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THE ROLE OF INTRACELLULAR GROWTH DURING THE SYSTEMIC SPREAD OF LISTERIA MONOCYTOGENES

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THE ROLE OF INTRACELLULAR GROWTH DURING THE SYSTEMIC SPREAD OF LISTERIA MONOCYTOGENES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

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2024

ABSTRACT OF DISSERTATION

THE ROLE OF INTRACELLULAR GROWTH DURING THE SYSTEMIC SPREAD OF LISTERIA MONOCYTOGENES

Listeria monocytogenes is a facultative intracellular pathogen transmitted through the consumption of contaminated food products. Typically, infections range from mild, self-limiting gastroenteritis to life-threatening systemic infections; however, the events that occur in the gut to allow for this spread are unclear.

The focus of my thesis aims to determine how *L. monocytogenes* escape the mesenteric lymph nodes (MLN), the final barrier to systemic spread for both commensal and pathogenic bacteria in the gut. I have shown that intracellular replication of *L. monocytogenes* in an as-yet-unidentified cell type is essential for the colonization and dissemination of the bacteria from the MLN. Intracellular replication protected *L. monocytogenes* from clearance by monocytes and neutrophils in the intestinal tissue, thereby promoting colonization of the MLN. I developed an *in vitro* assay to measure free lipoate concentration and determined that intestinal tissue had enough lipoate to support LplA2-dependent extracellular growth of *L. monocytogenes*, but exogenous lipoate in the MLN was severely limited. Thus, the bacteria could replicate only inside cells in the MLN, where they used LplA1 to scavenge lipoate from host peptides. I also found that intracellular replication is required for actin-based motility and cell-to-cell spread and showed that this intracellular function is vital for rapid exit from the MLN. Given these results, I developed a model of systemic spread in which *L. monocytogenes* must invade, escape the phagocytic vacuole, replicate, and undergo actin-based motility in the cytosol, in a critical cell type in the MLN that provides access to the bloodstream.

I focused my attention on lymph node stromal cells, specifically fibroblastic reticular cells (FRC) and blood endothelial cells (BEC), which make up the high endothelial venules within the MLN and could allow *L. monocytogenes* direct access into the blood. These cells comprise less than 1% of the lymph node cellularity, but I sort-purified these tiny subsets and developed *ex vivo* assays to show that *L. monocytogenes* could replicate exponentially, undergo actin-based motility, and induce an IFN-beta response within the cytosol of both FRC and BEC *in vitro*. Infected FRC and BEC also produced a robust chemokine and pro-inflammatory cytokine response during *in vitro* infection. Flow cytometric analysis confirmed that GFP+ *Lm* were associated with stromal subsets *in vivo* following foodborne infection of mice, and *ex vivo* cultures revealed that the *L. monocytogenes* associated with these cells were viable, replicating bacteria. In BEC in particular, we found using fluorescence microscopy that the number of intracellular bacteria increased over the course of a three-day infection. These results suggest that FRC and BEC could be an essential growth niche for *L. monocytogenes* in the MLN.

These additional data have refined my model. I predict that *Lm* invades perivascular FRC that surround the BEC in the T cell zone of the lymph node. Once taken up, *L. monocytogenes* replicate in the cytosol and use actin-based motility to spread into the underlying BEC which they cannot otherwise invade efficiently. Despite inefficient invasion, *L. monocytogenes* replicate much faster within BEC compared to FRC. I hypothesize that exponential replication of *L. monocytogenes* within these cells results in lysis or membrane damage that allows *L. monocytogenes* to escape extracellularly into the blood. Overall, this work lays the groundwork for future studies aimed at determining the key events that must occur in the MLN that permit the systemic spread of *L. monocytogenes*.

Keywords: Listeria monocytogenes, Stromal cells, Mesenteric Lymph Nodes, facultative foodborne pathogen, intracellular lifecycle

Jamila S. Tucker

03/20/2024

Date

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This work is dedicated to my mom.

I am the woman I am because of you; I love you!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

I. Foodborne Listeriosis

1.1 Bacterium and Transmission

Listeria monocytogenes (*L. monocytogenes* or *Lm*) are Gram-positive saprophytic bacteria that were first described in 1910 after isolation from the necrotic liver of a rabbit (Hülphers 1911). *L. monocytogenes* are facultatively anaerobic and are ubiquitous in the environment (Gray and Killinger 1966, Wieczorek, Dmowska et al. 2012). This bacterium is commonly isolated from soil, sewage, groundwater, and decaying vegetation (Thevenot, Dernburg et al. 2006). However, a significant amount of attention has focused on the ability of *L. monocytogenes* to survive in food processing plants, where the bacteria can withstand high salt concentrations (Holch, Webb et al. 2013), fluctuations in pH (4.4-9.6) (Holch, Webb et al. 2013), and low temperatures (-0.4°C to 45°C) (Liu 2006). Thus, survival under these extreme environmental conditions has made *L. monocytogenes* a threat to contaminate a variety of food products such as dairy products, meat products, vegetables, and seafood products (Ragon, Wirth et al. 2008, Leong, Alvarez-Ordonez et al. 2014, Zuber, Lakicevic et al. 2019).

1.2 Disease Epidemiology

L. monocytogenes was first isolated in humans in 1929 (Ansbacher, Borchardt et al. 1966). However, interest in the bacteria as a pathogen responsible for serious human infection did not arise until the 1980s, following several outbreaks attributed to improperly pasteurized milk (Klumpp and Loessner 2013). According to the CDC, *L. monocytogenes* has the lowest prevalence (0.27%) of all the foodborne pathogens but the highest hospitalizations (98%) and mortality rate (23%) (CDC 2023). Human listeriosis is commonly characterized in two forms, febrile gastroenteritis or severe invasive listeriosis (Buchanan, Gorris et al. 2017). Febrile gastroenteritis can manifest in immunocompetent individuals and is

characterized by fever, diarrhea, and headaches (Mateus, Silva et al. 2013). These infections are typically self-limiting and resolve within 2-3 days, resulting in an underreporting of cases (Matle, Mbatha et al. 2020). Invasive listeriosis occurs mostly in immunocompromised individuals and results in life-threatening infections such as sepsis, meningoencephalitis, and endocarditis (Doganay 2003). This severe form of infection accounts for over 90% of the hospitalizations and all deaths associated with the disease (Leong, Alvarez-Ordonez et al. 2014).

II. Intracellular Lifecycle

L. monocytogenes skillfully transitions from its saprophytic life in the soil to adopting a cytosolic form in mammalian cells through a variety of factors that promote bacterial invasion, phagosomal escape, replication, and spread into adjacent cells (Fig. 1.1). Two well-known surface proteins, internalin A (Gaillard and Finlay) and B (InlB) promote bacterial attachment and invasion into non-professional phagocytes (Lecuit, Ohayon et al. 1997, Bierne and Cossart 2002). Once internalized, *L. monocytogenes* quickly escapes the vacuole by secreting a cholesterol-dependent cytolysin, listeriolysin O (LLO), and phosphoinositide-specific phospholipases (PC-PLC and PI-PLC) (Gaillard, Berche et al. 1987, Marquis, Doshi et al. 1995, Marquis, Goldfine et al. 1997, Gedde, Higgins et al. 2000). In the cytosol, *L. monocytogenes* begins replicating and within hours after vacuolar escape, the actin-assembly inducing protein (ActA) mediates polymerization of host actin at one pole of the bacterium (Dabiri, Sanger et al. 1990). Using actin-based motility, *L. monocytogenes* move about the cell (Dabiri, Sanger et al. 1990, Sanger, Sanger et al. 1992, Theriot, Mitchison et al. 1992) and forms protrusions to spread to adjacent cells, avoiding the extracellular milieu (Tilney, DeRosier et al. 1992).

1.3 Regulation of Virulence Factors

The ability of *L. monocytogenes* to survive within a mammalian host cell is dependent on the alternative sigma factor, σ^B, and positive regulatory factor A protein (PrfA) regulation. Activation of σ^B is induced by a variety of environmental stresses such as low pH, low temperatures, osmotic shock, and high osmolarity (Becker, Cetin et al. 1998, Becker, Evans et al. 2000). In the gastrointestinal tract, the acidic conditions in the stomach and the osmotic stress and bile salts in the small intestine induce the expression of σ^B (O'Driscoll, Gahan et al. 1996, Wiedmann, Arvik et al. 1998, Sue, Fink et al. 2004, Sleator, Watson et al. 2009, Tiensuu, Guerreiro et al. 2019). This activation results in transcriptional modifications in the bacteria that allow for bacterial survival in these harsh conditions (Saklani-Jusforgues, Fontan et al. 2000, Toledo-Arana, Dussurget et al. 2009). Finally, σ^B also positively regulates *prfA* expression, the master regulator of virulence genes involved in the intracellular lifecycle (Mengaud, Dramsi et al. 1991, Chakraborty, Leimeister-Wachter et al. 1992, Dramsi, Kocks et al. 1993, Nadon, Bowen et al. 2002).

Apart from InlA and InlB, the genes that encode the key virulence factors (*prfA*, *hly, plcA, plcb, mpl and actA*) required for the intracellular lifecycle of *L. monocytogenes* are all linked on a chromosomal island, also known as the *Listeria* pathogenicity island 1 (Chakraborty, Hain et al. 2000, Vazquez-Boland, Kuhn et al. 2001). These genes were previously shown to be selectively induced inside the mammalian cell (Hevin, Morange et al. 1993, Hanawa, Yamamoto et al. 1995, Bubert, Sokolovic et al. 1999). It was later shown that this differential expression of virulence genes was due to PrfA levels (Renzoni, Cossart et al. 1999). *prfA* can be transcribed as either a monocistronic or a bicistronic transcript (Mengaud, Dramsi et al. 1991, Milohanic, Glaser et al. 2003). The initial monocistronic transcripts generate enough PrfA to activate the expression of *hly* and *plcA* (Freitag, Rong et al. 1993, Freitag and Portnoy 1994), whose gene products are essential for escape from the vacuole. The *plcA* promoter lies directly upstream of *prfA*, which results in a *plcA-prfA* bicistronic transcript. This process significantly

increases PrfA levels, resulting in the activation of genes such as, *actA* and *inlC*, virulence factors needed later in *L. monocytogenes* intracellular lifecycle.

1.4 Invasion

A. Phagocytosis

L. monocytogenes can be readily phagocytosed by macrophages, dendritic cells, and granulocytes. These cells can bind to microbe-associated molecular patterns (MAMPs) directly, using toll-like receptors and scavenger receptors, or indirectly, via Fc or complement receptors binding to opsonized targets. While opsonization of *L. monocytogenes* with immunoglobulins and complement factors has previously been shown to enhance phagocytosis, it is not required for efficient uptake of the bacteria (Kolb-Maurer, Gentschev et al. 2000, Thomas, Li et al. 2000). For example, complement receptor type 3 was shown to mediate the uptake and killing of C3-coated *L. monocytogenes* in inflammatory peritoneal macrophages (Drevets and Campbell 1991, Drevets, Canono et al. 1992). However, another study found that in the absence of opsinization, SR-AI/II, a scavenger receptor present on the surface of macrophages, contributes to the uptake of *L. monocytogenes* (Thomas, Li et al. 2000, Ishiguro, Naito et al. 2001). SR-AI/II knock-out animals exhibited increased susceptibility to infection and Kupffer cells displayed a reduced ability to phagocytose *L. monocytogenes* compared to wildtype animals (Ishiguro, Naito et al. 2001). More recently, PTEN, a phosphatase and tumor suppressor, was shown to be essential for the phagocytosis of *L. monocytogenes* by human and murine macrophages, as it enhances adherence of the bacteria to the cell surface (Glover, Schwardt et al. 2023). In fact, this study showed that this PTEN-dependent pathway of phagocytosis was more efficient than any PTEN-independent pathway.

B. Internalin-mediated uptake

L. monocytogenes can actively induce internalization into non-phagocytic cells, such as epithelial cells (Gaillard, Berche et al. 1987), endothelial cells (Drevets, Sawyer et al. 1995), lymphocytes (McElroy, Ashley et al. 2009), placental trophoblasts (Ireton 2007), hepatocytes (Dramsi, Biswas et al. 1995), and cardiomyocytes (Alonzo, Bobo et al. 2011), using receptor-mediated uptake. This is thought to be facilitated by a family of 25 internalin proteins, including InlA, InlB, InlC, InlF, and InlP, which have been shown to promote attachment (Bierne, Sabet et al. 2007), internalization of host cells (Mengaud, Ohayon et al. 1996, Bierne, Sabet et al. 2007, Ghosh, Halvorsen et al. 2018) , and cell-to-cell spread (Polle, Rigano et al. 2014, Dowd, Mortuza et al. 2020). Structurally, all internalins of *L. monocytogenes* share a leucine-rich repeat (LRR) domain that varies between 3 and 28 repeats (Marino, Braun et al. 2000, Bierne, Sabet et al. 2007). Another critical feature of these internalin proteins is the presence or absence of anchor regions. Most internalin proteins, like InlA, are covalently linked to the peptidoglycan through a carboxyl-terminal sequence “LPXTG” (Bierne, Sabet et al. 2007). However, other internalins like InlB lack this sequence and instead contain tandem repeats that facilitate non-covalent interactions with teichoic acids in the cell wall of *L. monocytogenes* (Bierne, Sabet et al. 2007, Sumrall, Shen et al. 2019, Sumrall, Schefer et al. 2020).

InlA and InlB are the primary invasins responsible for *L. monocytogenes* entry and have been best characterized in non-phagocytic cells. InlA specifically binds to the host cell receptor E-cadherin (Mengaud, Ohayon et al. 1996, Schubert, Urbanke et al. 2002), while InlB interacts with a tyrosine kinase, c-met (Shen, Naujokas et al. 2000, Niemann, Jager et al. 2007), with both receptors engaging the LRR regions on these proteins. Upon binding, InlA and InlB initiate a "zipper" mechanism triggering actin polymerization and cytoskeleton rearrangement, resulting in the envelopment of the bacterium and subsequent internalization (Ireton, Rigano et al. 2014, Radoshevich and Cossart 2018).

InlA and InlB are thought to be important during *L. monocytogenes* translocation across anatomical barriers during foodborne infection. In particular, InlA was shown to mediate *L. monocytogenes* transcytosis through the intestinal epithelium via goblet cells that harbor apically available E-cadherin (Lecuit, Vandormael-Pournin et al. 2001, Disson, Grayo et al. 2008, Nikitas, Deschamps et al. 2011). Notably, E-cadherin is exclusively accessible on the basolateral side of enterocytes. However, other studies have shown that *L. monocytogenes* may exploit the epithelial cell turnover process by binding to exposed E-cadherin when apoptotic enterocytes are extruded and detached (Pentecost, Otto et al. 2006). Similarly, InlB also mediates translocation across the intestinal epithelium barrier through interaction with c-met, a hepatocyte growth factor whose activation results in the disruption of cellular junctions (Birchmeier and Gherardi 1998, Shen, Naujokas et al. 2000). In addition to crossing the epithelial gut barrier, both InlA and InlB may also contribute to the invasion of the placental barrier. Studies have shown that the outer layer of placental villi, syncytiotrophoblast, also harbors apically accessible E-cadherin and c-met (Lecuit, Nelson et al. 2004, Gessain, Tsai et al. 2015). In fact, a study found that there was a severe defect in the colonization of the placentas and fetuses of pregnant gerbils and mice infected with *ΔinlA or ΔinlB L. monocytogenes* compared to wildtype (Disson, Grayo et al. 2008). Lastly, InlB is essential for mediating the invasion of endothelial cells, a cell type that forms a barrier between the vasculature and the underlying tissue (Parida S.K. 1998, Claesson-Welsh, Dejana et al. 2021). Previous studies have shown that the *L. monocytogenes* invasion of a variety of endothelial subsets such as human brain microvascular endothelial cells (Greiffenberg, Goebel et al. 1998) and human umbilical vein endothelial cells (Parida S.K. 1998) was InlB-dependent, suggesting that InlB may be involved in *L. monocytogenes* translocation into the vasculature at different anatomical sites.

1.5 Phagosomal Escape

Once internalized, intracellular bacterial pathogens can either replicate within the vacuole by preventing fusion with the phagolysosome or escape into the

cytosol before acidification of the vacuole takes place. *L. monocytogenes* readily escapes the vacuole compartment within 30 minutes by secreting LLO, a pore-forming hemolysin encoded by the *hly* gene (Cossart and Toledo-Arana 2008). This was the first identified virulence factor shown to be important for *L. monocytogenes* survival and proliferation within mammalian cells (Harvey and Faber 1941, Jenkins, Njoku-Obi et al. 1964, Gaillard, Berche et al. 1987, Kuhn, Kathariou et al. 1988, Portnoy, Jacks et al. 1988). LLO is functionally similar to streptolysin O, a cytolytic toxin secreted by *Streptococcus pyogenes,* both belonging to a family of cholesterol-dependent, pore-forming toxins (Geoffroy, Gaillard et al. 1987). This hemolysin is secreted as a soluble monomer that binds to cholesterol located in the host cell membrane. Following binding, it is thought that the LLO monomers oligomerize, forming a pre-pore ring that traverses through the membrane resulting in pore formation and the subsequent escape of the bacteria (Tweten, Parker et al. 2001). To prevent the destruction of the host plasma membrane and organelles, the activity of LLO is tightly restricted. For example, LLO has an activity range between pH 4.5 and 6.5, a range only achieved during the acidification of the phagosome, rendering the protein inactive in the cytosol (Geoffroy, Gaillard et al. 1987, Schuerch, Wilson-Kubalek et al. 2005)**.** Following *Listeria* escape from the vacuole, the inactive proteins undergo ubiquitylation and proteasomal degradation (Schnupf, Zhou et al. 2007).

In addition to the lysis of the vacuole, LLO has also been implicated in other cellular functions. It was previously shown LLO disrupts the host SUMOylation process, resulting in a reduction in SUMOylated proteins (Ribet and Cossart 2010). This was found to be beneficial to the bacterial infection, as an increase in SUMOylated proteins was shown to be detrimental to *L. monocytogenes* (Ribet and Cossart 2010). Additionally, LLO is also highly immunogenic. In fact, this protein was previously found to be the predominant epitope for both CD4 and CD8 T cells (Pamer, Harty et al. 1991). Many studies have shown that activation of the inflammasome, an intracellular immune sensor, occurs when LLO activates caspase-1 following the escape of *L. monocytogenes* from the vacuole, resulting in the production of IL-18 and IL-1β (Hara, Tsuchiya et al. 2008, Warren, Mao et

al. 2008, Meixenberger, Pache et al. 2010). Other cellular functions LLO has been implicated in include, the modification of histones (Hamon and Cossart 2011), mitochondrial fragmentation (Stavru and Cossart 2011) and the induction of autophagy (Birmingham, Canadien et al. 2007, Py, Lipinski et al. 2007, Meyer-Morse, Robbins et al. 2010).

Although the LLO hemolysin is thought to be pivotal for the disruption of the phagosome, it was previously shown that *Δhly L. monocytogenes* were still capable of escaping the vacuole within Henle 407 and HeLa cells, suggesting there were other products involved in vacuolar lysis (Marquis, Doshi et al. 1995). Subsequent studies revealed that *L. monocytogenes* also secretes two phospholipases, phosphatidylinositol-specific PlcA (Camilli, Goldfine et al. 1991, Mengaud, Dramsi et al. 1991) and phosphatidylcholine-specific PlcB (Vazquez-Boland, Kocks et al. 1992), to the rupture of the *Lm*-containing vacuole (Pizarro-Cerda, Kuhbacher et al. 2012). These phospholipases have an activity range between a pH of 5.5 - 8 and have been shown to effectively hydrolyze the phospholipids that constitute the vacuole membrane (Geoffroy, Raveneau et al. 1991, Goldfine, Johnston et al. 1993). Notably, functional analysis revealed that PlcA plays a minor role in the vacuolar escape, as a plcA-deficient mutant had only a slight reduction in the ability to escape the primary vacuole (Camilli, Tilney et al. 1993), and later studies showed that PlcA had no impact on cell-to-cell-spread (Smith, Marquis et al. 1995). In contrast, PlcB is critical in facilitating *L. monocytogenes* escape from both primary and double membrane vacuoles following cell-to-cell spread. Studies showed that *Δhly L. monocytogenes* were only able to escape the primary vacuole when PlcB was present (Marquis, Doshi et al. 1995). Additionally, there was an accumulation *of Δplcb L. monocytogenes* within the double membrane vacuole of J774 macrophages, indicating its role in cell-to-cell spread (Vazquez-Boland, Kocks et al. 1992).

