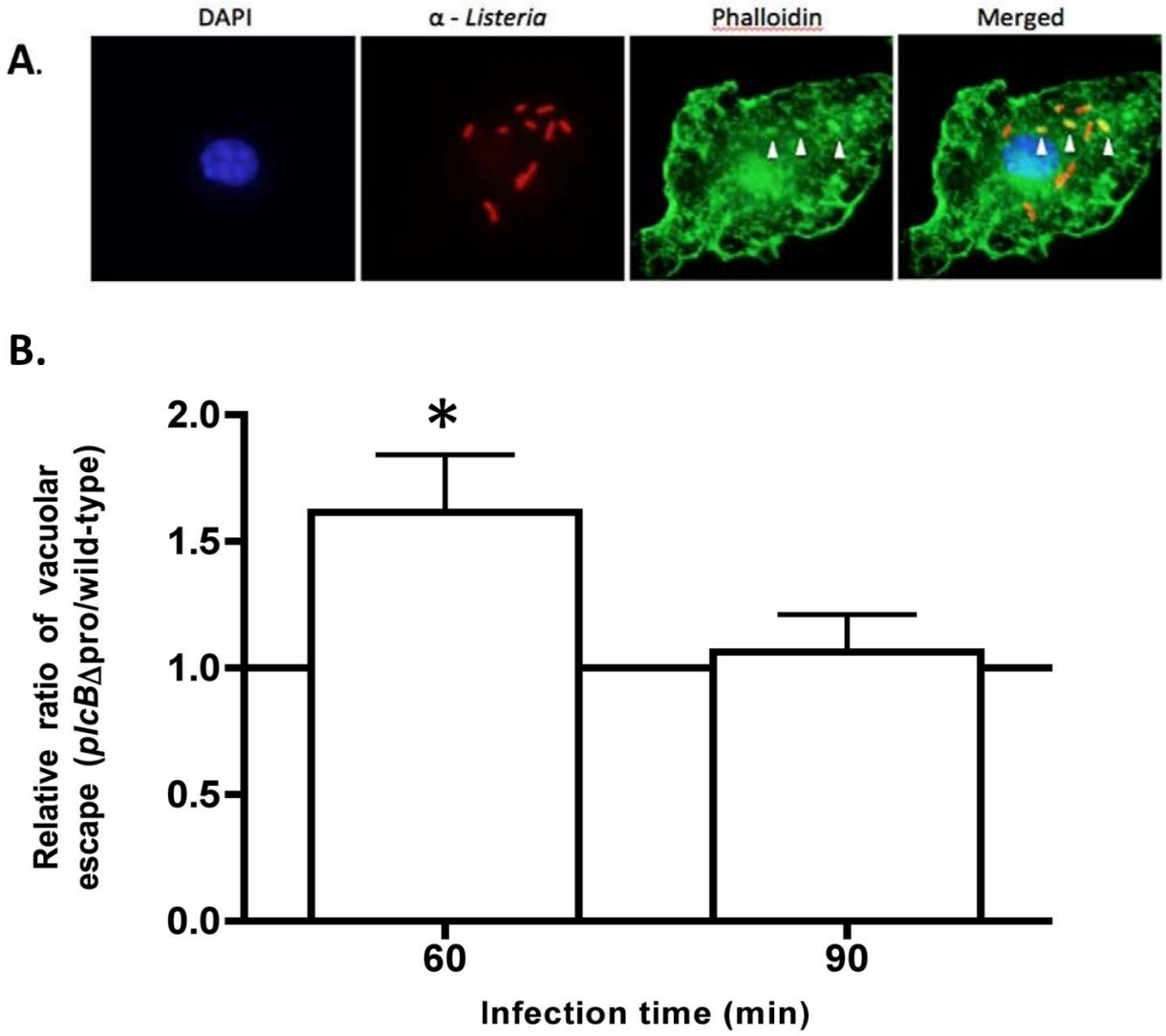

Figure 6. Ratio of *L. monocytogenes plcB*Δpro vacuolar escape versus wild-type *L. monocytogenes*. Macrophages were infected with either *L. monocytogenes inlA*^{m} or *inlA*^{m} *plcB*Δpro for 60 or 90 min. (A) The cells were then fixed and stained as previously described [47], with DAPI for nuclei, α-*Listeria* for bacteria, phalloidin for actin and then merged. (B) Bars represent mean ± SEM of the ratio of total *inlA*^{m} *plcB*Δpro /*L. monocytogenes inlA*^{m} escaped bacteria counted from 10-20 fields per experiment. Original data from independent experiments was arcsine transformed and analyzed by the paired Student's t test comparing *L. monocytogenes inlA*^{m} or *inlA*^{m} *plcB*Δpro vacuolar escape within each timepoint. *: p value of <0.05. n=5 for 60 min and n=3 for 90 min. Experiments performed by Delbert Abi Abdallah.