Extensive studies have revealed a critical role for LLO, PlcA, and PlcB in facilitating the vacuolar escape of *L. monocytogenes* into the cytosol. However,

recently, InlB, the internalin protein that binds the Met receptor on mammalian cells, has emerged as another potential mediator of phagosomal escape. Cain et al. demonstrated that InlB expedites the vascular escape of *L. monocytogenes* in HeLa cells, promoting the efficient recruitment of endosomal maturation mediators (Cain, Scortti et al. 2023). This suggested that InlB accelerates *L. monocytogenes* escape into the cytosol by directly facilitating the maturation of the vacuole.

1.6 Replication

Following escape from the phagocytic vacuole, *L. monocytogenes* must acquire nutrients and evade cytosolic immune defenses, such as autophagy to survive and replicate. *L. monocytogenes* are auxotrophic for many nutrients and must acquire them from the host, using over 330 transporters (Glaser, Frangeul et al. 2001). Carbon, one of the major essential nutrients required for replication, is acquired through multiple sources. *L. monocytogenes* utilizes a hexose phosphate transporter to transport glucose-1-phosphate and glucose-6-phosphate (Chico-Calero, Suarez et al. 2002), two compounds likely used as precursors for nucleic acid biosynthesis (Grubmuller, Schauer et al. 2014). The bacteria also transports host-derived glycerol, a carbon source shown to be essential for glycolysis and amino acid biosynthesis (Grubmuller, Schauer et al. 2014). Finally, it is thought that *L. monocytogenes* may utilize host-derived lipids as a third carbon source, particularly phosphatidylethanolamine. This hypothesis is supported by earlier research showing that an ethanolamine lyase-deficient mutant could not replicate within epithelial cells (Joseph, Przybilla et al. 2006).

Intracellular replication of *L. monocytogenes* is also dependent on nitrogen assimilation. Glutamine serves as the primary nitrogen source for the bacteria. It was previously shown that genes encoding glutamate synthase, a flavoprotein crucial for nitrogen assimilation, were upregulated during infection (Schreier 1993, Joseph, Przybilla et al. 2006). However, in the absence of glutamine, *Listeria* uses ammonia, ethanolamine, and arginine as alternative nitrogen sources (Tsai and Hodgson 2003, Kutzner, Kern et al. 2016). In addition to nitrogen assimilation, *L.*

monocytogenes must also scavenge for many essential cofactors, such as biotin, riboflavin, and lipoate, as the bacteria do not contain pathways for the *de novo* synthesis of these compounds (Premaratne, Lin et al. 1991, Phan-Thanh and Gormon 1997). Biotin and riboflavin are transported from the host cytosol through specific transporters dedicated to each compound (Karpowich, Song et al. 2015, Matern, Pedrolli et al. 2016). *L. monocytogenes* scavenges host-derived lipoate using two nonredundant lipoate ligase-like proteins, LplA1 and LplA2 (O'Riordan, Moors et al. 2003, Keeney, Stuckey et al. 2007, Christensen, Hagar et al. 2011). LplA2 is dispensable for cytosolic growth but important for acquiring exogenous free lipoate in the extracellular environment (Keeney, Stuckey et al. 2007). However, LplA1, possessing a higher affinity for lipoate, is required in the cytosol, where available lipoate is bound to host peptides (Keeney, Stuckey et al. 2007, Christensen, Hagar et al. 2011). Many studies have shown that in the absence of biotin, riboflavin and lipoate, intracellular replication of *L. monocytogenes* is inhibited (O'Riordan, Moors et al. 2003, Rivera-Lugo, Light et al. 2022).

In addition to nutrient availability, the intracellular growth of *L. monocytogenes* also varies from cell type to cell type. For example, *L. monocytogenes* was previously shown to have a doubling rate of approximately 60 minutes within the cytosol of J774 macrophages, murine embryonic fibroblasts and Henle 407 epithelial cells (Portnoy, Jacks et al. 1988). In contrast, another study showed that the doubling time of *L. monocytogenes* within a kidney epithelial cell line was only 3 hours (Ortega, Koslover et al. 2019). While this variability in growth within different cell types could be due to lack of nutrients, another possibility could be delayed escape from the vacuole. Portnoy et al. previously showed that the doubling time of *Δhly L. monocytogenes* increased to 4 hours in J774 macrophages and embryonic fibroblasts when the bacteria could not efficiently escape the vacuole (Portnoy, Jacks et al. 1988). Thus, the absence/lack of intracellular replication of *L. monocytogenes* in some cell types could be due to inefficient vacuolar escape.

1.7 Cell-to-Cell Spread

L. monocytogenes, as well as other intracellular bacterial pathogens like *Shigella* and *Rickettsia*, have the ability to use actin-based motility to move around the cytosol and spread into adjacent cells, avoiding immune detection (Tilney and Portnoy 1989, Gouin, Gantelet et al. 1999). This motility and cell-to-cell spread is mediated by a single actin recruitment surface protein, ActA (Domann, Wehland et al. 1992, Kocks, Gouin et al. 1992, Kocks, Marchand et al. 1995). This protein has three functional regions: An N-terminal region that promotes actin filament nucleation through interactions with the host Arp2/3 complex; a C-terminal region that contains a non-covalently linked membrane anchor; and a central region that contains four proline-rich repeats that bind ENA/VASP family proteins (Chakraborty, Ebel et al. 1995, Niebuhr, Ebel et al. 1997, Welch, Rosenblatt et al. 1998, Pistor, Grobe et al. 2000).

Once in the cytosol, the expression of ActA is uniform around the surface of *L. monocytogenes*, which actively recruits actin filaments, creating the appearance of an actin cloud enveloping the bacteria (Tilney, Connelly et al. 1990). As the infection progresses, ActA polarizes to one pole of the bacterium. The formation of actin comet tail is initiated by the activation of the Arp2/3 complex bound to the N terminus of ActA (Welch, Rosenblatt et al. 1998). This complex stimulates the nucleation of new actin filaments (Winter, Podtelejnikov et al. 1997). Once an actin monomer is bound at the actin-binding site of ActA, ENA/VASP, bound at the proline-rich repeat domain of ActA will bind profilin, an actin monomer binding protein (Goldberg 2001). The activated Arp2/3 complex then mediates the addition of actin monomers, brought by profilin, to the barbed ends and caps the actin filament (Goldberg 2001). As the actin filament extends from the bacteria, Arp2/3 is released by ActA and replaced by a new Arp2/3 complex, repeating the process and forming the actin comet tail (Goldberg 2001). This actin tail gives the bacteria motility, allowing it to propel through the cytosol (Pistor, Grobe et al. 2000). Upon contact with the host cell membrane, *L. monocytogenes* creates a protrusion that can extend into the cytoplasm of the adjacent cell (Robbins, Barth et al. 1999).

InlC facilitates the creation of this membrane protrusion by interacting with the scaffolding protein, Tuba, resulting in reduced cortical tension and relaxed cellular junctions (Rajabian, Gavicherla et al. 2009, Ireton 2013). Following protrusion, the neighboring cell can engulf the protrusion, enclosing the bacteria within a double membrane vacuole until *L. monocytogenes* escape into the cytosol of the new cell (Gedde, Higgins et al. 2000).

III. Gastrointestinal Stage of Infection

L. monocytogenes is transmitted orally through the consumption of contaminated food products. Following colonization of the gut, *L. monocytogenes* disseminates from the mesenteric lymph nodes causing life-threatening systemic infecions. For decades, investigators utilized the intravenous model of infection, which mimics systemic listeriosis but completely bypasses the gut phase of infection. Consequently, key events that occur in the gut that ultimately lead to the systemic spread of *L. monocytogenes* have been severely understudied. In this section, I will review the recent advancements that have allowed for reliable ways to study the gastrointestinal phase of infection and our current understanding of the events that occur following the oral transmission of *L. monocytogenes.*

1.8 Infectious Dose

The infectious dose for humans and primates is estimated to range between 10^6- 10^7 CFU (Farber, Ross et al. 1996, Smith, Takeuchi et al. 2008). However, a dose as low as 10^5 CFU can be infectious for immunocompromised individuals (Farber, Ross et al. 1996). In contrast, mice, rats, and gerbils require extremely high doses ranging between 10^9-10^{10} CFU to establish intestinal infection (D'Orazio 2014). This disparity in doses is largely dependent upon the species-specificity of internalin proteins, InlA and InlB and will be discussed in more detail in the following section.

1.9 Animal Models

The ideal animal model should mimic every facet of human listeriosis. Yet, the existing animal models for *L. monocytogenes* infections have limitations due to technical challenges. These limitations include the need for a high infectious dose to establish infection and species-specific interactions between the internalin proteins, InlA and InlB, and their respective host receptors, E-cadherin and Met (Mengaud, Ohayon et al. 1996, Shen, Naujokas et al. 2000). Guinea pigs were previously used to show that the MLN was a critical bottleneck during *L. monocytogenes* infection, with only 1 in every 10^2-10^3 bacteria overcoming this barrier (Melton-Witt, Rafelski et al. 2012). Additionally, this model is thought to be valuable for understanding the maternal-fetal transmission of *L. monocytogenes*, as guinea pigs and human placentas are similar in architecture (Leiser and Kaufmann 1994). However, this model's limitation is any potential pathway requiring InlB-mediated uptake, as the Met receptor in guinea pigs cannot effectively bind to InlB due to a specific amino acid change in the receptor. In contrast, gerbils were previously found to be a suitable model for *L. monocytogenes* infections, mimicking both the human intestinal infection and systemic diseases such as spontaneous abortions and rhombencephalitis (Blanot, Joly et al. 1997, Disson, Grayo et al. 2008). However, due to the lack of reagents and tools, this model has not gained favor.

Listeriosis in non-human primates was previously found to recapitulate the clinical symptoms of sporadic *L. monocytogenes* infections (Smith, Takeuchi et al. 2003, Lemoy, Lopes et al. 2012). Though this is ideal, non-human primates are extremely expensive, resulting in most studies having a small number of animals. This limitation significantly impedes the range of feasible research questions within a study, making a smaller animal model more favorable. Mice, due to their size, affordability, and the availability of reagents, are an ideal model. However, this model faces challenges such as a high infectious dose to establish intestinal infections, which vary based on the mouse strain background (Czuprynski, Faith et al. 2003, Bou Ghanem, Jones et al. 2012), and the lack of specificity of InlA to

murine E-cadherin (Lecuit, Vandormael-Pournin et al. 2001). To combat these limitations and enhance the binding of InlA and E-cadherin, the Lecuit group engineered transgenic and knockout mice expressing humanized E-cadherin (Lecuit, Vandormael-Pournin et al. 2001, Disson, Nikitas et al. 2009). Conversely, Wollert, et al. developed a mouse-adapted *L. monocytogenes* strain expressing a murinized InlA ($InlA^m$), allowing *L. monocytogenes* to bind both murine E- and N-cadherin with high affinity (Wollert, Pasche et al. 2007). These adaptations have significantly improved the mouse model, rendering it a preferred model for *L. monocytogenes* oral infections.

1.10 Foodborne Infection

Only in recent decades has research shifted to studying the *L. monocytogenes* oral infection. This is largely due to the recent advancements of humanized mice and mouse-adapted *L. monocytogenes* strains (Lecuit, Vandormael-Pournin et al. 2001, Wollert, Pasche et al. 2007, Disson, Nikitas et al. 2009). Yet, the method of inoculum delivery to the animal remains a challenge. *L. monocytogenes* can be added directly to the drinking water. Though, it is difficult to determine the amount of bacteria consumed by the animal. Oral gavage, a needle used to ensure the inoculum reaches the stomach, is also a common method utilized by investigators. However, this method is user-dependent and could inadvertently cause early systemic spread due to potential esophageal trauma from the needle (O'Donnell, Pham et al. 2015). The most reproducible model of oral transmission involves the consumption of contaminated food, which closely resembles human transmission (Bou Ghanem, Myers-Morales et al. 2013, Bou Ghanem, Myers-Morales et al. 2013). In this natural feeding model, developed by our lab, mice undergo a 16-24 hour fasting period and are placed on wire floors to prevent coprophagia. Following the fast, they are fed small bread pieces saturated in butter containing 10^6-10^8 *L. monocytogenes*. This method allows for doses similar to that of humans, is not user-dependent, and has been demonstrated to be reproducible (Bou Ghanem, Myers-Morales et al. 2013, Bou Ghanem, Myers-Morales et al. 2013)

1.11 Intestinal Infection

A. Survival in the gastrointestinal lumen

Humans and mice are highly resistant to oral *L. monocytogenes* infections, necessitating the need for a higher infectious dose (Marco, Prats et al. 1992). This resistance is likely attributed to the multiple physical and chemical defenses in the gastrointestinal tract that foodborne pathogens must overcome. Once ingested, *L. monocytogenes* first encounters the acidic pH of the stomach, which kills most of the oral inoculum (Brandl, Plitas et al. 2007, Jiang, Olesen et al. 2010). The small fraction that survives is thought to undergo transcriptional modifications in response to the harsh stomach and duodenal environments, increasing bacterial resistance to low pH and enhancing viability (O'Driscoll, Gahan et al. 1996, Saklani-Jusforgues, Fontan et al. 2000, Wemekamp-Kamphuis, Wouters et al. 2002, Toledo-Arana, Dussurget et al. 2009). *L. monocytogenes* also upregulates bile salt hydrolases to withstand the high concentration of bile salts in the duodenum (Begley, Gahan et al. 2002, Dussurget, Cabanes et al. 2002, Begley, Sleator et al. 2005). Additionally, the bacteria must resist expulsion via intestinal peristalsis. This is achieved by bacterial attachment to the intestinal epithelium using the ActA protein (Travier, Guadagnini et al. 2013) and the adhesion protein, LAP (Jaradat and Bhunia 2002). Finally, the gut microbiota can significantly influence *L. monocytogenes* colonization in the gut lumen. For example, *Lactobacilli* and *Bifidobacterium* have been shown to inhibit *L. monocytogenes* invasion through the secretion of anti-bacterial factors *in vitro* (Corr, Gahan et al. 2007). Additionally, the microbiota produces signals during infection that result in increased mucus production (Johansson, Jakobsson et al. 2015) and secretion of anti-microbial peptides (Brandl, Plitas et al. 2008) by enterocytes. However, some strains of *L. monocytogenes* combat the microbiota defenses by secreting Listeriolysin S, which kills competing commensal bacteria, allowing for the luminal expansion of *Listeria* (Quereda, Dussurget et al. 2016).

B. Transcytosis across the intestinal epithelium

L. monocytogenes traverses the intestinal epithelium using three mechanisms, InlA-mediated transcytosis, LAP-mediated translocation, and M-cell-mediated transcytosis (Drolia and Bhunia 2019). The primary route of intestinal invasion is thought to involve InlA, which binds to the transiently exposed E-cadherin on extruding enterocytes(Lecuit, Dramsi et al. 1999, Lecuit, Vandormael-Pournin et al. 2001, Pentecost, Otto et al. 2006, Nikitas, Deschamps et al. 2011) or goblet cells (Nikitas, Deschamps et al. 2011). This interaction triggers endocytosis by the enterocytes, and the bacteria are rapidly transcytosed in an LLO independent manner across the epithelium and released into the underlying lamina propria (Nikitas, Deschamps et al. 2011). This process has been shown to involve *L. monocytogenes* hijacking the E-cadherin apical-basal recycling pathway (Kim, Fevre et al. 2021). Though InlA-mediated transcytosis is believed to be the predominant pathway of intestinal invasion, it was previously shown that InlA was not required to establish intestinal infection (Lecuit, Vandormael-Pournin et al. 2001, Bou Ghanem, Jones et al. 2012).

During LAP-mediated translocation, *L. monocytogenes* surface protein LAP binds to the luminally expressed Hsp60 protein on enterocytes (Drolia, Tenguria et al. 2018). This interaction activates the myosin light-chain kinase, which disrupts the tight junctional proteins, claudin-1, occludin, and E-cadherin (Drolia, Tenguria et al. 2018). This ultimately permits neighboring enterocytes to open, allowing *L. monocytogenes* to translocate between the cells and enter the lamina propria (Drolia, Tenguria et al. 2018, Drolia and Bhunia 2019). This mechanism also facilitates InlA-dependent invasion by providing access to E-cadherin. Lastly, *L. monocytogenes* can transcytose through M-cells, specialized enterocytes that monitor the intestinal lumen for antigens (Hase, Kawano et al. 2009). *L. monocytogenes* is believed to transcytose through M cells within the vacuole, though this mechanism is not well established (Drolia and Bhunia 2019).

C. Colonization of the lamina propria

Many studies have shown that *L. monocytogenes* colonizes the large intestine more efficiently than the small intestine. For example, Disson et al. observed increased invasion of *L. monocytogenes* in the colon compared to the ileum of humanized mice (Disson, Grayo et al. 2008). Similarly, we showed that there was a 10-fold increase in the bacterial burden of the colon compared to the ileum following infection with mouse-adapted *L. monocytogenes* (Bou Ghanem, Jones et al. 2012). This increased colonization of the colon might be attributed to the abundance of goblet cells, the primary site of intestinal invasion, being more numerous there, or the fact that the murine cecum is relatively large, serving as a reservoir for the bacteria. Additionally, Peyer's patches, specialized lymphoid follicles, are found underlying M-cells in the small intestine. Thus, the bacteria are likely phagocytosed following transcytosis through these cells. In fact, Disson et al. showed that once *L. monocytogenes* transcytose through M cells they are rapidly taken up by CX3CR1^{int} mononuclear phagocytes in the Peyer's patches, triggering a cytokine cascade that renders E-cadherin inaccessible on the apical surface, preventing further intestinal invasion of the small intestine (Disson, Bleriot et al. 2018).

Efficient colonization of the lamina propria is also dependent on whether *L. monocytogenes* are intracellular or extracellular. Our previous studies found that InlA was required for *L. monocytogenes* to persist within the lamina propria by 60 hours post-infection (Bou Ghanem, Jones et al. 2012). A subsequent study by Jones et al. showed that while intracellular replication was not required for *L. monocytogenes* to establish an infection in the colon, it was required for the bacteria to persist within this tissue by three days post-infection (Jones, Bussell et al. 2015). These studies together suggest that invasion of into a unknown cell type is vital for *L. monocytogenes* survival in the lamina propria.

D. Mononuclear Phagocytes

Mononuclear phagocytes (MP) are a heterogeneous population of cells that include, blood circulating monocytes, tissue-resident macrophages, and antigen-presenting dendritic cells (DC) (Merad, Sathe et al. 2013, Ginhoux and Jung 2014, Varol, Mildner et al. 2015). Together, these innate cells play a pivotal role in pathogen clearance, induction of the adaptive immune response, and tissue repair (Ginhoux and Jung 2014). MP cells, located in the intestinal lamina propria, are the first cells that interact with *L. monocytogenes* following foodborne infection and are therefore thought to be the primary intracellular growth niche for the bacteria. The heterogeneity among these cells, coupled with the lack of distinguishable surface markers and rapidly changing nomenclature has made comparing the function of these cells across different studies quite challenging. Nonetheless, below I will discuss our current understanding of the differentiation and nomenclature of each of these intestinal MP subsets and delve into our current knowledge of their role during a *L. monocytogenes* infection.

1. Monocytes

Monocytes originate from a common myeloid progenitor in the bone marrow, which then generates these cells via two independent pathways (Akashi, Traver et al. 2000, Manz, Miyamoto et al. 2002). One pathway arises from granulocyte-monocyte progenitors, while the other originates from monocyte-dendritic cell progenitors (Fogg, Sibon et al. 2006, Auffray, Fogg et al. 2009, Yanez, Coetzee et al. 2017). In both mice and humans, there are two major subsets of circulating monocytes: Classical monocytes, identified as Ly6C^{hi} CD43^{-} in mice and CD14^{+} CD16^{-} in humans; and Non-classical monocytes recognized as Ly6C^{lo} CD43^{+} in mice and CD14^{lo} CD16^{+} in humans (Geissmann, Jung et al. 2003, Auffray, Fogg et al. 2007, Zhu, Thomas et al. 2016). Nonclassical monocytes, known for their wound healing and anti-inflammatory phenotype, have a half-life of approximately 2 days in the blood (Yona, Kim et al. 2013, Thomas, Tacke et al.

2015). In contrast, classical or Ly6C^{hi} monocytes have a half-life of only 20 hours at steady state and are commonly associated with inflammation due to their ability to rapidly egress from the bone marrow and differentiate into macrophages in infected tissues, though this process can occur in steady state as well (Sunderkotter, Nikolic et al. 2004, Yona, Kim et al. 2013, Bain and Mowat 2014, Joeris, Muller-Luda et al. 2017). Previously, we showed that Ly6C^{hi} monocytes quickly infiltrate the gut following a *L. monocytogenes* foodborne infection and are the predominant cell type associated with the bacteria in the intestinal tissue and MLN at 2- and 3- days post-infection (Jones and D'Orazio 2017). However, despite this interaction, *L. monocytogenes* does not efficiently invade or grow exponentially within these cells (Jones and D'Orazio 2017). This suggests that monocytes may serve an alternative role during the gut phase of a *L. monocytogenes* infection.

2. Macrophages

Intestinal tissue-resident macrophages initially originate from an embryonic mesenchymal stem cell precursor but are constantly replenished by bone marrow-derived monocytes, specifically Ly6C^{hi} monocytes (Tamoutounour, Henri et al. 2012, Bain and Mowat 2014, Bain and Schridde 2018). Typically, classical resident macrophages are distinguished by their cell surface expression of CD11b, CD64, MERTK, MHCII, CX$_3$CR1, and F4/80. However, the level of surface expression of each marker may vary based on the subset and developmental stage of the macrophage (Schulz, Jaensson et al. 2009, Bain and Mowat 2014, Bain and Schridde 2018). Upon reaching the lamina propria, monocytes undergo local differentiation into various intermediate subsets through a process known as “waterfall differentiation” before ultimately maturing into bona fide resident macrophages (Tamoutounour, Henri et al. 2012, Bain, Scott et al. 2013). In mice, this process starts with monocytes initially acquiring MHCII expression, then losing Ly6C expression, and finally upregulating CX$_3$CR1 expression, resulting in the development of a CX$_3$CR1^{hi}MHCII^{hi}Ly6C^{lo} resident macrophage population (Zigmond and Jung 2013, Bain and Mowat 2014). While this resident phenotype

is widely accepted throughout the literature, a recent study has revealed an additional intestinal macrophage subset (Koscso, Kurapati et al. 2020). Initially thought to be a monocyte-derived dendritic cell, this migratory MP subset originates from Ly6C^{hi} monocytes but deviates from the typical tissue-resident macrophage pathway, gaining the expression of *Ccr7* and only expressing CX_3CR1 intermediately (Koscso, Kurapati et al. 2020). To date, not much is known about the role of intestinal macrophage subsets during a *L. monocytogenes* foodborne infection. However, macrophages at other anatomical sites have previously been shown to rapidly take up *L. monocytogenes* upon entrance into the tissue (Conlan 1996, Cousens and Wing 2000, Aichele, Zinke et al. 2003) . Thus, it is possible that intestinal macrophages could have a similar phenotype in the lamina propria.

3. Dendritic Cells

Like macrophages, intestinal DC can originate from multiple precursors, including a DC progenitor in the bone marrow or a circulating monocyte (Persson, Scott et al. 2013, Cerovic, Bain et al. 2014, Scott, Wright et al. 2016). It was previously believed that intestinal DC constituted a homogeneous population, primarily identified by the expression of CD103. However, recent literature has demonstrated that this MP subset can be further divided based on their expression of CD103 and CD11b, resulting in four distinct populations: CD103$^+$CD11b$^-$, CD103$^+$CD11b$^+$, CD103$^-$CD11b$^-$, and CD103$^-$CD11b$^+$ (Cerovic, Houston et al. 2013, Scott, Bain et al. 2015). All four of these DC subsets have been shown to migrate from the lamina propria to the MLN and induce the adaptive immune response through antigen presentation (Cerovic, Houston et al. 2013, Cerovic, Houston et al. 2015, Scott, Bain et al. 2015). Previously, we showed that while bone marrow-derived DC can serve as an intracellular growth niche for *L. monocytogenes*, primary CD103$^+$ and CD103$^-$ DC populations isolated from the gut were unable to support the growth of the bacteria (Jones, Smith et al. 2017). However, despite this finding, a recent study has shown that DC may still play a

critical role in MLN colonization. This study observed a notable reduction in *L. monocytogenes* burden within the MLN of Batf3$^{-/-}$ animals compared to WT animals following foodborne infection (Imperato, Xu et al. 2020). Together these studies suggest that although DC do not serve as a replicative niche for *L. monocytogenes* they could still play a role in the infection process by facilitating the transportation of the bacteria from the lamina propria to the MLN.

1.12 Dissemination from the Lamina Propria

Unpublished data generated by our lab suggest that *L. monocytogenes* may disseminate from the lamina propria using one of three mechanisms: free-floating through the lymphatics, attached to the cell surface, or inside a migratory cell. Indeed, a preliminary study by our group used confocal microscopy to show that extracellular and cell-associated *L. monocytogenes* could be found within the afferent lymphatics of the MLN (Jones 2017, Nowacki 2024). Additionally, Jones et al. found that intracellular replication was vital for colonization of the MLN, as Δ*lplA1 L. monocytogenes,* a strain deficient in intracellular replication, had a severe defect in colonizing the MLN by three days post-infection compared to wildtype bacteria (Jones, Bussell et al. 2015). This study suggests that for efficient dissemination to the MLN*, L. monocytogenes* must replicate within a cell, either in the lamina propria or upon reaching the MLN. Indeed, both CD103^{+} DC and CX$_3$CR1^{int} MP cells can sample pathogens from the intestinal lumen and migrate to the MLN (Niess, Brand et al. 2005, Schulz, Jaensson et al. 2009, McDole, Wheeler et al. 2012, Diehl, Longman et al. 2013, Farache, Koren et al. 2013). Although DC are known to not support the growth of *L. monocytogenes*, it remains possible that the CX$_3$XR1 MP cells could, and both subsets could contribute to transporting the bacteria to the lymph nodes, resulting in the bacteria gaining access to another cell type (Jones, Smith et al. 2017). Nonetheless, the specific route of dissemination of *L. monocytogenes* from the lamina propria remains understudied.

IV. Mesenteric Lymph Nodes

The MLN serves as the final barrier that prevents the systemic spread of commensal and pathogenic bacteria. Melton-Witt et al. previously showed, using signature-tagged *L. monocytogenes*, that only 1 in every 100-1000 bacteria passes this bottleneck (Melton-Witt, Rafelski et al. 2012). However, studies investigating how *L. monocytogenes* traverses through the node and the mechanisms employed by the bacteria to overcome this barrier are nonexistent. In this section, I will review the function and architecture of lymph node stromal cells and our current understanding of the MLN *L. monocytogenes* infection.

1.13 Architecture

The MLN are secondary lymphoid organs responsible for draining both the small and large intestine via lymphatic vessels. Their primary functions include constant immune surveillance and the initiation of the adaptive immune response. Structurally, they are comprised of four distinct regions: the subcapsular sinus (SCS), where lymph enters the node via afferent vessels; the underlying cortex containing B cell follicles; the centrally located paracortex, where the T cells reside; and the medulla that reconnects to circulation via efferent lymphatic vessels (Fig. 1.2). This architecture is critical for creating an environment where immune cells can interact and generate an appropriate and prolonged immune response (Roozendaal, Mebius et al. 2008). Lymph node stromal cells (LNSC) are solely responsible for creating this 3D structure and maintaining the homeostasis of the node by secreting homeostatic cytokines and survival factors necessary for adequate adaptive immune responses (Sixt, Kanazawa et al. 2005, Chang and Turley 2015). Despite this pivotal role in orchestrating a functional immune system, these cells are often overlooked. Yet, they are perfectly positioned to interact with pathogens as they traverse through the lymph node, suggesting these cells could play a substantial role during infections. LNSC are all $CD45^{Neg}$ and can be subdivided into four major subsets, lymphoid endothelial cells (LEC), fibroblastic reticular cells (FRC), blood endothelial cells (BEC), and double negative cells

(DNC), based on the surface expression of CD31 and podoplanin (gp38) (Fletcher, Malhotra et al. 2011, Koning and Mebius 2012).

A. Lymphoid Endothelial Cells

LEC, indentified by their expression of both CD31 and gp38, line the lymphatic vessels and sinuses within the node and are the first and last subset to interact with lymph-borne antigens and antigen-bearing cells. Consisting of two subsets, floor (fLEC) and ceiling (cLEC) LEC, these cells are responsible for maintaining SCS macrophages through the secretion of CSF-1 and RANK-L (Camara, Cordeiro et al. 2019, Mondor, Baratin et al. 2019) and facilitating the passage of dendritic cells (Jalkanen and Salmi 2020) and free antigen (<70 kilodaltons) (Gretz, Norbury et al. 2000, Rantakari, Auvinen et al. 2015) into the underlying parenchyma. In the medullary sinus, LEC also support neutrophil accumulation (Takeda, Hollmen et al. 2019) and lymphocyte egress (Fujimoto, He et al. 2020). Though research investigating the role of these cells during bacterial infections is limited, there is growing evidence suggesting that LEC may be an important growth niche for many viral pathogens (Gao, Deng et al. 2003, Fiorentini, Luganini et al. 2011, Bryant-Hudson, Chucair-Elliott et al. 2013, Choi, Park et al. 2020). Additionally, these cells were previously shown to produce a robust chemokine response against herpes simplex virus (Gregory, Walter et al. 2017), lymphocytic choriomeningitis virus (Rodda, Lu et al. 2018), and human cytomegalovirus (Fiorentini, Luganini et al. 2011). Thus, it is possible these cells may play a similar role during bacterial infections as well.

B. Fibroblastic Reticular Cells

$CD31^{-}gp38^{+}$ FRC are located in nearly every lymph node compartment and are the most abundant of the stromal subsets. These cells can be further divided based on their anatomical location or the primary cell type they interact with. For

example, T-cell zone FRC are primarily located in the cortex and are known to secrete chemokines that support T-cell survival and recruitment, such as CCL19 and CCL21 (Link, Vogt et al. 2007, Chai, Onder et al. 2013). In contrast, T-B border reticular cells support the accumulation of plasma B cells by secreting APRIL and CXCL12 (Huang, Rivas-Caicedo et al. 2018, Zhang, Tech et al. 2018). Other subsets identified using single-cell RNA sequencing include follicular dendritic cells located within B cell follicles, marginal zone reticular cells directly underlying the fLEC of the SCS, perivascular reticular cells that surround high endothelial venules, and medullary reticular cells found within the medullary sinus (Rodda, Lu et al. 2018, Kapoor, Muller et al. 2021). FRC are also the most well-studied stromal subset in the context of viral infections. These cells were previously shown to be a key target for several viral infections, such as Ebola and lymphocytic choriomeningitis virus in lymphoid tissues (Davis, Anderson et al. 1997, Mueller, Matloubian et al. 2007, Steele, Anderson et al. 2009, Ng, Nayak et al. 2012, Twenhafel, Mattix et al. 2013). Additionally, during helminth infections, FRC secrete survival factors that support B cell function and antibody production (Dubey, Lebon et al. 2016). However, like their stromal counterparts, their role during bacterial infections is unknown.

C. Blood Endothelial Cells

BEC, as their name suggests, form the inner lining of the vasculature within the lymph node and are commonly identified as $CD31^{+}gp38^{-}$ stromal cells. Previously, this subset was broadly categorized into high endothelial venule (HEV)-BEC and non-HEV BEC. However, recent single-cell RNA sequencing analysis revealed that these cells can be further divided into nine subsets, including arterial BEC, capillary BEC, venous BEC, and medullary BEC (Brulois, Rajaraman et al. 2020). However, the majority of research has focused only on BEC that line the HEV. HEV-BEC are responsible for facilitating the migration of lymphocytes into the node through the secretion of CCL21 and expression of adhesion molecules, GlyCAM-1, MadCAM-1, and ICAM-1 (Low, Hirakawa et al.

2018). Unlike LEC and FRC, the specific role of BEC in any infection remains largely unknown. Instead, studies have honed in on the involvement of BEC and HEV during leukocyte extravasation (Cahill, Frost et al. 1976, Bjerknes, Cheng et al. 1986, Bargatze and Butcher 1993, Butcher and Picker 1996).

D. Double Negative Cells

DNC, identified by their lack of any distinguishable marker, are the most understudied of the LNSC subsets and their location within the node is unknown. However, it is believed that these cells are functionally different from FRC. A previous study found that when both cell types were challenged with an inflammatory stimulus, the FRC cytokine and chemokine response significantly differed from DNC (Severino, Palomino et al. 2017). Additionally, this study demonstrated increased migration of dendritic cells towards FRC treated with TNF-α + IL-1β compared to similarly treated DNC. Apart from these observations, the functional characteristics of the DNC remain unknown and will require significant investigation.

1.14 Infection in the Lymph Node

Little is known about the early events of the *L. monocytogenes* infection within the MLN or how the bacteria trafficks within the node. However, previous studies have highlighted the importance of DC in T cell priming and the development of a robust CD8 T cell response to control the infection. For example, it was previously shown that *L. monocytogenes* induces a Th1 response following oral infection, implicating the involvement of $CD11b^{-}CD103^{+}$ DC in T cell priming within the MLN (Romagnoli, Fu et al. 2017). Additionally, multiple studies have demonstrated that the *L. monocytogenes*-specific CD8 T cell response reaches its peak at approximately eight days post-infection, further suggesting the involvement of DC to some extent during the infection process (Pope, Kim et al. 2001, Sheridan, Pham et al. 2014). Apart from their role in priming CD8 T cells,

DC have also been implicated in contributing to the bacterial burden within the MLN. Imperato et al. recently showed that there is a significant decrease in the *L. monocytogenes* burden within the MLN of Batf3$^{-/-}$ animals compared to WT animals following foodborne infection, suggesting that dendritic cells are necessary for the colonization of the MLN (Imperato, Xu et al. 2020). Surprisingly, we previously showed that intracellular replication of *L. monocytogenes* was also required for colonization of the MLN and the subsequent spread to the spleen and liver, but that this growth niche was not a CD103^{+} or CD103^{-} DC (Jones, Bussell et al. 2015, Jones, Smith et al. 2017). This indicates the possibility that there is another critical cellular niche within the MLN that is required for *L. monocytogenes* survival, though to date no study has identified any cell type within the MLN that could serve this role.

V. Systemic Spread of *L. monocytogenes*

L. monocytogenes causes severe life-threatening systemic diseases in susceptible individuals. However, our knowledge of the mechanisms employed by *L. monocytogenes* that allow entrance into the blood is limited. In this section, I will outline the two possible routes *L. monocytogenes* may use to disseminate beyond the gut.

1.15 Spread Through the Portal Vein

It was previously shown that *L. monocytogenes* clones could rapidly seed the liver without infecting the spleen following the oral infection of guinea pigs (Melton-Witt, Rafelski et al. 2012). This suggests that there is an alternative route of dissemination from the gut to the liver. The portal vein drains blood from the gastrointestinal tract, spleen, and gallbladder directly into the liver, constituting about 75% of the liver's blood flow (Corness, McHugh et al. 2006). It is also known that antigens and microbial products, such as LPS, from the intestines are continuously delivered to the liver (Crispe 2009). Therefore, it is possible *L.*

monocytogenes could directly invade venous capillaries within the intestine and spread systemically. However, this route of systemic spread is thought to only occur if the infectious dose is overwhelmingly large or when there is trauma to the esophagus during intragastric inoculation, as we do not see such early systemic spread in our natural feeding model when doses do not exceed 10^9 CFU (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015).

1.16 Spread Through the Lymphatics

The second pathway, likely the predominant route of systemic spread during human listeriosis, is through the MLN. This dissemination from the MLN might occur through the efferent vessels, where the bacteria could either float through freely or associate with an exiting migratory cell. Efferent vessels drain directly into the thoracic duct, the largest lymphatic channel that connects to the left subclavian and left internal jugular veins (Null, Arbor et al. 2023). Consequently, this would provide *L. monocytogenes* direct access to the blood and peripheral organs. Alternatively, *L. monocytogenes* could directly invade the capillaries within the node. The MLN is vascularized by a single arteriole that enters the medulla and branches into capillary beds and specialized postcapillary venules like HEV (Benahmed, Ely et al. 2012). Thus, there is also a possibility that *L. monocytogenes* could enter the circulation through this mechanism.

VI. Overall Hypothesis

My dissertation aims to identify key events in the MLN that result in the systemic spread of *L. monocytogenes*. The MLN acts as the final barrier in the gut intended to prevent both commensal and pathogenic bacteria from entering the bloodstream. However, the MLN phase of infection is significantly understudied. We previously showed that intracellular replication of *L. monocytogenes* was necessary for efficient colonization of the MLN and the subsequent spread to peripheral organs (Jones, Bussell et al. 2015). This finding led to the hypothesis that this critical growth niche is likely a cell type within the MLN that would allow *L. monocytogenes* easy access to the blood.

To determine why intracellular replication was required for colonization of the MLN and identify this critical cellular niche, I performed the following studies to:

Chapter 3: investigate why intracellular replication of *L. monocytogenes* was critical for colonization of the MLN

Chapter 4: determine if resident and migratory lamina propria macrophages serve as an intracellular growth niche for L. monocytogenes

Chapter 5: determine if lymph node stromal cells serve as an intracellular growth niche for L. monocytogenes

Chapter 6: characterize the mechanism of systemic spread of L. monocytogenes and the involvement of lymph node stromal cells in this dissemination

FIGURES

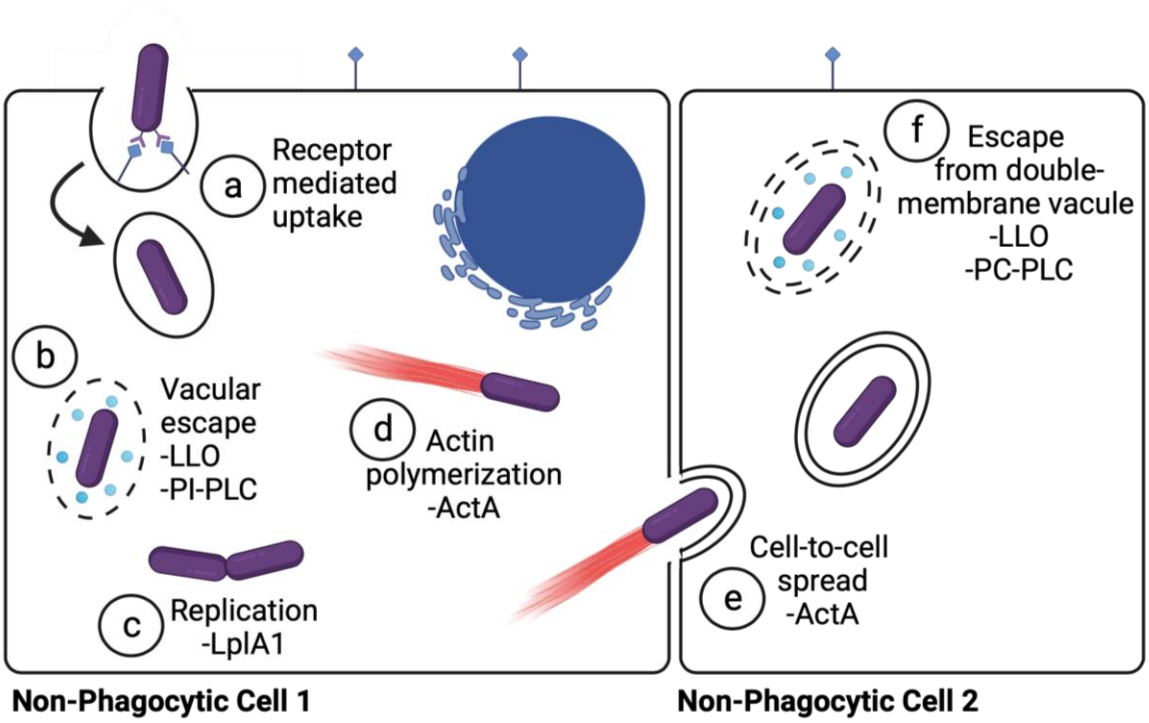

Figure 1.1: Intracellular lifecycle of *L. monocytogenes*. (a) *L. monocytogenes* invades non-phagocytic cells using receptor-mediated uptake. (b) The bacteria escape the vacuole before lysosomal fusion by by secreting the pore-forming toxin, listeriolysin O (LLO), and phosphatidylinositide phospholipase (PI-PLC). (c) Once in the cytosol, *L. monocytogenes* replicates, (d) polymerizes host actin to form actin tails that propel the bacteria forward and (e) promotes cell-to-cell spread. Escape from the double-membraned vacuole in the neighboring cell is mediated by the secretion of LLO and phosphatidylcholine phospholipase (PC-PLC). Image was generated with BioRender.