Mitochondrial dynamics is less affected by the *plcB*Δpro mutant than by wild type bacteria.

The microarray data also revealed a differential expression pattern for mitochondria genes related to respiratory complexes II to V, showing significant up-regulation of multiple genes within these complexes in wild-type but not in mutant-infected cells. It has previously been demonstrated that *L. monocytogenes* induces transient mitochondrial fragmentation as a strategy to promote its intracellular growth [55,56]. Therefore, we speculated that the *plcB*Δpro mutant was not as efficient as wild-type *L. monocytogenes* in interfering with cellular physiology to promote its intracellular growth. For this purpose, HeLa cells were infected with wild-type or the mutant strain of *L. monocytogenes,* stained with fluorescent Mito Tracker, fixed and examined at 90 minutes post-infection. Based on three independent experiments the mean number of cells with fragmented mitochondria was significantly lower in mutant versus wild-type infected cells (Fig. 7), indicating that the mutant is less efficient than wild-type *L. monocytogenes* in causing mitochondria fragmentation, possibly contributing to its attenuation.

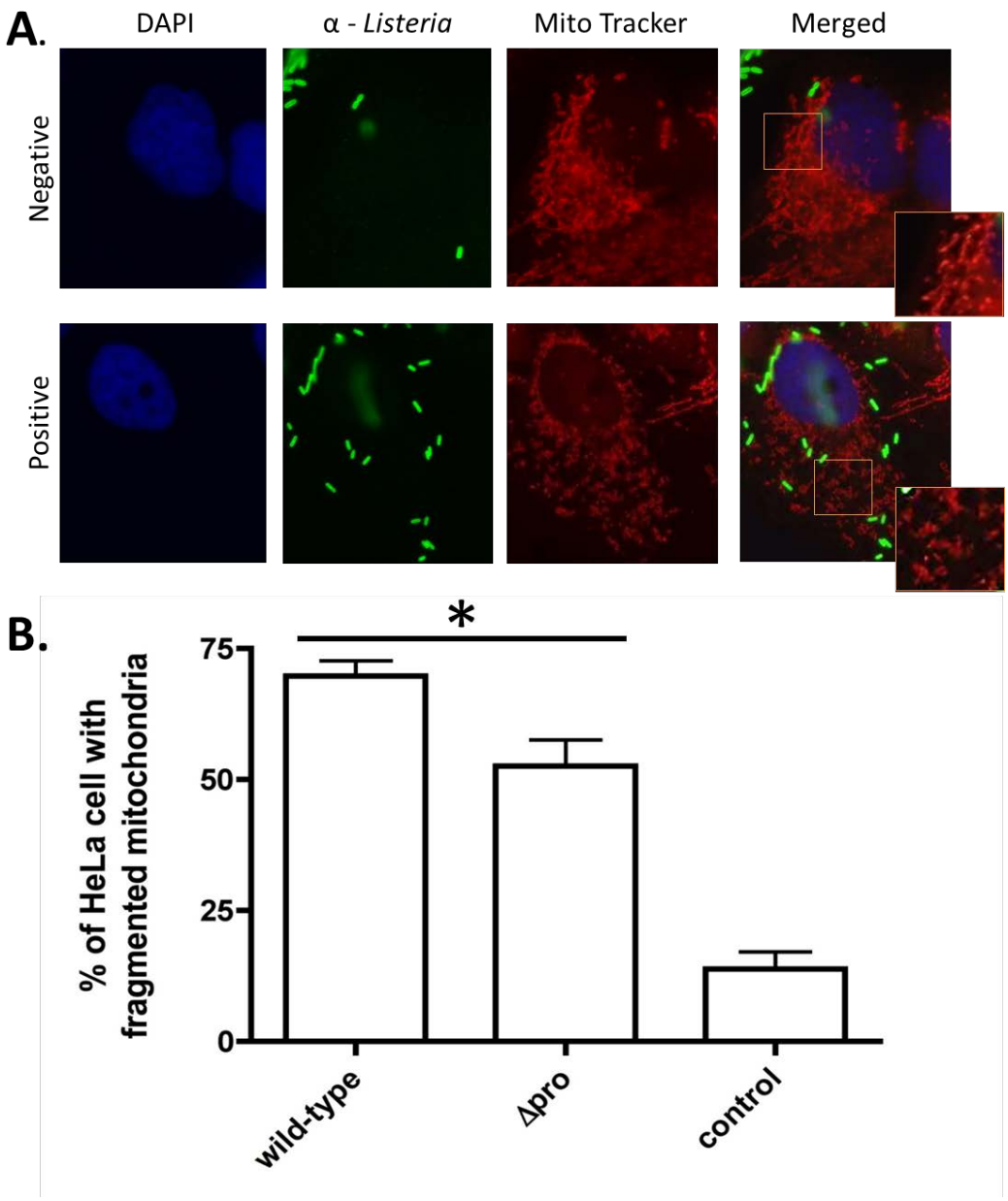

Figure 7. Percentage of HeLa cells with fragmented mitochondria after infection with *L. monocytogenes plcB*Δpro or wild-type *L. monocytogenes*. HeLa cells were infected with either *L. monocytogenes inlA*^{m} or *inlA*^{m} *plcB*Δpro for 90 min. (A) The cells were fixed and stained with DAPI for nuclei, α-*Listeria* for *inlA*^{m} *plcB*Δpro, and Mito Tracker for mitochondria. The negative row demonstrates normal mitochondria and the positive row demonstrates mitochondrial fragmentation. (B) Bar graph representing the mean ± SEM of percentage of HeLa cells with mitochondrial fragmentation after infection with *L. monocytogenes inlA*^{m}, *inlA*^{m} *plcB*Δpro or uninfected control. Data were analyzed by the paired Student's t test comparing cells infected with either *L. monocytogenes inlA*^{m} or *inlA*^{m} *plcB*Δpro. *: p value of <0.05 for three independent experiments. Experiments performed by Delbert Abi Abdallah.