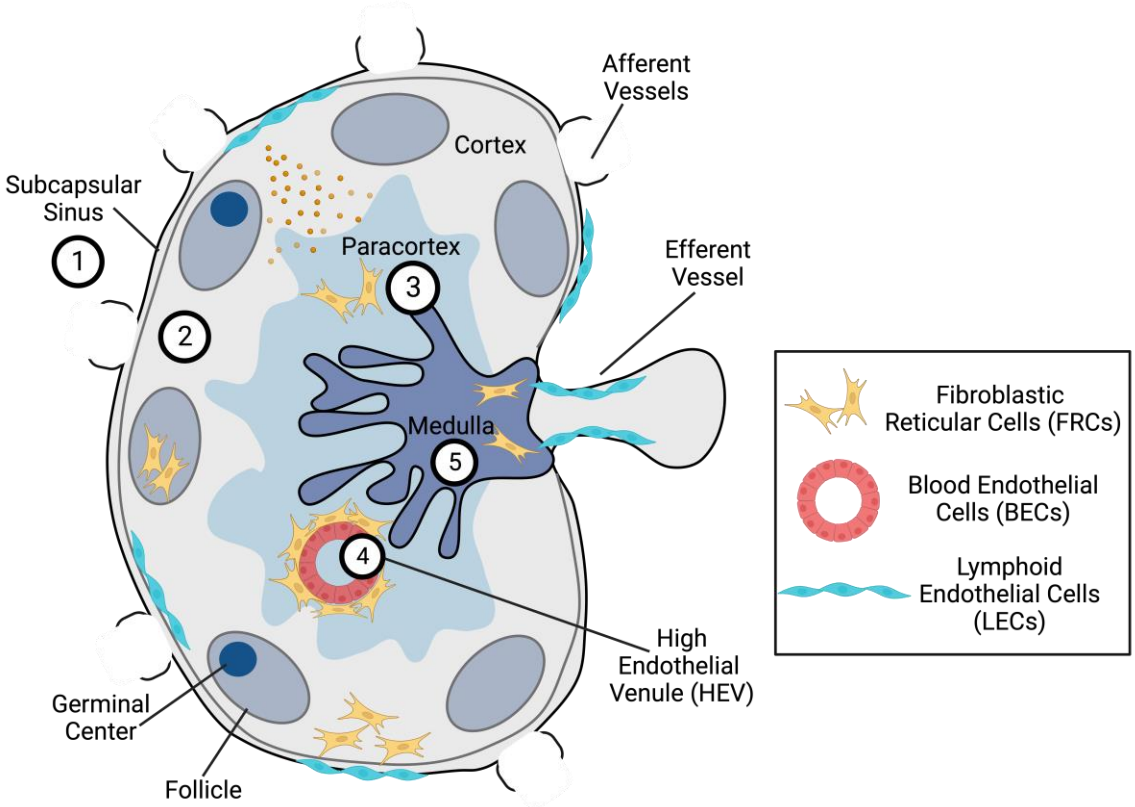

Figure 1.2: Architecture of the lymph node. The lymph node is compartmentalized by stromal subsets. 1) The afferent lymphatic vessels drain into the subcapsular sinus. LEC line all lymphatic vessels and sinuses and distribute incoming antigen across the node. 2) FRC are located below the subcaspsular sinus and form the B cell follicles and cortex of the lymph node. 3) In the paracortex, FRC guide T cells and dendritic cells by secreting CCL19 and CCL21. 4) HEV are located throughout the paracortex, and facilitate immune cell entry via the expression of adhesion molecules and are composed of an outer layer of perivascular FRC and an inner layer of BEC. 5) In the medulla, where the lymph is collected before exiting through the efferent vessels, FRC and LEC produce chemokines to maintain immune cells. Image was generated with BioRender.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacteria

All *L. monocytogenes* strains used in this study are listed in Table 2.1. *L. monocytogenes* were cultured in either Improved Minimal Media (IMM) (Phan-Thanh and Gormon 1997) or Brain Heart Infusion (BHI) broth (Difco). IMM was prepared from concentrated stocks and then used within two weeks. Each strain was grown to stationary phase at either 30°C (for *in vivo* infection of mice) or 37°C (for *in vitro* assays) and then aliquots were prepared, stored at -80°C, and thawed prior to use as described (Jones and D'Orazio 2013). Variants of the integrative plasmid pIMC3 (Monk, Casey et al. 2008) were used to complement the deletions and confer IPTG-induced (1 mM final concentration) resistance to either kanamycin (Kan) (50 µg/mL) or erythromycin (Ery) (5 µg/mL).

2.2 Construction of Bacterial Strains

The mouse-adapted, $InlA^m$-expressing variant of EGDe (strain SD2000) was described previously (Jones, Bussell et al. 2015). *ΔlplA1 L. monocytogenes* (strain SD2301, kanamycin-resistant) and the complemented *lplA1+* strain (SD2302, Ery^R) were previously derived from *L. monocytogenes* SD2000 and validated (Jones, Bussell et al. 2015). PAM300 *ΔactA,* also derived from *L. monocytogenes* EGDe, was provided by Jose Vazquez-Boland (The University of Edinburgh) and then used to generate the mouse-adapted ($InlA^m$-expressing) variant, strain SD2151, by allelic exchange using pTM2 (Jones, Bussell et al. 2015). To complement the *ΔactA* mutation, a 2.4-kb DNA fragment flanking *actA* was amplified from *L. monocytogenes* SD2000 chromosomal DNA using Platinum SuperFi II Mastermix (Invitrogen) using the following primers: Forward, 5'-CAGGAATTGGGGATCGCTTCCACTCACAGAG-3', Reverse, 5'-CAAAGCATAATGGAGCTCCATACCTAGAACCACC-3'. The resulting PCR

product was subcloned into pIMC3Kan to generate plasmid pTA01 and electroporated into PAM300-InlA^{m}*ΔactA* to generate the kanamycin-resistant strain SD2154. The empty vector pIMC3Ery was electroporated into PAM300-InlA^{m}*ΔactA* to generate the erythromycin-resistant strain SD2152. All chromosomal manipulations were confirmed by whole genome sequencing (Illumina) with variant calling (MiGS, Pittsburgh). Whole genome sequencing revealed an additional single nucleotide polymorphism (I42T) in the PAM300-InlA^{m}*ΔactA plcB* gene. Cell-to-cell spread, which requires a functional PlcB protein (Schluter, Domann et al. 1998) was not altered compared to wildtype EGDe *L. monocytogenes* as determined by plaque assay (see Fig. 3.9). The constitutively GFP expressing variant, SD2710 and control strain with empty vector pPL2 integrated, SD2001, were also previously derived from *L. monocytogenes* SD2000 and validated (Jones, Bussell et al. 2015).

2.3 Lipoate Starvation

To ensure that *L. monocytogenes* had depleted all intracellular reserves of lipoate, bacteria were first grown overnight in 50 mL of BHI. The stationary phase cultures were centrifuged at 5,000 *x g* for 10 minutes, washed once with phosphate-buffered saline (PBS; Life Technologies cat. # 14190-144), suspended in 1 mL of IMM lacking lipoate (Keeney, Stuckey et al. 2007), and inoculated into 200 mL of IMM media lacking lipoate. The culture was incubated for 16-24 hours with orbital shaking at either 30˚C or 37˚C, and growth was monitored using optical density at 600 nm (OD_{600}). Once the bacteria reached stationary phase, aliquots were prepared as previously described (Bou Ghanem, Myers-Morales et al. 2013).

2.4 Mice

Four-week-old female BALBc/By/J (BALB) mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in a specific pathogen-free (SPF) facility at The University of Kentucky. For foodborne infections, the mice were adapted to an ASBL-2 room with a 14-hour light cycle (7 PM to 9 AM) for at least two weeks and then used in experiments when they were between 6 to 10 weeks old. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. For depletion of neutrophils and monocytes, mice were given intraperitoneal (i.p) injections of 200 µg Ultra-LEAF purified anti-mouse Ly-6G/Ly-6C GR1 antibodies (Biolegend; clone RB6-8C5) one day before and one day after *L. monocytogenes* infection. Control mice received i.p injections of 200 µg Ultra-LEAF purified rat IgG2b (Biolegend; clone KTK-4530). For *in vivo* gentamicin treatment, mice were given an i.p injection of gentamicin (40 mg/kg diluted in PBS, total volume of 200 µL) every 8 hours (starting 30 min post-infection in Fig. 3.7 and 45 h post-infection in Fig. 6.4), until tissues were harvested; control mice received PBS alone. This dosing regimen was based on the pharmacokinetics of gentamicin clearance in mice reported in Onyeji et al. (Onyeji, Nicolau et al. 2000) and predicts a Cmax of 74 mg/L and a half-life of 0.6 hours.

2.5 Foodborne and Intravenous Infection of Mice

Mice were infected using the natural feeding model as described (Bou Ghanem, Myers-Morales et al. 2013, Bou Ghanem, Myers-Morales et al. 2013). Briefly, mice were placed in cages with raised wire floors for the duration of the experiment to prevent coprophagy and food was withheld for 16-20 hours. Aliquots of *L. monocytogenes* were thawed in either BHI or IMM for 1.5 hours at 30°C. Bacteria suspended in PBS were used to saturate 3 mm bread pieces (Kroger), followed by the addition of 2 µl of melted salted butter (Kroger). For co-infections,

two strains of *L. monocytogenes* were mixed and added to a single bread piece at the same volume. Mice were fed the *L. monocytogenes*-contaminated bread at the beginning of their dark cycle, and chow was returned immediately after infection. For i.v. infections, *L. monocytogenes* were aseptically injected into the lateral tail vein in a total volume of 200 µl.

2.6 Bacterial Loads in Tissue Homogenates

Ileums (the terminal third of the small intestine) and colons were harvested aseptically, the intestinal contents were expelled and then the tissues were flushed with 8 mL of sterile PBS (Bou Ghanem, Myers-Morales et al. 2013). Tissues were cut longitudinally with sterile microdissection scissors, cut laterally into smaller fragments, and homogenized (VWR® 200 homogenizer at setting 5) for 1 minute in 2 mL of sterile water. Spleens and livers were harvested aseptically and homogenized in 5 mL of sterile water for 30 seconds on setting 4. Serial dilutions of tissue homogenates were prepared, plated on BHI agar (Difco) plates with appropriate antibiotics, and incubated at 37°C for 24 hours. For co-infections, competitive index ratios were determined by dividing the number of CFU recovered for the mutant strain by the number of CFU recovered for the wild type (complemented) strain. If no CFU were recovered for one of the strains, the limit of detection for that tissue was used for calculations; if no bacteria of either strain were detected, the mouse was not included in the competitive index (CI) graph.

2.7 Isolation of Intestinal Lamina Propria Cells

Myeloid cells were isolated from the lamina propria as previously described (Koscso and Bogunovic 2016). The small and large intestine (ileum and colon) was cut into three pieces, stool was removed using forceps, and small scissors were used to make a longitudinal incision to expose the lumen. Mucus was removed by

shaking the tissue in complete Hanks' Balanced Salt Solution (HBSS) (Ca^{2+}/Mg^{2+}-free HBSS supplemented with 2% FBS), and epithelial cells were removed by three incubations in complete HBSS with dithiothreitol (20 min.) followed by EDTA (40 min.) at 37°C. The remaining tissue was cut into 1-2 mm pieces using small scissors and then digested in 8 ml RPMI supplemented with 2% FBS, collagenase type IV (840 U/ml) and DNAse (120 U/ml) in a 6-well plate for 1 hour at 37°C/7% CO_2. Finally, any undigested tissue was homogenized by repeatedly passing it through an 18-G needle attached to a 5 ml syringe until completely dissociated (Koscso and Bogunovic 2016).

2.8 Enzymatic Digestion of Lymph Nodes

Mesenteric lymph nodes that drain the small intestine (sMLN) and colon (cMLN) were aseptically harvested as described (Houston, Cerovic et al. 2016), cut into small pieces using a sterile scalpel, and placed into 4 mL of RPMI 1640 media (Life Technologies cat. # 21870-084) supplemented with 20 μM HEPES and 5% fetal bovine serum (FBS). Collagenase IV (300 U/mL; Worthington) and DNase I (120 U/mL; Worthington) were added and the samples were incubated for 30 minutes at 37°C with orbital shaking (250 RPM) in a 50 mL conical tube containing a sterile 2 cm stir bar. A portion of each sample was removed for CFU determination, and the remainder was filtered through a nylon cell strainer (VWR; 40 μm pore size) and stained for flow cytometry.

For stromal cell isolation, the MLN or peripheral lymph nodes (PLN, axillary, brachial and inguinal) were harvested. Lymph nodes were pierced using a sterile 25G needle and placed in 2 mL of freshly made media consisting of RPMI 1640 supplemented with 0.6 mg/mL Dispase II (0.9 U/mg; Roche), 0.2 mg/mL Collagenase P (1.9 U/mg; Roche), and 0.1 mg DNase I (120 U/mL; Worthington) and digested as previously described (Fletcher, Malhotra et al. 2011). Following digestion, cells were filtered through a nylon cell strainer and prepared for cell sorting or stained for flow cytometry.

2.9 Antibodies and Flow Cytometry

Prior to antibody staining, cell number and viability were assessed by trypan blue staining. Single cell suspensions were incubated with fluorescently conjugated monoclonal antibodies specific for the following cell surface markers: CD3 (clone 145-2C11), CD11b (clone M1/70), and CD19 (clone 1D3) from eBioscience; CD16/32 (Fc Block; clone 93), Ly6C (clone HK 1.4), Ly6G (clone 1A8), F4/80 (clone BM8), CD11c (clone N418), CD103 (clone 2P7), MHC II (clone M5/114.15.2), CD45 (clone 30-F11), CD31 (clone 390), and podoplanin (clone PMab-1) from BioLegend. Cells were fixed with 10% neutral buffered formalin (VWR cat. #16004-128) prior to analysis. Flow cytometry data were acquired using either a LSRII cytometer (BD Biosciences) or a FACSymphony (BD Biosciences) and analyzed using FlowJo software (Version 10). The percentage of *Listeria*-associated (GFP+) cells (shown in Fig. 4.9) in each population was determined by using cells from mice infected with *L. monocytogenes* SD2001 as a negative gating control as described previously (Jones, Bussell et al. 2015, Jones and D'Orazio 2017, Jones, Smith et al. 2017).

2.10 Cell Sorting

Due to the low yield of LNSC, cells pooled from three animals were used for each experiment. Prior to sorting, $CD45^{neg}$ cells were enriched using magnetic bead separation. MLN cells were incubated with a R-Phycoerythrin (PE)-conjugated anti-CD45 antibody for 30 minutes on ice. Cells were washed with cold buffer and then incubated with anti-PE magnetic particles (BD Biosciences cat. #557899) for 30 minutes at 4°C. $CD45^{neg}$ cells were enriched by placing a tube containing the stained cells against a magnet for 8 minutes and collecting the supernatant three times. The cells were then further stained and subsequently purified using an iCyt Sorter (Sony). Sorted stromal populations were suspended

in Minimal Essential Media (cat. # 10370-021; Gibco) supplemented with 10% FBS.

2.11 Infection of LNSC *ex vivo*

Sorted cells were counted and viability was assessed prior to seeding in a half-area 96-well flat bottom dishes (Corning) (3×10^4/well). Cells were left to recover for at least 30 minutes at 37°C in 7% CO_2 prior to infection. Following the recovery period, cells were infected at an MOI of 10. In each experiment, the plated cells were used to analyze infection at two time points; no technical replicates were plated.

2.12 Antibiotic Pre-Treatment for Dysbiosis

Mice were given the following antibiotics orally (direct injection into the mouth using a syringe) or via i.p injections: a single oral dose of 20 mg streptomycin (OmniPur cat. #3810-74-0) suspended in PBS, a single dose of an antibiotic cocktail (MNVC) consisting of 0.7 mg metronidazole (Sigma Aldrich cat. #M1547), 3.5 mg neomycin (Sigma Aldrich cat. #N5285), 3.5 mg vancomycin (Sigma Aldrich cat. #V2002) orally and 0.2 mg clindamycin (Sigma Aldrich cat. #C5269), or i.p injections 0.2 mg clindamycin suspended in PBS. Before mice were fed *L. monocytogenes*-contaminated bread pieces, fecal samples were collected from mice to verify the disruption of the gut microbiota (less diversity with an Enterobacteriaceae “bloom” visible in an aerobic culture). Fecal pellets were weighed and suspended at 150 mg/mL in sterile water. Serial dilutions were prepared and plated on BHI agar.

2.13 Lipoate Assay

Frozen aliquots of lipoate-starved *L. monocytogenes* were thawed and cultured in IMM lacking lipoate at 37˚C for 1.5 hours. The MLN, ileum (the terminal third of the small intestine) and colon were harvested aseptically, and the intestinal contents were expelled and then the intestinal tissue were flushed with 8 mL of sterile PBS (Bou Ghanem, Myers-Morales et al. 2013). Tissues were harvested from an uninfected mouse and homogenized in 2 mL of IMM media lacking lipoate using a sterile glass tissue grinder. Homogenates were added to a 96-well round bottom plate (150 µL/well) and inoculated with 2 x 10^2 CFU of *L. monocytogenes* suspended in 15 µL of IMM (-). For control wells, lipoic acid (Sigma Aldrich cat. #T5625) suspended in IMM was added at a final concentration of 2.5 nM. Plates were incubated at 37°C with orbital shaking (200 RPM), and bacterial growth was monitored both by measuring OD_{600} and by plating serial dilutions of *L. monocytogenes* on the BHI agar.

2.14 qRT-PCR

Frozen aliquots of lipoate-starved *L. monocytogenes* were thawed and cultured in IMM(-) at 37˚C for 1.5 hours. *L. monocytogenes* were suspended in 5 mL of Listeria synthetic medium (iLSM) as previously described (Whiteley, Garelis et al. 2017) that was supplemented with reduced glutathione (Sigma cat. #G6013) (iLSM-GSH) (Portman, Dubensky et al. 2017) and incubated at 37°C. At indicated time points, samples were suspended in 300 µL of TRIzol Reagent (Invitrogen). RNA was isolated from samples following the manufacturer's guidelines and then cDNA was prepared from 500 ng of isolated RNA using the Superscript IV First-Strand Synthesis System (Invitrogen). cDNA (10 ng), 2X SYBR green (Applied Biosystems), and forward and reverse primers (10 µM) were added to a MicroAMP Fast 96-well reaction plate (Applied biosystems) in triplicate. Products were amplified using a StepOnePlus™ Real-Time PCR System (Applied Biosystems).

To determine induced *actA* transcript levels, samples were normalized to the 1-hour time point. Fold changes were determined using the 2–ΔΔCT method, as previously described (Livak and Schmittgen 2001).

2.15 *In vitro* Cell Culture

To generate macrophages, femurs were asceptically harvested from BALB mice and bone marrow cells were suspended in BMM-10 media (Dulbecco's Modified Eagle Medium (DMEM) (cat. # 11960-051; gibco) supplemented with 2.5 mM L-glutamine, 10% FBS, and 10% L929 conditioned medium in 24-well dishes (Corning) that contained sterile round coverslips (12 mm diameter) and incubated at 37˚C in 7% CO_2. Cells were used on day 6 of culture when cells had differentiated into bone marrow-derived macrophages (BMDM) and coverslips were 80-90% confluent (~5 x 10^5 cells/coverslip). L2 fibroblasts were suspended in L2 media (DMEM supplemented with 2.5 mM L-glutamine, 10% FBS, and penicillin/streptomycin) in 100 mm dishes (Corning) and incubated at 37˚C in 7% CO_2. SVEC4-10 cells, a transformed murine blood endothelial cell line (ATCC, cat #CRL-2181) were suspended in SVEC media (DMEM supplemented with 5 mM L-glutamine, 10% FBS, and penicillin/streptomycin) in 100 mm dishes and incubated at 37˚C in 7% CO_2 (O'Connell and Edidin 1990). Caco-2 cells were suspended in Caco-2 media (Minimum Essential Medium (MEM) (cat. # 10370-021; gibco) supplemented with 2.5 mM L-glutamine, 10% FBS, 2.5mM sodium pyruvate and penicillin/streptomycin) in 100 mm dishes and incubated at 37˚C in 7% CO_2.

2.16 BMDM Infection

BMDM were infected with lipoate-starved SD2301 and SD2302 *L. monocytogenes* at an MOI=0.1 and plates were centrifuged for 5 minutes at 300 *x g* to synchronize infection. At one-hour post-infection, cells were washed three

times with pre-warmed PBS (37°C), suspended in media containing 15 µg/mL gentamicin, and incubated at 37°C in 7% CO_2 for 15 hours. Following incubation, cells were fixed with 10% neutral buffered formalin for 30 minutes and stained for microscopy.

2.17 Intracellular Growth Assay

SVEC4-10 cells were seeded at 1 × 10^5/well in 24-well dishes (Corning). Primary stromal cells were seeded at 3 × 10^4cells/well in half area 96-well flat bottom dishes (Corning, cat. #3696). Aliquots of *L. monocytogenes* were thawed, incubated shaking at 37°C in BHI broth for 1.5 h, washed once in PBS (gibco cat. #14190-144), and then used to infect cells at a multiplicity of infection (MOI) of 10. Plates were centrifuged for 5 min at 300 *x g* to synchronize infection. Extracellular bacteria were removed 1 h later by washing cells 3 times with pre-warmed (37˚C) PBS and then suspended in media containing 10 µg/ml gentamicin for at least 20 min. At each timepoint, cells were washed once and either sterile water was added to the wells with vigorous pipetting up and down or coverslips were harvested and placed in sterile H_2O and then vortexed. Following cell lysis, serial dilutions were prepared and plated on BHI agar. For analysis of invasion efficiency, the percentage of each inoculum internalized at 1 hour post-infection was calculated by dividing the number of CFU recovered at each time point by the total number of CFU added to each well (% invasion).

2.18 Microscopy

For phallodin (F-actin) staining, cells were fixed with 10% neutral buffered formalin for 30 minutes. Fixed cells were washed three times with PBS then permeabilized using Tris-buffered saline (TBS) supplemented with 1% bovine serum albumin (BSA; ThermoFisher) and 0.1% Triton X-100 (Sigma) for 30

minutes at room temperature. Permeabilized cells were incubated with Listeria O Antiserum Poly (Invitrogen) diluted 1:100 in TBS+BSA and goat anti-rabbit IgG-Texas Red-X phalloidin (Thermo Fisher) diluted 1:100 in TBS+BSA for 30 minutes on ice. The cells were washed eight times in TBS-TX (TBS supplemented with 0.1% Triton X-100) and then incubated for 45 minutes on ice with a secondary goat anti-rabbit IgG-conjugated to Alexa 488 (Invitrogen) diluted 1:200 in TBS+BSA. Cells were washed eight times in TBS-TX, followed by eight washes with TBS alone and mounted under coverslips with ProLong Diamond antifade with DAPI.

For differential “in/out” staining cells were washed three times with cold buffer (Ca^{2+}/Mg^{2+}-free HBSS/1% FBS/1 mM EDTA) and then incubated for 20 min on ice with polyclonal anti-*Listeria* antibodies (BD Difco cat. # 223021) that were diluted 1:100 in PBS containing 3% bovine serum albumin (BSA; ThermoFisher # A11037). Following incubation, the cells were washed with cold buffer and then incubated with goat anti-rabbit IgG–Texas Red diluted 1:200 in PBS + BSA for 20 min on ice. Air-dried slides were fixed with formalin for 10 min at 4°C, washed with PBS, and mounted under coverslips with ProLong Diamond antifade (Molecular Probes). Following staining, cells were visualized using a Nikon A1R Confocal System with a 100X oil immersion objective and analyzed with Nikon NIS Elements AR software (version 4.50).

2.19 Plaque Assay

Plaque assays were performed as previously described (Marquis 2006). L2 fibroblasts and SVEC4-10 cells were grown to confluency overnight in a 6-well plate (~1 x 10^6 cells/well) at 37˚C in 7% CO_2. Cells were washed with warm PBS and infected with either *L. monocytogenes* grown in BHI or in IMM lacking lipoate at multiple dilutions (1:10, 1:100, or 1:1000) and the plates were centrifuged for 5 minutes at 300 *x g* to synchronize infection. One hour later, cells were washed three times with warm PBS, and suspended in medium containing 0.7% agarose and 10 µg/ml gentamicin. Following a four-day incubation at 37˚C/7% CO_2, each

well was overlayed with medium containing 0.7% agarose and 0.5% neutral red and then incubated for 6-8 hours at 37˚C/7% CO_2. Plaques were photographed after incubation and measured with a digital ruler (Adobe Photoshop). All assays were carried out in duplicate with strain *Lm* SD2000 as the positive control. To determine plaque size, the following equation was used: Plaque size = Surface area of mutant strain ÷ Surface area of reference strain.

2.20 Multiplex Immunoassay

Supernatants were collected after each intracellular growth assay and stored at -80˚C until used for subsequent Luminex assays. A custom mouse 32-plex Procartaplex kit was purchased from Life Technologies and used according to manufacturer's recommendations. The plate was read using a Luminex 200 and data was analyzed using the ProcartaPlex analysis App (ThermoFisher).

2.21 LDH Assay

SVEC4-10 and primary LNSC supernantents collected from intracellular growth assays described above were used to determine cytotoxicity. LDH release was assessed using CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen). For positive control wells, one hour prior to the indicated timepoints, 50 µL of media was removed from each well and replaced with an equal amount of lysis buffer. After incubation, one-tenth volume (50 µL) of the supernatant was collected, mixed with 50 µL of reaction mix and added to a 96-well flat-bottom plate (Corning). The plate was vortexed gently and incubated at room temperature in the dark for 30 min. Stop solution was added to each well and the absorbance (490 and 680 nm) was measured using a microplate reader. To determine LDH activity, the OD_{490} was subtracted from the OD_{680}. Percent cytotoxicity was calculated using the following equation:

$$\% \text{ Cytotoxicity} = \frac{\text{infected sample LDH activity} - \text{negative sample LDH activity}}{\text{Max lysis} - \text{infected sample LDH activity}}$$

2.22 Statistics

Statistical analyses were performed using Prism for Macintosh (version 9; GraphPad). The specific tests used for each experiment are indicated in the figure legends. P values of < 0.05 were considered significant and are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Table 2.1: Plasmids, strains and primers

	Description	Antibiotic Resistance[1]	Source/ Reference
Plasmids			
pIMC3ery	Site-specific integration vector with IPTG-induced expression of Ery	Cm,Ery	(Monk et al. 2008)
pIMC3kan	Site-specific integration vector with IPTG-induced expression of Kan	Cm,Kan	(Monk et al. 2008)
pGJ-cGFP	P*help* from pIMC3 subcloned into SacI/EagI-digested pAD_1-cGFP (constitutive expression of GFP)	Cm	(Jones et al., 2015)
pTA01	a 2.4-kb DNA fragment flanking *actA of Lm SD2000* subcloned into pIMC3kan	Cm,Kan	(Tucker et al., 2023)
pTM2	InlAm subcloned into PstI/BamHI-pKSV7 (temperature sensitive shuttle vector)	Cm,Ery	(Jones et al., 2015)
pTML1	lplA1 plus 988 bp of upstream DNA from *Lm* $InlA^m$ in SacI/PstI- digested pIMC3ery	Cm,Ery	(Jones et al., 2015)
Bacterial Strains			
Lm SD2000	*Lm* EGDe-CG *ΔinlA*::pTM2 ($InlA^m$)	None	(Jones et al., 2015)
Lm SD2001	*Lm SD2000*::pIMC3*kan*	Cm,Kan	(Jones et al., 2015)
Lm SD2301	*ΔlplA1 SD2000*::pIMC3*kan*	Cm,Kan	(Jones et al., 2015)
Lm SD2302	*ΔlplA1 SD2000*::pTML1 *(+ lplA1)*	Cm,Ery	(Jones et al., 2015)
Lm SD2151	*ΔactA PAM300*::pTM2 ($InlA^m$)	Cm,Ery	(Tucker et al., 2023)
Lm SD2154	*Lm SD2151*:: pTA01 *(+ actA)*	Cm,Kan	(Tucker et al., 2023)
Lm SD2710	*Lm SD2000*::pGJ-cGFP	Cm	(Jones et al., 2015)
PAM300	*Lm* EGDe *ΔactA*	None	J. Vazquez-Boland-U. of Edinburgh
Lm SD2801	*Lm* EGDe-CG *ΔInlA*::pIMC3*kan*	Kan	Grant Jones
Lm SD3003	*Lm* EGDe-CG *ΔInlAInlB*::pIMC*3ery*	Ery	Tanya Meyers
Primers	**Sequence (5'-3')**		
actA FWD	5'-CAGGAATTGGGGATCGCTTCCACTCACAGAG-3'		
actA REV	5'-CAAAGCATAATGGAGCTCCATACCTAGAACCACC-3'		

[1]Cm, chloramphenicol; Ery, erythromycin; Kan, kanamycin

CHAPTER 3. EGRESS OF LISTERIA MONOCYTOGENES FROM MESENTERIC LYMPH NODES DEPENDS ON INTRACELLULAR REPLICATION AND CELL-TO-CELL SPREAD

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I. Summary

The mesenteric lymph nodes (MLN) function as a barrier to systemic spread for both commensal and pathogenic bacteria in the gut. *Listeria monocytogenes*, a facultative intracellular foodborne pathogen, readily overcomes this barrier and spreads into the bloodstream, causing life-threatening systemic infections. We show here that intracellular replication protected *L. monocytogenes* from clearance by monocytes and neutrophils and promoted colonization of the small intestine-draining MLN (sMLN) but was not required for dissemination to the colon-draining MLN (cMLN). Intestinal tissue had enough free lipoate to support LplA2-dependent extracellular growth of *L. monocytogenes*, but exogenous lipoate in the MLN was severely limited, and so the bacteria could replicate only inside cells, where they used LplA1 to scavenge lipoate from host peptides. When foodborne infection was manipulated to allow *ΔlplA1 L. monocytogenes* to colonize the MLN to the same extent as wild-type bacteria, the mutant was still never recovered in the spleen or liver of any animal. We found that intracellular replication in the MLN promoted actin-based motility and cell-to-cell spread of *L. monocytogenes* and that rapid efficient exit from the MLN was *actA* dependent. We conclude that intracellular replication of *L. monocytogenes* in intestinal tissues is not essential and serves primarily to amplify bacterial burdens above a critical threshold needed to efficiently colonize the cMLN. In contrast, intracellular replication in the MLN is

absolutely required for further systemic spread and serves primarily to promote ActA-mediated cell-to-cell spread.

II. Introduction

Listeria monocytogenes are highly adaptive Gram-positive bacteria that can readily switch from a saprophytic lifecycle in the environment to intracellular growth in the cytosol of eukaryotic cells. *L. monocytogenes* are typically transmitted to humans through the consumption of contaminated ready-to-eat food products with disease severity ranging from mild, self-limiting gastroenteritis to more severe infections of the bloodstream, brain, and placenta (Bartt 2000, Ooi and Lorber 2005, Charlier, Perrodeau et al. 2017). Immunocompromised individuals have a higher risk for developing the life-threatening systemic infections, and account for the high mortality rates (20 to 30%) associated with recent outbreaks of listeriosis (McCollum, Cronquist et al. 2013, Smith, Tau et al. 2019).

Foodborne *L. monocytogenes* must overcome multiple bottlenecks in the gut to enter the bloodstream. Most of an oral inoculum is either killed in the stomach (Brandl, Plitas et al. 2007) or shed in the feces of experimentally infected mice (Hardy, Margolis et al. 2006, Bou Ghanem, Jones et al. 2012). The small percentage of *L. monocytogenes* that survive passage through the stomach and duodenum are presumed to undergo transcriptional modifications induced by exposure to low pH and high osmolarity (O'Driscoll, Gahan et al. 1996, Saklani-Jusforgues, Fontan et al. 2000, Wemekamp-Kamphuis, Wouters et al. 2002, Toledo-Arana, Dussurget et al. 2009). Despite this adaptation to the gut environment, the frequency of epithelial invasion is still thought to be relatively low. For example, it was estimated that only 1 in 10^6 *L. monocytogenes* in the intestinal lumen crossed the mucosal barrier in a guinea pig model of oral infection (Melton-Witt, Rafelski et al. 2012). Once in the intestinal lamina propria, the bacteria can re-invade epithelial cells from the basolateral side (Gaillard and Finlay 1996), interact with infiltrating immune cells (Jones and D'Orazio 2017, Jones, Smith et al. 2017) or transit to the mesenteric lymph nodes (MLN), secondary lymphoid

tissues that are critical for immunosurveillance and the induction of adaptive immune responses. The MLN also function as an anatomical barrier that prevents both commensal bacteria and orally acquired pathogens from accessing peripheral organs (Macpherson and Smith 2006). *L. monocytogenes* use an unknown mechanism to overcome this barrier with at least some portion of orally-acquired bacteria entering the bloodstream to seed the spleen and liver by 48 hours post-infection. (Bou Ghanem, Jones et al. 2012). In guinea pigs infected with signature-tagged *L. monocytogenes,* only 1 in every 100 to 1,000 bacteria passed through this bottleneck (Melton-Witt, Rafelski et al. 2012).

L. monocytogenes readily invade a variety of different mammalian cell types by either triggering phagocytosis (Drevets, Canono et al. 1992) or using the "zipper" mechanism of specific receptor-ligand binding (Lecuit, Ohayon et al. 1997). Once internalized, *L. monocytogenes* quickly escape from the vacuole by secreting a cholesterol-dependent cytolysin and a phosphoinositide-specific phospholipase (Gaillard, Berche et al. 1987, Marquis, Doshi et al. 1995, Marquis, Goldfine et al. 1997, Gedde, Higgins et al. 2000). In the cytosol, *L. monocytogenes* quickly begin to replicate, and within hours after vacuolar escape, the surface protein ActA mediates polymerization of host actin at one pole of the bacterium (Dabiri, Sanger et al. 1990). *L. monocytogenes* use actin-based motility to move about the cell (Dabiri, Sanger et al. 1990, Sanger, Sanger et al. 1992, Theriot, Mitchison et al. 1992) and form protrusions to spread to adjacent cells without encountering the extracellular milieu (Tilney and Portnoy 1989).

It is well-established that the deletion of virulence factors critical for the intracellular life cycle significantly reduces the virulence of *L. monocytogenes* during the systemic phase of infection (Camilli, Tilney et al. 1993, Jones and Portnoy 1994, Lecuit, Ohayon et al. 1997, Glomski, Decatur et al. 2003, Peters, Domann et al. 2003). However, intracellular *L. monocytogenes* comprised only about 10% of the bacterial burden in the gut after foodborne transmission (Jones, Bussell et al. 2015), leading us to question whether intracellular replication was required at all during the early gut phase of infection. The cytosol of mammalian

cells is rich in nutrients that *L. monocytogenes* cannot synthesize *de novo* (Marquis, Bouwer et al. 1993) and the bacteria are well-adapted to scavenge these host factors. Lipoate, an essential cofactor required for the metabolism and growth of *L. monocytogenes,* can be found in the host either extracellularly in its free form or bound to peptides inside the cytosol (Akiba, Matsugo et al. 1998, Perham 2000). *L. monocytogenes* acquires this nutrient using two nonredundant lipoate ligase-like (LplA) proteins (O'Riordan, Moors et al. 2003, Keeney, Stuckey et al. 2007, Christensen, Hagar et al. 2011). LplA1 is required for intracellular growth, where only cytosolic lipoyl-peptides are present, while LplA2 is dispensable for cytosolic growth but important for acquiring exogenous free lipoate in the extracellular environment (Keeney, Stuckey et al. 2007). LplA1-deficient *L. monocytogenes* can invade cells, escape from the vacuole, and survive in the cytosol, but they do not replicate (Keeney, Stuckey et al. 2007, Christensen, Hagar et al. 2011). Therefore, *ΔlplA1 L. monocytogenes* are an effective tool to assess the importance of intracellular replication which could be important simply for increasing bacterial numbers or could serve to promote ActA-mediated cell-to-cell-spread.

We previously showed that *L. monocytogenes* burdens were highest in the colon following foodborne transmission, with infection of the ileum being much more transient (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015). *ΔlplA1 L. monocytogenes* were unable to persist in the colon and they did not subsequently colonize the MLN as well as wildtype bacteria three days post-infection (Jones, Bussell et al. 2015). In this study we ask whether *ΔlplA1 L. monocytogenes* are unable to reach the MLN, or can they not survive and replicate in the MLN once they get there? We use multiple approaches to show that intracellular replication is not needed for *L. monocytogenes* to disseminate to the MLN, but it does enhance survival in the gut tissue by allowing the bacteria to avoid immune clearance by phagocytes. We also show that the MLN environment does not have enough free lipoate to promote the extracellular growth of *L. monocytogenes* and that systemic spread beyond the MLN is dependent on both intracellular replication and cell-to-cell spread.

III. Results

Depleting monocytes and neutrophils improved colonization of LplA1-deficient *L. monocytogenes* in the intestinal tissue and MLN.

LplA1 deletion mutant (*ΔlplA1) L. monocytogenes* that are unable to replicate inside mammalian cells do not colonize the MLN efficiently (Jones, Bussell et al. 2015). We hypothesized that intracellular replication could promote survival in the gut tissue including the epithelium and the underlying lamina propria by protecting *L. monocytogenes* from clearance by phagocytes. Circulating neutrophils (polymorphonuclear cells; PMN) released from the bone marrow are the first myeloid-derived phagocytes to infiltrate infectious foci and kill *L. monocytogenes* in the spleen (Rogers and Unanue 1993), liver (Pitts, Combs et al. 2018) and gut (Jones and D'Orazio 2017). Shortly thereafter, Ly6C^{hi} monocytes infiltrate the gut and eventually become the predominant cell type associated with *L. monocytogenes* in both the intestinal tissue and MLN at two to three days post-infection (Jones and D'Orazio 2017). To determine if *ΔlplA1 L. monocytogenes* could persist in the gut and colonize the MLN more efficiently if these phagocytes were depleted, we used an antibody treatment (clone GR1) that targets both monocytes and neutrophils. Mice were fed a 1:1 mixture of mouse-adapted (InlA^{m}-expressing (Wollert, Pasche et al. 2007)), lipoate-starved *ΔlplA1 L. monocytogenes* and the complemented strain (*lplA1+*) and total bacterial loads were determined three days post-infection (Fig. 3.1A). The anti-GR1 antibody treatment was effective (Fig. 3.1B), resulting in nearly complete removal of neutrophils and a marked reduction of monocytes (Fig. 3.1C, D).