The *plcB*Δpro mutant does not enhance ubiquitination of bacteria within the host cytosol.**

Conjugation of ubiquitin on bacterial surfaces followed by proteasomal degradation is a defense mechanism used by mammalian cells to target both gram-negative and gram-positive bacteria within the host cytosol [57]. *L. monocytogenes* avoids ubiquitination through actin based motility and this phenomenon is dependent on de novo bacterial protein synthesis [57]. Our microarray data revealed that the ubiquitin mediated proteolysis pathway was significantly up regulated at the 3 hour time point in the mutant infection, but not the wild-type infection. Based on the transcriptional data and reduced cytosolic virulence factors displayed by the mutant we hypothesized that the mutant could be undergoing increased ubiquitination and ultimately proteasome degradation. We used the FK2 antibody, which interacts with mono- and poly-ubiquitinated proteins but not free ubiquitin [58], to determine if increased ubiquitination was occurring during mutant bacterial infection of macrophages (Fig. 8). Ubiquitin positive bacteria at 4 hrs post infection were not significantly different in mutant or wild-type infected cells. These results reveal that the mutant does not enhance ubiquitination of bacteria within macrophage cytosol, but it does not exclude the possibility that ubiquitination of specific host proteins is enhanced in mutant-infected cells.

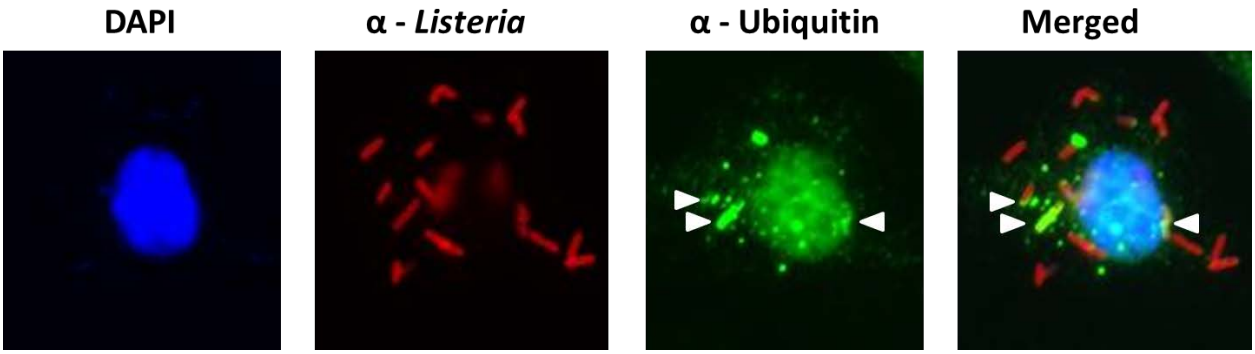

Figure 8. Ubiquitination of *L. monocytogenes* within macrophage host cells. Macrophages were infected with either *L. monocytogenes* $inlA^m$ or $inlA^m$ *plcB*Δpro for 4 hours. The cells were fixed and stained with DAPI for nuclei, α-*Listeria* for bacteria, and α-ubiquitin for ubiquitinated proteins. The white arrows indicate ubiquitinated proteins surrounding intracellular *L. monocytogenes*.