We expected *ΔlplA1 L. monocytogenes* to have a severe colonization defect in the intestines of isotype control-treated animals three days post-infection (Jones, Bussell et al. 2015), and indeed, in most animals, no mutant *ΔlplA1* bacteria were recovered (Fig. 3.1E). In the large intestine, the GR1 antibody treatment resulted in greater recovery of all *L. monocytogenes* and notably increased the number of

animals in which the *ΔlplA1* mutant was detected in both the colon tissue and the colon-draining cMLN (Fig. 3.1E). In contrast, *ΔlplA1 L. monocytogenes* persisted better in the ileum after the anti-GR1 antibody treatment, but only one animal had increased colonization in the small intestine-draining lymph nodes (sMLN), while *WT L. monocytogenes* burdens increased in every animal (Fig. 3.1E). Although depletion of monocytes and PMN enhanced colonization of the *ΔlplA1* mutant, *lplA1+ L. monocytogenes* still outcompeted the mutant in every compartment of the gut (Fig. 3.2). These results show that *ΔlplA1 L. monocytogenes* can reach the cMLN, albeit inefficiently, when major subsets of infiltrating phagocytes are depleted. This suggests the existence of a mechanism for dissemination that does not require intracellular replication, potentially via migration of extracellular bacteria in lymphatic fluid. However, these data also indicate an important role for intracellular replication to amplify bacterial burdens in the gut since the *ΔlplA1 L. monocytogenes* did not reach the same level as wildtype, even with the anti-GR1 treatment.

Increasing the inoculum can overcome the intestinal bottleneck that prevents *ΔlplA1 L. monocytogenes* from colonizing the MLN.

If intracellular replication serves primarily as a means to amplify the number of bacteria above some critical threshold needed to efficiently reach the MLN, then increasing the inoculum size should also result in enhanced colonization of the *ΔlplA1* mutant in the MLN. For the experiments shown in Fig. 1, mice were fed a total of 5×10^8 CFU and we were concerned that the use of doses higher than that might promote non-physiologic modes of rapid hematogenous spread (Lecuit, Vandormael-Pournin et al. 2001, Gajendran, Mittrucker et al. 2007, Wollert, Pasche et al. 2007, D'Orazio 2014). Becattini et al. showed that C57BL/6 mice pretreated with various antibiotic regimens to disrupt the gut microbiota could be given as few as 10^2 CFU of *L. monocytogenes* and still establish intestinal infection (Becattini, Littmann et al. 2017). We were unable to repeat their observations when

we treated BALB/cByJ (BALB) mice with single injections of either streptomycin alone (Fig. 3.3A) or a cocktail containing metronidazole, neomycin, vancomycin, and clindamycin (Fig. 3.3B). Neither of these treatments increased the susceptibility of mice fed 10^8 CFU *L. monocytogenes* compared to PBS-treated control animals. However, two sequential doses of clindamycin resulted in a 100-fold increase in bacterial burden in the colon two days post-infection (Fig. 3.3C). This suggested that we could feed clindamycin-pretreated BALB mice as little as 10^6 CFU, 100-fold less than our standard dose.

To determine if increasing the amount of *ΔlplA1* mutant relative to the *lplA1+* inoculum would result in enhanced colonization of the MLN, mice were pretreated with clindamycin, co-infected with various ratios of *ΔlplA1* and *lplA1+ L. monocytogenes,* and bacterial burdens were determined three days post-infection (Fig. 3.4A). As a control, one group of antibiotic-treated mice was fed a 1:1 mixture of the two strains; this resulted in *lplA1+ L. monocytogenes* outcompeting the mutant in each tissue, similar to what we had observed in untreated animals (Figs. 3.4B and 3.2). When mice were fed 10-fold more mutant *L. monocytogenes* than *lplA1+*, no significant differences in competitive index values were observed (Fig. 3.4B). However, when 100-fold more *ΔlplA1 L. monocytogenes* was used, the colonization defect in the sMLN was significantly reduced. In the large intestine, the number of *ΔlplA1 L. monocytogenes* recovered in the colon and cMLN increased significantly (Fig. 3.5A) even though the mutant continued to be outcompeted by *lplA1+ L. monocytogenes* (Fig 3.4D).

We sought to improve the antibiotic pretreatment model to further increase the ratio between the co-infecting *L. monocytogenes* strains, which required us to administer even lower doses of the *lplA1+ L. monocytogenes*. We reasoned that intestinal dysbiosis was not maintained in our mice because they were housed in standard SPF conditions and therefore, were exposed to environmental bacteria that could re-colonize the gut. To test this, mice were treated with clindamycin and immediately placed in sterile cages and given sterile food and water to limit exposure to environmental microbiota. We found that this regimen did further

increase the susceptibility of mice (Fig. 3.5B), allowing us to increase the mutant:complemented strain ratio to 10,000:1. In the colon and cMLN, *lplA1+ L. monocytogenes* no longer outcompeted the *ΔlplA1* mutant (Fig. 3.4E) and the two strains were recovered in nearly identical amounts when 10,000-fold more mutant was fed to mice (Fig. 3.5C). However, in the small intestine, no further increase in *ΔlplA1 L. monocytogenes* burden was observed (Fig. 3.4C). These results confirmed that intracellular replication was not required to reach the cMLN but could promote dissemination by allowing *L. monocytogenes* to achieve bacterial numbers required for cMLN colonization.

Exogenous lipoate availability is severely limited in the MLN.

Another possibility to explain the MLN colonization defect was that extracellular *ΔlplA1 L. monocytogenes* that can reach the MLN are unable to persist and replicate there due to a lack of essential nutrients. Host-derived lipoate is mostly protein-bound in the cell but is also found as free exogenous lipoate in the extracellular space of some tissues (Keeney, Stuckey et al. 2007). The *ΔlplA1* mutant can acquire free lipoate from the extracellular environment using LplA2 but cannot utilize lipoyl-peptides in the cytosol of mammalian cells (Christensen, Hagar et al. 2011). We hypothesized that the availability of exogenous free lipoate in the MLN could be lower than in the intestinal tissue, hindering the extracellular growth of *L. monocytogenes* and thereby causing a colonization defect for the *ΔlplA1* mutant. The concentration of lipoyl-peptides in the kidney, brain, spleen, and liver of cows, rats, and rabbits was previously determined using enzymatic methods (Akiba, Matsugo et al. 1998). Additionally, the total lipoate concentration found in the liver, urine, and blood of humans was determined using an *in vitro* assay to measure the growth of the lipoate-dependent eukaryotic protozoan, *Tetrahymena thermophila* (Baker, Deangelis et al. 1998). However, the concentration of lipoate in the intestinal tissue and MLN has not been reported.

To determine if there was sufficient exogenous free lipoate in the MLN to support the extracellular growth of *ΔlplA1 mutant L. monocytogenes,* we developed an *in vitro* assay to indirectly measure free lipoate availability in tissue homogenates. The ileum, colon, sMLN and cMLN were harvested from uninfected mice, homogenized in Improved Minimal Media (IMM) (Phan-Thanh and Gormon 1997) lacking lipoate, and then inoculated with *L. monocytogenes* that had been cultured in lipoate-free media to deplete intracellular lipoate reserves (Fig. 3.6A). Lipoate-starved *L. monocytogenes* have a long lag phase (~12-14 hours) (O'Riordan, Moors et al. 2003, Jones, Bussell et al. 2015); therefore, an initial absorbance measurement was taken immediately post-inoculation and the growth of each strain was determined 20 hours later.

The complemented strain that could use both LplA1 and LplA2 to acquire lipoate grew exponentially in every gut tissue homogenate as assessed by both optical density (Fig. 3.6B) and CFU (Fig. 3.6C). In contrast, *ΔlplA1 L. monocytogenes* grew only in the ileum and colon homogenates and did not replicate efficiently in the MLN. To ensure that the lack of growth was due solely to limited free lipoate in the MLN, the tissue homogenates were spiked with 2.5 nM lipoate, a concentration known to support the growth of *L. monocytogenes* in IMM (Jones, Bussell et al. 2015). This resulted in maximal growth of both *lplA1*+ and *ΔlplA1 L.* monocytogenes in every tissue (Fig. 3.6D), suggesting that the tissue homogenates did not contain any inhibitory substances. Notably, the *ΔlplA1 mutant L. monocytogenes* did not have any growth defect compared to *lplA1*+ *L. monocytogenes* in the ileum homogenates (Fig. 3.6B and 3.6C). These data suggest that there may be a gradient of available exogenous free lipoate in the gut, with the highest concentration in the small intestine, less available in the large intestine, and the least amount in the draining MLN. Furthermore, these results indicate that exponential growth of *L. monocytogenes* in the MLN is likely only to occur intracellularly, where the bacteria can use LplA1 to scavenge lipoate from the host cell cytosol. Thus, extracellular *L. monocytogenes* that avoid phagocytosis can persist in the gut tissue but cannot replicate in the MLN.

Intracellular replication was absolutely required for *L. monocytogenes* to spread from the MLN to the spleen and liver.

When we manipulated oral infections using either larger inocula or phagocyte depletion, colonization of *ΔlplA1 L. monocytogenes* increased, in some cases to nearly the same level as *lplA1*+ bacteria (see Fig. 3.1E and Fig. 3.4C). However, *ΔlplA1 L. monocytogenes* was never recovered in the spleens or livers of mice fed 10,000-fold more *ΔlplA1* mutant than *lplA1*+ *L. monocytogenes* (Fig. 3.7A) or the spleens of anti-GR1 antibody-treated animals (Fig. 3.7B). These data either suggest that intracellular replication is absolutely required for *L. monocytogenes* to exit the MLN, or that intracellular replication is essential for survival in the tissue after reaching the spleen or liver.

Keeney et al. previously showed that LplA1-deficient *L. monocytogenes* administered intraperitoneally had a significant growth defect in the spleens and livers of C57BL/6 mice (Keeney, Stuckey et al. 2007). Since that study examined only a single time point (3 days post-infection), it was not clear if *ΔlplA1 L. monocytogenes* did not grow at all or if it just grew slower than wild type bacteria. To test this, we used the intravenous (i.v.) model to bypass the gut phase of infection and ensure that the vast majority of the inoculum would be present in either the spleen or liver within 15 minutes of administration (Mackaness 1962). Mice were coinfected with lipoate-starved *ΔlplA1* and *lplA1*+ *L. monocytogenes*, the total CFU burden in the spleen and liver was determined at 30 minutes post-infection, and then growth was assessed at later time points. At 8 hours post-infection, both *L. monocytogenes* strains had decreased in number in the spleen and liver (Fig. 3.7C). This was likely due to a loss of extracellular bacteria killed by resident and infiltrating phagocytes. By 24 hours post-infection, *lplA1*+ *L. monocytogenes* increased by three logs while the mutant only had a modest amount of growth in the spleen. However, both strains grew exponentially in the liver by 24 hours post-infection (Fig. 3.7C). These data suggest that both the

spleen and liver contain enough free exogenous lipoate to support some growth of extracellular *ΔlplA1 L. monocytogenes*.

To confirm that the increase in *ΔlplA1 L. monocytogenes* at 24 hours post-infection was due to extracellular growth, we treated mice with gentamicin every 8 hours (40 mg/kg i.p.) to kill extracellular *L. monocytogenes,* and bacterial burdens in the spleen and liver were compared to control mice that received PBS injections. As expected, we observed an initial decrease of both strains at 8 hours with a small residual burden of gentamicin-protected intracellular bacteria (Fig. 3.7D). The intracellular (gentamicin-protected) *lplA1+ L. monocytogenes* increased only slightly while the total bacterial burden, which presumably represents extracellular growth, increased significantly (Fig. 3.7D). In contrast, there was no net growth of *ΔlplA1 L. monocytogenes* at 24 hours post-infection in the presence of gentamicin. Together, these results indicate that the spleen and liver can support the growth of extracellular *ΔlplA1 L. monocytogenes,* which suggests that the absence of bacteria in the spleen and liver following foodborne infection is due to an inability to disseminate out of the MLN.

LplA1-deficient *L. monocytogenes* did not form actin tails.

Since the lplA1 mutant did not exit the MLN even when present in high numbers, we next tested the idea that intracellular replication was needed to promote cell-to-cell spread in the MLN. Soon after *L. monocytogenes* begin replicating in the host cell cytosol, they express ActA at one pole (Kocks, Hellio et al. 1993), a critical step in actin-based motility. Microscopic analysis of dividing *L. monocytogenes* showed that ActA and F-actin were absent at the point of septum formation and the authors predicted that polarization of surface-exposed ActA and the subsequent formation of actin tails would be directly linked to cell division (Kocks, Hellio et al. 1993). *ΔlplA1 L. monocytogenes* can access the host cell

cytosol but do not replicate, so we hypothesized that they would not form actin tails and that cell-to-cell spread was essential for egress from the MLN.

To first determine if our lipoate-starved strains were expressing *actA*, we measured mRNA levels after growth in a defined minimal medium (iLSM) supplemented with reduced glutathione. Growth in this synthetic media mimics the conditions *L. monocytogenes* experience in the mammalian cytosol and results in induction of *actA* expression (Portman, Dubensky et al. 2017). As shown in Fig. 3.8A, *actA* was induced during *in vitro* growth and there was no significant difference in *actA* expression for *ΔlplA1* and *lplA1+ L. monocytogenes* over a 16-hour period. Next, we infected bone marrow-derived macrophages with lipoate-starved bacteria and examined the cells 16 hours post-infection to quantify the presence of actin filaments associated with *L. monocytogenes* (Fig. 3.8B). When *L. monocytogenes* first enter the cytosol, they become coated with actin; this has been referred to as an actin "cloud" (Tilney, DeRosier et al. 1992). At a later timepoint, presumably after the bacteria begin replicating, the actin re-distributes to one pole (the "tail") and motility initiates (Kocks, Gouin et al. 1992, Smith, Portnoy et al. 1995). At 16 hours post-infection all of the intracellular *ΔlplA1 L. monocytogenes* were associated with actin clouds, and no actin tails were observed (Fig 3.8C). Consistent with the prediction that actin tail formation begins as *L. monocytogenes* replicate in the cytosol, we did observe one *lplA1+* bacterium that had not yet completed cell division with two polar actin tails (Fig. 3.8D). Thus, the lack of intracellular replication by *ΔlplA1 L. monocytogenes* likely prevented the polar redistribution of ActA on the surface of the bacteria and the subsequent formation of actin tails.

To verify that *ΔlplA1 L. monocytogenes* had a defect in cell-to-cell spread, we performed plaque assays. L2 fibroblasts were infected with lipoate-starved *ΔlplA1, lplA1+* and $InlA^m$-expressing *EGDe* (wild type*) L. monocytogenes* for one hour and plaque sizes were measured four days post-infection. We found that *lplA1+ L. monocytogenes* created plaques similar to *wildtype L. monocytogenes* (Fig 3.8E). However, the lipoate-starved *ΔlplA1 mutant* formed plaques that were

100-fold smaller, suggesting a severe defect in the ability to spread cell-to-cell. O'Riordan et al. previously reported that *ΔlplA1 L. monocytogenes* did form small plaques within L2 fibroblasts (O'Riordan, Moors et al. 2003). However, the *L. monocytogenes* used in that plaque assay were grown on BHI slants, not in minimal media lacking lipoate. We repeated the plaque assay using mid-log phase *ΔlplA1 L. monocytogenes* grown in BHI broth and observed tiny plaques 11-fold smaller than the wildtype plaques (Fig. 3.8E). These results suggested that *L. monocytogenes* could replicate in the cytosol and spread cell-to-cell until any intracellular lipoate reserves in the bacteria were depleted, and then intracellular growth and cell-to-cell spread were aborted.

ActA-deficient *L. monocytogenes* had a severe colonization defect in the spleen and liver following foodborne infection.

If *ΔlplA1 L. monocytogenes* do not exit the MLN primarily because they do not complete actin-based motility and spread cell-to-cell, then ActA-deficient *L. monocytogenes* should have a similar phenotype during foodborne infection. To test this, mice were co-infected with a 1:1 mixture of mouse-adapted ($InlA^m$-expressing) *ΔactA mutant* and *actA*+ complemented *L. monocytogenes,* and tissue burdens were determined at two- and three-days post-infection. Consistent with the hypothesis that cell-to-cell spread is not needed for colonization of the gut tissue, the two strains were recovered in similar numbers in both the ileum and the colon (Fig. 3.9B and 3.10). However, *ΔactA L. monocytogenes* had a significant defect colonizing the sMLN and were delayed in reaching maximal CFU burdens the cMLN. At two days post-infection, none of the *ΔactA* bacteria were detected in the spleen or liver. By three days post-infection, the mutant bacteria had reached the spleens and livers of a few animals, but there was 100-fold less in the spleen and 1000-fold less in the liver compared to wild type *L. monocytogenes* (Fig. 3.9B and 3.10), suggesting a significant delay in egress from the MLN. Thus, *actA*-

dependent cell-to-cell spread was important to achieve high bacterial burdens in the MLN and for rapid subsequent dissemination to the spleen and liver.

IV. Discussion

The primary function of the MLN is to serve as a barrier that prevents commensal and foodborne pathogens from entering the bloodstream (Macpherson and Uhr 2004, Voedisch, Koenecke et al. 2009). *L. monocytogenes* can readily overcome this bottleneck to cause life-threatening systemic infections and we show here that exiting the MLN depends on both intracellular replication and ActA-dependent cell-to-cell spread. Neither of these intracellular functions were absolutely required for intestinal colonization, but could promote persistence in the gut by allowing the bacteria to avoid clearance by phagocytes and to increase in number. However, once *L. monocytogenes* reach the MLN, there is an insufficient concentration of exogenous free lipoate to support extracellular growth, so continued growth requires LplA1-dependent intracellular replication. Our data suggest that cytosolic *L. monocytogenes* must then use ActA-mediated cell-to-cell spread to access an as-yet-unknown cell type to spread systemically to the spleen and liver.

We found notable differences in the importance of intracellular replication and cell-to-cell spread for colonizing the MLN. Although the results of the lipoate assay suggest that the ileum contains a much higher concentration of free lipoate than the colon which would support extracellular growth of *L. monocytogenes* there seem to be very few extracellular bacteria in the small intestines. Although not absolutely required, both intracellular replication and cell-to-cell-spread appear to be important for getting past some innate immune or anatomic bottleneck to traffic from ileal mucosa to the sMLN. In contrast, when we used either the phagocyte depletion or the increased inoculum approach to modify the infection, there was a significant increase in *L. monocytogenes* in the colon and cMLN which suggests that there was a greater extracellular burden in these tissues that was vulnerable to phagocyte clearance. It is not yet clear whether that is simply a reflection of the

generally larger bacterial burdens in the colon compared to the ileum following foodborne transmission in mice (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015) or if there are different subsets of phagocytes present in these two tissues. For example, it was previously shown that macrophages and $CD103^{+}CD11b^{-}$ dendritic cells are more abundant in the colon than in the small intestine (Nagashima, Yoshida et al. 2005, Denning, Norris et al. 2011, Ogino, Nishimura et al. 2013). Our studies also suggested that there may be a gradient of free lipoate in the gut, with the small intestine having a much higher concentration than the colon (Fig. 3.6C). The resident microbiota of the large intestine is also more abundant and more diverse than the small intestine (Kho and Lal 2018). Therefore, when we used antibiotic pretreatment to promote intestinal colonization of *L. monocytogenes* by reducing bacterial competition at the intestinal mucosa, it was not surprising to find a bigger difference in the colon than the ileum, given the differing microbiota composition.

Our data suggest that intracellular replication and cell-to-cell spread are much more important for colonizing the sMLN than the cMLN. It is possible that the route used to cross the intestinal mucosa determines the types of phagocytes that *L. monocytogenes* will encounter enroute to the MLN. *L. monocytogenes* efficiently invade the intestinal epithelium through the interaction of InlA binding to luminally exposed E-cadherin on goblet cells (Lecuit, Vandormael-Pournin et al. 2001, Nikitas, Deschamps et al. 2011) or epithelial cells adjacent to extruding cells, and are quickly translocated across the epithelium to the underlying lamina propria (Nikitas, Deschamps et al. 2011, Kim, Fevre et al. 2021) where they will encounter infiltrating phagocytes. However, *L. monocytogenes* can also be taken up by M cells (Jensen, Harty et al. 1998, Chiba, Nagai et al. 2011) and since M cells are found overlying Peyer's patches in the small intestine, this will result in the bacteria interacting with the resident immune cells that are part of these lymphoid structures. M cells in the colon can be induced during inflammation but are less well-organized into distinct structures (Bennett, Parnell et al. 2016), and it is likely that bacteria that translocate across these cells will encounter different subsets of phagocytes. Another possible reason for the importance of the intracellular niche

in the sMLN could be the relatively smaller number of *L. monocytogenes* found in the ileum compared with the colon. Disson et al. found that *L. monocytogenes* entry into the Peyer's patches triggers a signal cascade that results in accelerated epithelial cell renewal with inaccessibility of E-cadherin, thereby blocking further InlA-dependent translocation of *L. monocytogenes* (Disson, Bleriot et al. 2018). Therefore, if there are fewer bacteria able to invade the ileum and increased colonization of *L. monocytogenes* due to the loss of intestinal epithelial integrity in the colon, one could speculate that there simply may not be enough bacteria to reach the threshold required to colonize the sMLN.

The early events that occur immediately after *L. monocytogenes* enter the MLN are still unclear. Using the i.v. inoculation model, *L. monocytogenes* are rapidly filtered from the blood into the splenic marginal sinus (Hardy, Francis et al. 2004, Neuenhahn, Kerksiek et al. 2006, Hardy, Chu et al. 2009, Edelson, Bradstreet et al. 2011, Mitchell, Brzoza-Lewis et al. 2011). This compartment, which may closely resemble the subcapsular sinus in the MLN, contains resident macrophages that trap *L. monocytogenes* as they enter the spleen (Conlan 1996, Aichele, Zinke et al. 2003). Previous studies have suggested that *L. monocytogenes* are then transported by dendritic cells from the marginal sinus to the periarteriolar lymphoid sheaths for antigen presentation to T cells (Berg, Crossley et al. 2005, Aoshi, Zinselmeyer et al. 2008). It is possible that dendritic cells in the MLN have a similar function and transport *L. monocytogenes* from the subcapsular sinus to the MLN cortex, which is primarily populated by T cells. We previously showed that *L. monocytogenes* can invade dendritic cells and survive for several hours, but then decrease in number, suggestive of intracellular killing (Jones, Smith et al. 2017). Once in the cortex, dendritic cells will present degraded *L. monocytogenes* antigens to T cells and that will promote induction of cell-mediated immunity. But given the proximity, a few *L. monocytogenes* could also spread from dendritic cells into high endothelial venules where the bacteria could use ActA-dependent cell-to-cell spread to enter blood endothelial cells. Our working model posits that exponential growth in a stromal cell type such as blood endothelial cells could result in cell damage and release of extracellular bacteria

into the bloodstream. Future studies will aim to explore the path taken by *L. monocytogenes* to transit through the MLN, identifying each of the cell types that serve as an intracellular niche for the bacteria to promote cell-to-cell spread.

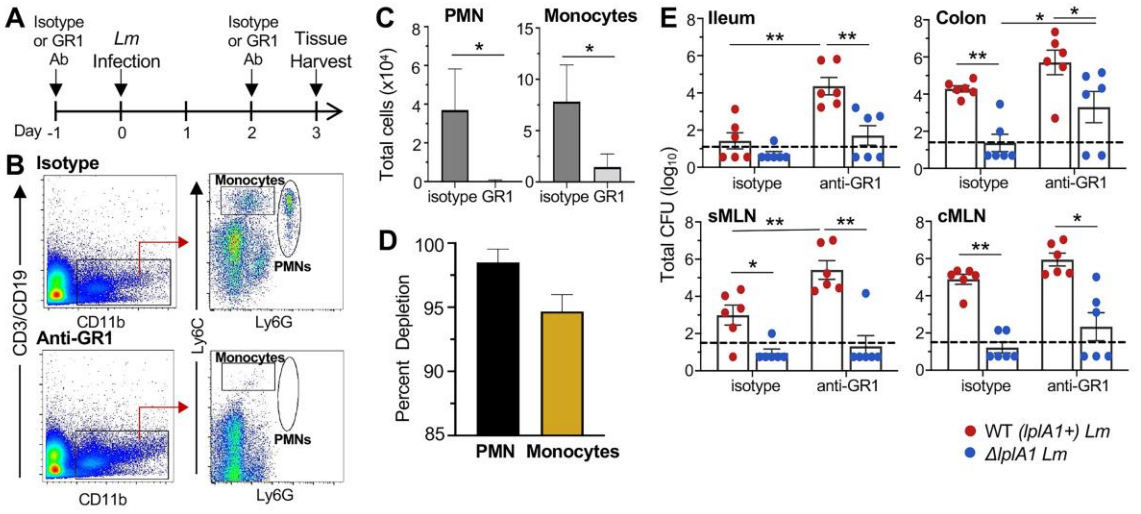

Figure 3.1: Depletion of PMN and monocytes during foodborne infection results in increased colonization of *ΔlplA1 L. monocytogenes* in tissues. (A) Female BALB/cByJ mice were treated with anti-GR1 or isotype control antibodies and co-infected with a 1:1 mixture of lipoate-starved *L. monocytogenes* SD2302-Ery^R (WT *lplA1+)* and SD2301-Kan^R (*ΔlplA1),* totaling ~5 x 10^8 CFU. (B) Gating scheme and representative dot plots showing depletion of PMN and monocytes in the colon draining MLN (cMLN) after anti-GR1 treatment. Cells were pre-gated to deplete lymphocytes (CD3$^+$/CD19$^+$). The total number of cells (C) and the percent depletion (D) of PMN and monocytes in the cMLN for a representative experiment (n=3) are shown; bars indicate mean values ± SEM. (E) Total *L. monocytogenes* CFU recovered from tissues three days post-infection. Dashed lines indicate limit of detection. Data pooled from two separate experiments; bars indicate mean values (± SEM). Small intestine draining lymph nodes are denoted as “sMLN”. Statistical significance was determined by two tailed Mann-Whitney analysis.

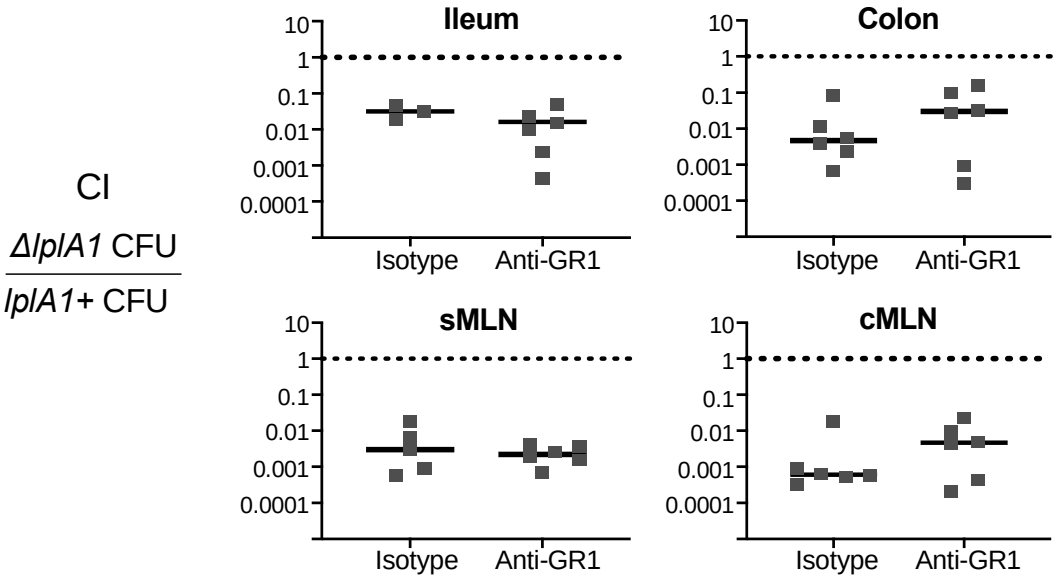

Figure 3.2: Wildtype *L. monocytogenes* still outcompete LplA1-deficient bacteria even when monocytes and PMN are depleted. BALB/cByJ mice were treated with an anti-GR1 antibody then co-infected with a 1:1 mixture of lipoate-starved, mouse-adapted *L. monocytogenes* SD2302-Ery^R (lplA1+) and SD2301-Kan^R (ΔlplA1), totaling ~5 x 10^8 CFU. CFU recovered from tissues three days post-infection is expressed as a competitive index (CI); horizontal lines indicate median values. Dotted line indicates a hypothetical recovery of CFU in a 1:1 ratio. Raw CFU values for these infections are shown in Fig. 3.1.

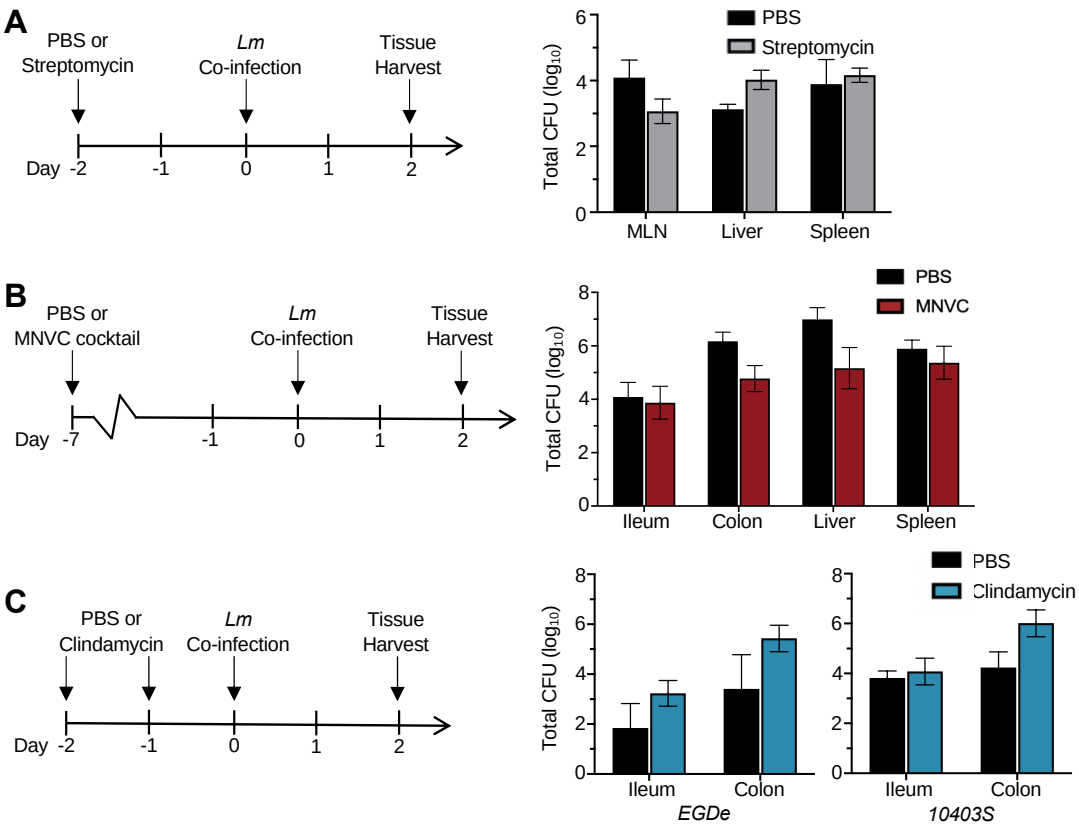

Figure 3.3: Treatment with clindamycin increases susceptibility to oral *L. monocytogenes* infection. BALB/cByJ mice were treated with three different antibiotic regimens. Each panel displays the treatment timeline on the left and the CFU recovered from tissues two days after feeding mice 10^8 CFU of *L. monocytogenes* on the right. (A) Mice (n=4) were given a single dose of streptomycin (20 mg, orally) and infected with *L. monocytogenes* SD2000. (B) Mice (n=3) were treated with a single dose of an antibiotic cocktail (MNVC; 0.7 mg metronidazole, 3.5 mg neomycin, and 3.5 mg vancomycin orally plus 0.2 mg clindamycin i.p.) and then infected with *L. monocytogenes* SD2001 (SD2000-Ery^R). (C) Mice (n=3) were treated with two doses of 0.2 mg clindamycin (i.p) and infected with either strain 10403s or SD2900 (EGDe-Kan^R). Bars indicate mean values (± SEM). *Data shown in Fig. 3.3 generated by co-author, Jessica Ferrel.

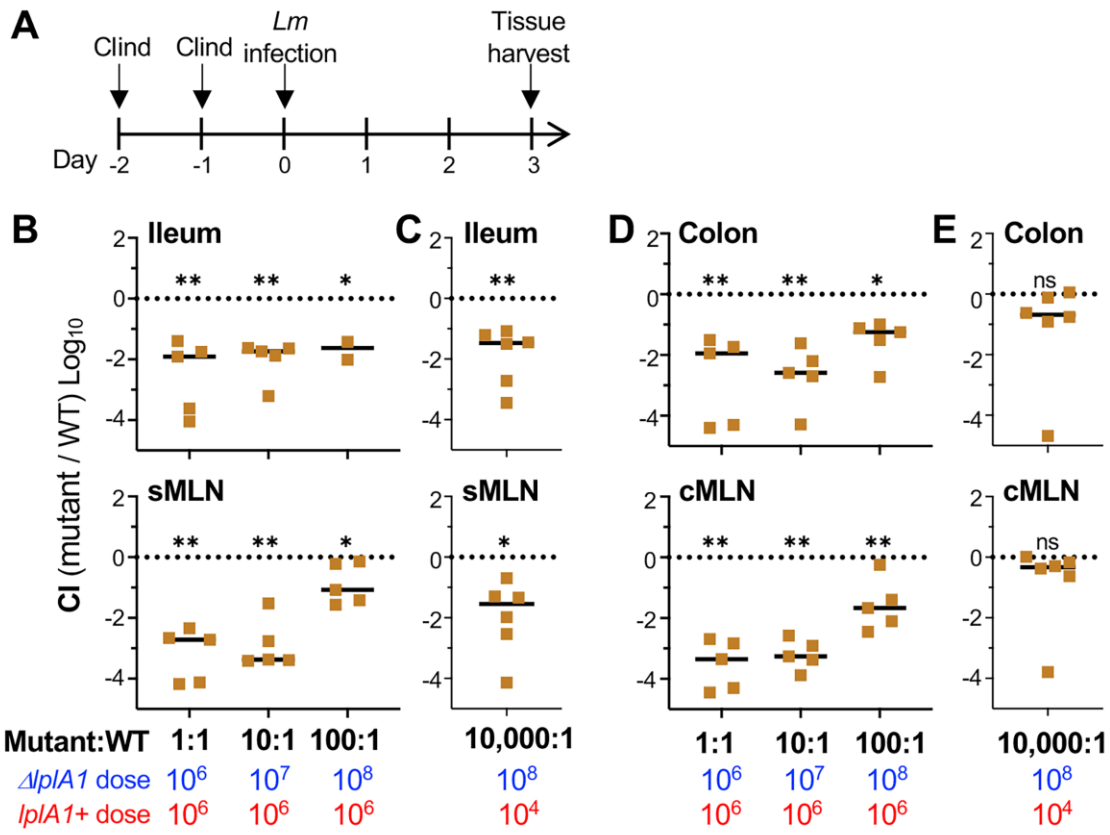

Figure 3.4: Increasing the inoculum can overcome the intestinal bottleneck that prevents *ΔlplA1 L. monocytogenes* from colonizing the MLN. (A) BALB/cByJ mice were given 0.2 mg of clindamycin (clind) i.p. and then co-infected with 5 x 10^4 to 5 x 10^6 CFU of lipoate-staved SD2302-Ery^R (WT) and 5 x 10^6 to 5 x 10^8 CFU of lipoate-starved SD2301-Kan^R (mutant). (B) The ratio of *ΔlplA1* to *lplA1+* *L. monocytogenes* recovered from tissues three days post-infection is expressed as a competitive index (CI). (C) Mice were treated as above but maintained in sterile caging to ensure prolonged dysbiosis of the gut microbiota. Pooled data from two separate experiments is shown; horizontal lines indicate median values. Dotted line at 0 indicates CFU recovery at the input 1:1 ratio. Statistical significance determined by two tailed Mann-Whitney analysis, comparing CI median to the hypothetical value of 0.

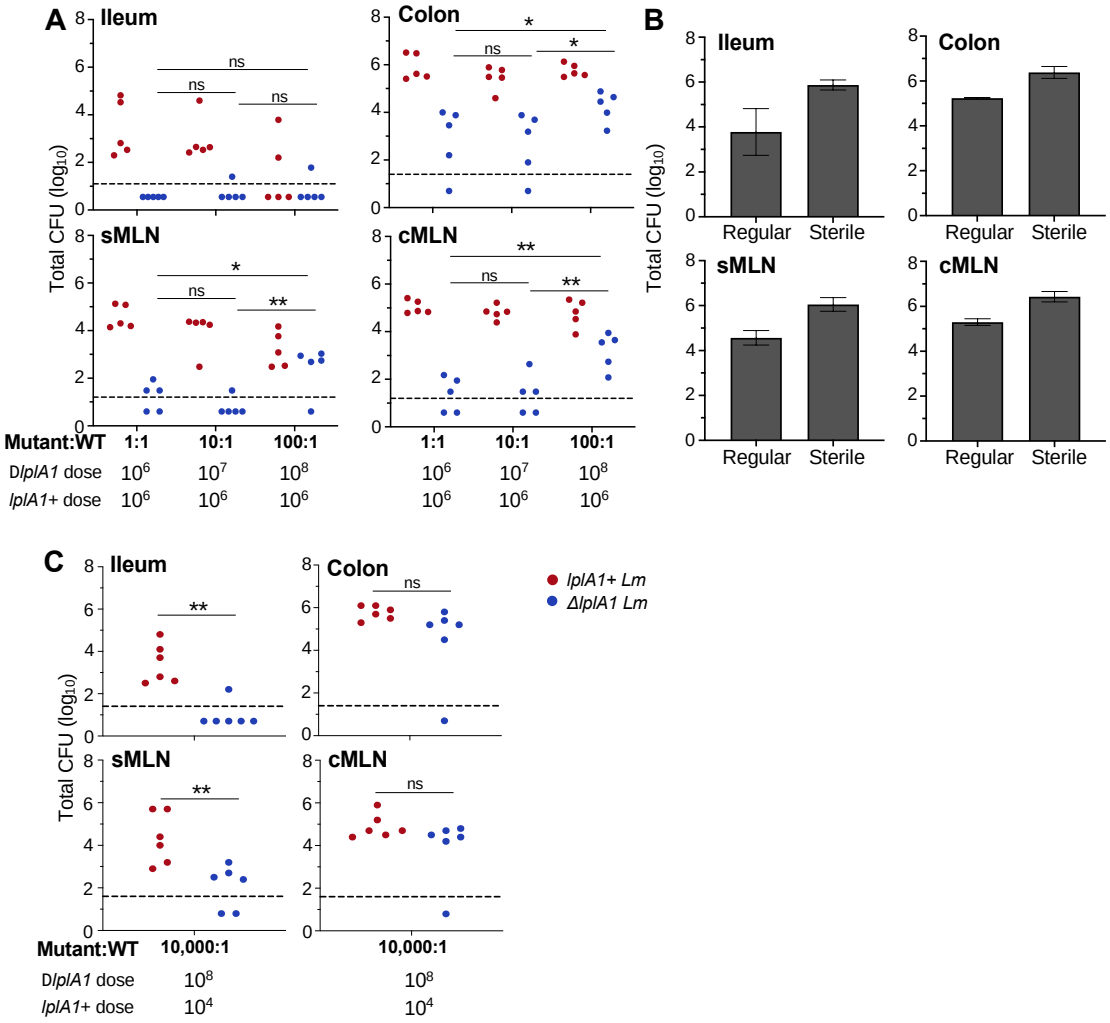

Figure 3.5: Primary data to support the results shown in Figure 3.4. BALB/cByJ mice were treated i.p. with 0.2 mg clindamycin as shown in Fig. 2A and L. monocytogenes CFU were determined in tissues harvested three days post-infection. Bars indicate mean values (± SEM). Dashed lines indicate limit of detection for each tissue. (A) Mice were co-infected with 10^6 CFU of lipoate-staved SD2302 (lplA1+ Lm) and 10^6 to 10^8 CFU of lipoate-starved SD2301 (ΔlplA1 Lm). Statistical significance was determined by ANOVA and Tukey's Multiple Comparison test. (B) Mice (n=3) were maintained in either standard SPF caging (Regular) or in autoclaved cages with sterile chow & water (Sterile) and infected with 10^5 CFU of lplA1+ L. monocytogenes. (C) Mice -were coinfected with 10^4 CFU of SD2302 and10^8 CFU of SD2301. Statistical significance determined by two-tailed Mann-Whitney test.