CHAPTER FOUR:

DISCUSSION

The results of this study show that a *L. monocytogenes* mutant that has lost the ability to regulate the activity of PC-PLC is attenuated *in vivo* and has a deleterious effect on wild-type bacteria during co-infection. Interestingly, this deleterious effect was observed specifically in the liver of infected mice. We hypothesized that the *plcB*Δpro mutant enhances the innate immune response to infection. However, the data did not support this hypothesis. Nevertheless, differences were observed in the intracellular life cycle of the mutant and the impact of infection on mitochondrial integrity.

We observed that constitutive secretion of active PC-PLC by *L. monocytogenes plcB*Δpro enhances the efficiency of bacterial escape from primary vacuoles in macrophages. In retrospect, it is not surprising that *L. monocytogenes plcB*Δpro is more efficient at escaping vacuoles than wild-type strain, considering that PC-PLC would start hydrolyzing vacuolar membrane phospholipids as the vacuole is being formed as opposed to when the vacuole becomes acidified at a later time point [59]. However, this effect seems to be cell type dependent as the efficiency of escape from vacuoles was not enhanced in human epithelial cells [54]. It is conceivable that a more rapid transit from the vacuole to the cytosol in macrophages influences the innate immunity-related signaling pathways that are induced by *L. monocytogenes*. Accordingly, 32-33% more genes related to the Toll-like receptor signaling pathway and MAPK signaling pathway were activated in wild-type-infected cells compared to mutant infected cells at 3 hours post-infection. Yet, the cytosolic signaling pathways such as the

NOD-like signaling pathway, the cytosolic DNA signaling pathway, and the RIG-1 like receptor signaling pathway were activated to similar levels in wild-type and mutant infected cells. Although down-regulation of the immune response to infection can be beneficial in some instances, it remains to be determined whether or not attenuation of the *L. monocytogenes plcB*Δpro mutant is due to a decrease in the activation of immune signaling pathways associated with vacuolar bacteria.

L. monocytogenes has been shown to cause mitochondrial fragmentation, affecting host cell homeostasis in favor of intracellular growth of bacteria [55,56]. Accordingly, our microarray data indicated that expression of genes related to mitochondrial respiratory complexes II to V were up-regulated during infection with wild-type bacteria suggesting mitochondrial dysfunction. In mutant infected cells, only genes related to mitochondrial respiratory complex I were up-regulated indicating a much lower degree of mitochondrial dysfunction. Moreover, mitochondrial fragmentation was significantly less prevalent in cells infected with the mutant strain than with wild-type strain. Mitochondrial fragmentation is dependent on LLO, but there is no evidence that LLO made by *L. monocytogenes plcB*Δpro lacks functionality as mutant bacteria show an increased rate of escape from vacuoles, which is in large part mediated by LLO. Perhaps active PC-PLC interacts with LLO in the cytosol of infected cells, preventing it from causing mitochondrial damage. Alternatively, PC-PLC might modify the mitochondrial membrane in a way that reduces the effect of LLO. These early host-pathogen interactions should be more closely examined to elucidate the effect that PC-PLC may play in altered cellular response to infection.

Neutrophils can play a significant role in the killing of extracellular bacteria [27,29]; but their contribution to controlling intracellular *Listeria monocytogenes* infections within the liver is

debated. Neutrophils are known to lyse *L. monocytogenes* infected hepatocytes within foci of infection [30]. Also, studies have demonstrated that after i.v. infection in mice neutrophil populations within the liver increase in direct relation to a reduction in *L. monocytogenes* recovered from the liver during the first 6 hours post-infection; yet these studies use nearly 3 logs the bacterial dose used in our experiments [27]. It has been demonstrated that depletion of neutrophils has drastic effects on survivability of immunocompetent mice early in infection, with this effect completely abrogated by day 4 post-infection [29]; however, this study was performed using anti-Gr-1 antibodies (clone RB6-8C5) which has been shown to deplete neutrophils and monocytes [60]. Thus, mice treated with RB6-8C5 demonstrate immune responses based on altered leukocyte population beyond solely neutrophil depletion. A recent study demonstrates that specific depletion of neutrophils, with the Ly6G-specific antibody 1A8, does not significantly alter survivability of mice i.v. infected with *L. monocytogenes* [61]. Furthermore, when neutrophil-depleted mice are infected with either low or high doses of *Listeria monocytogenes*, ~10^4 CFU or ~3.5 x 10^4 CFU respectively, only the high doses have been shown to significantly increase mortality [62]. This suggests low doses of *L. monocytogenes* in mice, such as in our study, would be more significantly affected by leukocytes other than neutrophils. Accordingly, mice infected with the attenuated mutant strain had a significant increase in the number of liver macrophages at day 2 post-infection, which might have contributed more significantly than neutrophils to clearance of the bacteria.

The lack of significant neutrophil populations in mutant infected mice, unlike wild-type infected mice, at day 3 post-infection is an interesting finding. It is possible that the constitutively secreted PC-PLC caused an earlier influx of neutrophils within the liver, leading to an earlier decline in the overall population that became apparent by day 3 post-infection or that

these neutrophils present demonstrated increased efficiency at killing mutant bacteria over wild-type, allowing fewer cells to overcome the hepatic infection. However, we did not detect any difference at days 1 and 2 post-infection. Another possibility for the decrease in neutrophil population is that the mutant strain is cytotoxic to neutrophils. PC-PLC is able to hydrolyze all phospholipids within mammalian cells [59,63]. Massive release of activated PC-PLC from multiple vacuoles into the host cytoplasm has been shown to be cytotoxic to J774 cells *in vitro* [37]. While this mutant, with constant secretion of active PC-PLC, is not cytotoxic *in vitro*, compromised host cell membrane integrity is seen in a cell-to-cell spread dependent manner [43]. The effect of increased amounts of active PC-PLC within neutrophils *in vivo* is unknown. Future studies of neutrophil recruitment should focus on earlier time points post infection and viability of cells.

The lack of interferon γ (IFN-γ) and interleukin 12 (IL-12) production, two of the primary inflammatory cytokines in *L. monocytogenes* infection, in mutant infected mice is interesting. Despite the abundance of natural killer (NK) cells, one producer of IFN-y, recruited to the liver during infection with the mutant we see a significant decrease in production of this cytokine at day 3 post-inoculation. IFN-γ is needed to induce resistance to *L. monocytogenes* in immunocompetent mice through increased microbicidal activity against *L. monocytogenes* through production of reactive nitrogen and oxygen intermediates [64,65], so our lack of IFN-γ would be predicted to aid in persistence of mutant infection. Despite a significant increase of dendritic cells at day 3 post-inoculation of mice with the mutant, a lack of IL-12 would suggest that macrophages are not being activated [64]. This discrepancy suggests that there is another mechanism, independent of macrophage and T cell activation, responsible for attenuation of this mutant strain.

Our transcriptional response data also does not explain the attenuation of the mutant from an immune gene regulation perspective. It showed many similarities among genes up-regulated by mutant and wild-type infection, with few specific differences. An important finding that we can conclude from our microarray data is that mutant infection of macrophages does trigger a primary cytosolic response. Seven host genes have been identified to be up regulated as part of the primary cytosolic response during *L. monocytogenes* infection in macrophages, independent of secondary signaling and vacuolar response [48]. Five of the seven genes indicated in primary cytosolic response, IFNβ, PEL1, IFIT1, IFIT2, and IFIT3, are up regulated in macrophages during mutant infection at 6 and 9 hours post infection, while myeloid differentiation primary response 116 (MYD116) and thymidylate kinase family (TYK1) genes are not significantly up regulated. This evidence shows that the mutant is eliciting a host response specific to cytosol target genes within the overall immune gene up regulation. Taking into account the similar patterns in up regulation of immune response genes in both mutant and wild-type infection, one should consider that the microarray was performed in BALB/c bone-marrow derived macrophages only and, as discussed, LLO and PC-PLC can have drastically different essential functions within different cell types. Performing additional experiments in different cell lines could result in altered transcriptional profiles and *in vivo* examination of gene regulation would be much more informative, considering the complexity of the immune response.

In this study we examined the attenuation of *L. monocytogenes* secreting constitutively active PC-PLC by the *plcB*Δpro mutant. Efficiency of vacuolar escape and decreased mitochondrial fragmentation were demonstrated during mutant infection *in vitro*. Further, murine inoculation results in attenuation of mutant bacteria and co-infected wild-type bacteria without increased cellular immune response and with decreased inflammatory cytokines, IFN-γ

and IL-12. *In vitro* analysis of transcriptional response reveals few immune-related gene regulation changes in BMMΦ infection between mutant and wild-type strains. These combined results suggest that early host response to infection with the *plcB*Δpro mutant is responsible for reduced bacterial survivability within the mouse liver, independent of inflammatory response.

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