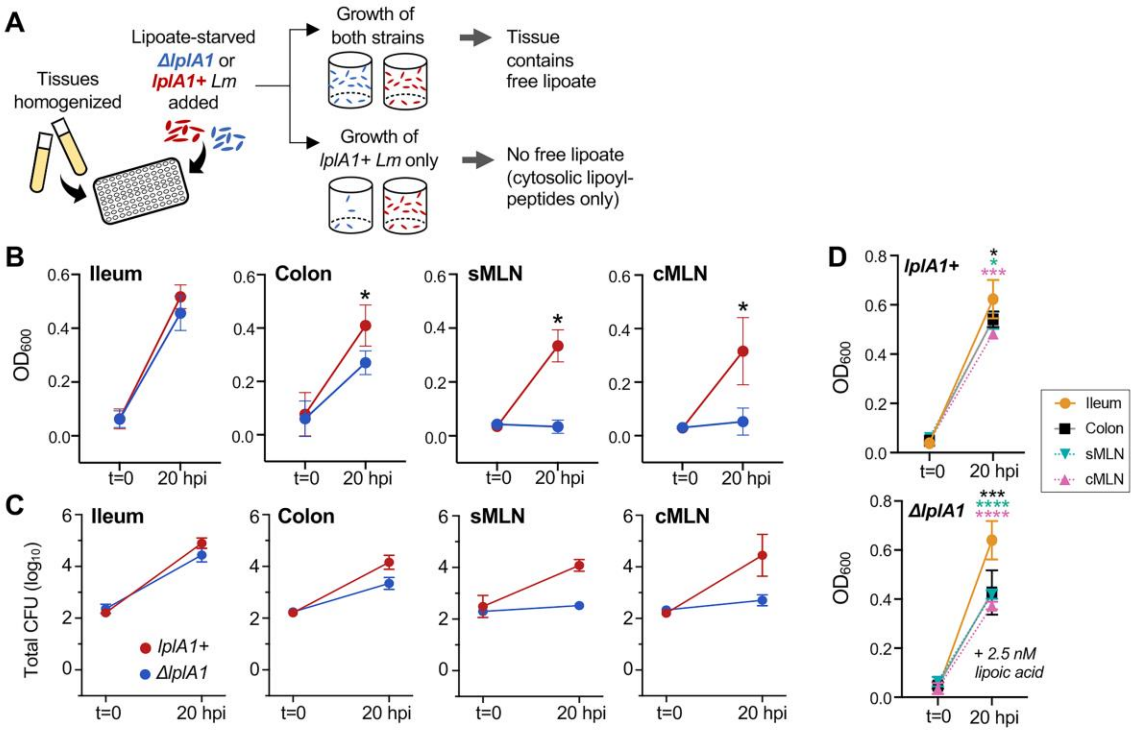

Figure 3.6: Exogenous free lipoate is abundant in intestinal tissue but severely limited in the MLN. (A) Tissues harvested from uninfected BALB/cByJ mice were homogenized in IMM(-) media and inoculated with lipoate-starved SD2301 (*ΔlplA1)* or SD2302 (*lplA1+).* For all panels, mean values (± SEM) of the replicates tested in three separate experiments are shown. Free lipoate concentration was assessed in gut tissues by either (B) measuring optical density at 600 nm or (C) determining CFU. Statistical significance determined by paired t-tests. (D) Overnight growth of lipoate-starved *lplA1(+)* and *ΔlplA1 L. monocytogenes* cultured in IMM media with 2.5 nM lipoic acid was assessed by optical density. Means significantly different than the mean value for ileum homogenate as assessed by ANOVA with Tukey's Multiple Comparison Test are shown.

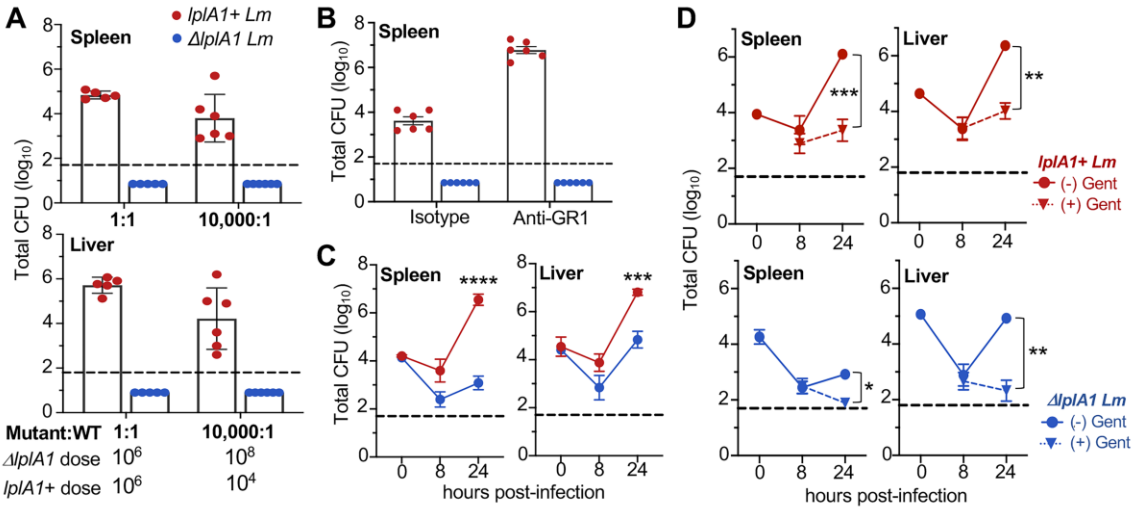

Figure 3.7: Intracellular replication is absolutely required for *L. monocytogenes* to spread from the MLN to the spleen and liver. Female BALB/cByJ mice were co-infected orally (A, B) or intravenously (C, D) with a mixture of lipoate-starved, mouse-adapted *L. monocytogenes* SD2302-SD2301-Kan^R (*ΔlplA1)* and total CFU recovered from spleen and liver was determi (0.2 mg, i.p.) was given to cause dysbiosis of the gut microbiota and then mice were co-infected with 5 x 10^4 to 5 x 10^6 CFU of *lplA1*+ and 5 x 10^6 to 5 x 10^8 CFU of *ΔlplA1 L. monocytogenes. (*B) Mice were given anti-GR1 to deplete phagocytes or isotype control antibody and co-infected with a 1:1 mixture *of L. monocytogenes* totaling ~5 x 10^8 CFU. In panels (A) and (B), total *L. monocytogenes* CFU recovered from tissues three days post-infection. Dashed lines indicate limit of detection and bars indicate mean values (± SEM). (C) Mice were co-infected (i.v.) with total of ~1 x 10^5 *L. monocytogenes* and total CFU for each strain was determined at indicated time points. (D) Mice were treated with PBS or gentamicin (40 mg/kg i.p.) every 8 hours. In (C) and (D), pooled data from two separate experiments (n=6) were analyzed by ANOVA with Tukey's Multiple Comparison test**.**

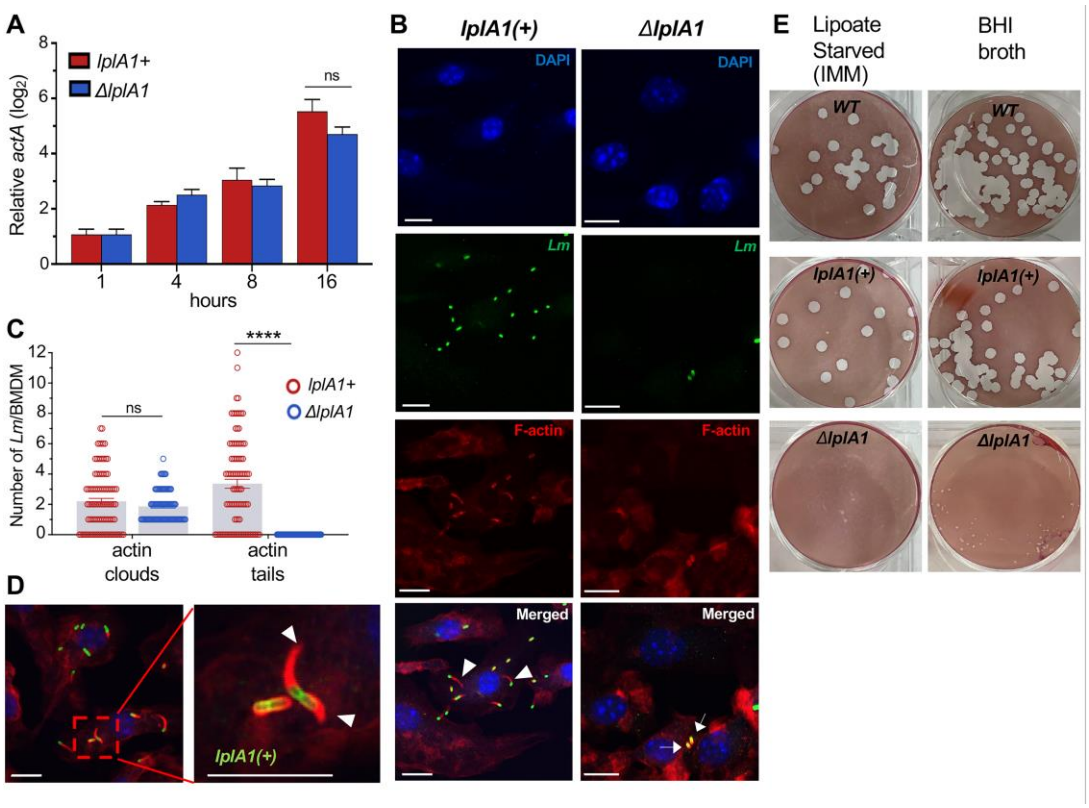

Figure 3.8: Intracellular *ΔlplA1* mutant *L. monocytogenes* do not form actin tails. (A) *actA* mRNA expression in lipoate-starved *L. monocytogenes* SD2302 (*lplA1+)* and SD2301 (*ΔlplA1)* grown in iLSM supplemented with glutathione. Bars indicate mean -ΔΔCT values (± SEM) for triplicate samples from one of two independent experiments. (B, C, D) Bone marrow-derived macrophages (BMDM) were infected with *lplA1(+)* and *ΔlplA1 L. monocytogenes* at MOI=0.1, fixed at 16 hpi and stained with DAPI (BMDM nucleus), phalloidin (F-actin) and *anti-Listeria* antibody (*Lm*). (B) Representative images show actin tails (arrowheads) and actin clouds (arrows). (C) Symbols indicate the number of *L. monocytogenes* in observed with actin clouds or tails in 100 cells counted. bars indicate means (± SEM). Data from one of two independent experiment are shown. (D) Image of a replicating *lplA1(+) L. monocytogenes* with two polar actin tails. Scale bar, 10μm. (E) L2 fibroblasts were infected with *EGDe SD2000 (WT), lplA1+, and ΔlplA1 L. monocytogenes* cultured in either IMM lacking lipoate (left images) or BHI (right images). Live cells were stained with 0.5% neural red 4 dpi to visualize plaque formation. Statistical significance determined by two tailed Mann-Whitney analysis.

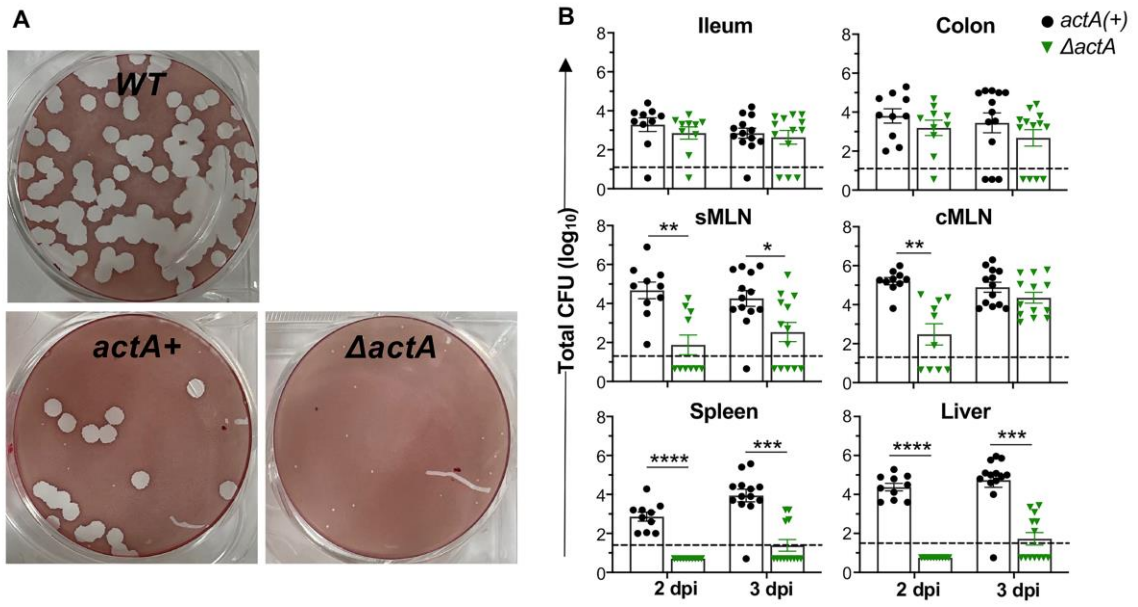

Figure 3.9: *ΔactA L. monocytogenes* has a severe colonization defect in the spleen and liver. (A) Phenotypes of mouse-adapted *L. monocytogenes* SD2154 (*actA*+) and SD2152 (*ΔactA)* strains were verified by plaque assay in L2 fibroblasts. Plaque sizes were compared to *EGDe SD2000 L. monocytogenes (WT)*. (B) BALB/cByJ mice were co-infected with a 1:1 mixture of mouse-adapted *L. monocytogenes* SD2154 (*actA*+) and SD2152 (*ΔactA*), totaling ~5 x 10^8 CFU. The total amount of each strain was determined 2- and 3-days post-infection (dpi). Data is pooled from three separate experiments. Statistical significance determined by two tailed Mann-Whitney analysis. Bars indicate mean values (± SEM). Dashed lines indicate limit of detection.

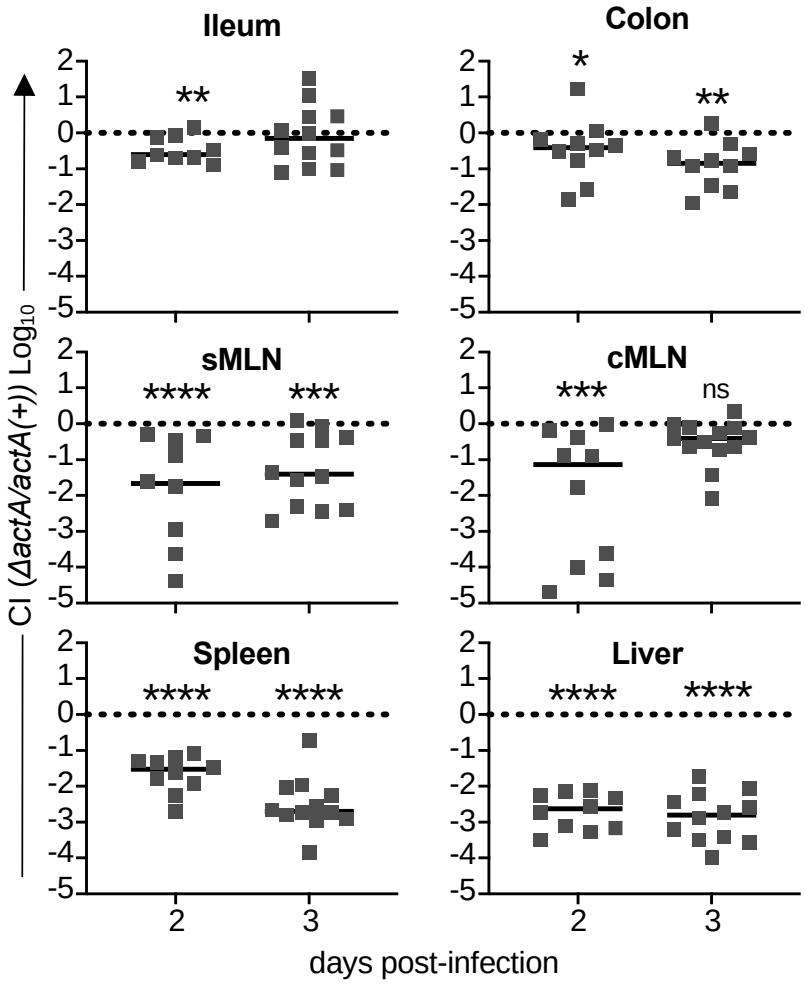

Figure 3.10: *actA(+) L. monocytogenes* outcompetes *ΔactA L. monocytogenes* in the spleen and liver. BALB/cByJ mice were co-infected with a 1:1 mixture of mouse-adapted *L. monocytogenes* SD2154 (actA(+)) and SD2152 (ΔactA), totaling ~5 x 10^8 CFU. CFU recovered from tissues two- and three-days post-infection is expressed as a competitive index (CI); horizontal lines indicate median values. Dotted line at 0 indicates a hypothetical recovery of CFU in a 1:1 ratio. Statistical significance determined by two tailed Mann-Whitney analysis, comparing CI median to the hypothetical value of 0. Raw CFU values for these pooled data (n=10-12) are shown in Fig. 3.9.

CHAPTER 4. DETERMINING THE ROLE OF GUT MYELOID CELLS DURING *IN VITRO L. MONOCYTOGENES* INFECTION

**This work was completed with the help of Alyssa Franklin, an IBS rotation student. Alyssa Franklin performed one of the immunoassays (Fig. 4.3). All other experiments, figure generation, and writing were completed by me.

I. Summary

Extensive research has highlighted the pivotal involvement of myeloid cells, particularly macrophages, during various aspects of a *Listeria monocytogenes* foodborne infection. In our prior work, we identified that there is an unknown critical cellular growth niche essential for the escape from mesenteric lymph nodes (MLN). These findings lead to the hypothesis that MLN macrophages or transitional mononuclear phagocytes (MP) could be among these critical cell types. Here, we show that a migratory CX_3CR1^{int} MP subset does not efficiently internalize *L. monocytogenes*, despite being recently classified as true macrophages. Additionally, we found that both primary MLN macrophages and MP cells produce significant amounts of IFNαβ and IL-18 during *in vitro L. monocytogenes* infection, indicating the presence of cytosolic bacteria within these subsets. This further suggests the potential of these myeloid subsets to act as a replicative niche for the *L. monocytogenes in vivo*.

II. Introduction

Listeria monocytogenes, a facultative intracellular foodborne pathogen, is readily taken up by phagocytes. Once inside the phagocytic vacuole, it quickly escapes using a cytolysin and phospholipases, and then replicates within the cytosol (Gaillard, Berche et al. 1987, Marquis, Doshi et al. 1995, Marquis, Goldfine et al. 1997). *L. monocytogenes* were initially believed to have the ability to infect and replicate within virtually any cell type, but further research highlighted that the activation state of phagocytic cells determines their ability to support growth. For

example, early studies by Mackaness demonstrated that macrophages developed resistance to the *L. monocytogenes* infection over time (Mackaness 1962). Later studies revealed that it was the exposure to the cytokines IFN-y and TNFα that activated these macrophages, leading to the inability of the bacteria to escape the vacuole and inhibition of *Listeria* growth (Biroum 1977, Kiderlen, Kaufmann et al. 1984, Shaughnessy and Swanson 2007). Despite this, macrophages are still thought to be the predominant growth niche for *L. monocytogenes* early during foodborne infection.

Macrophages create a cellular network within tissues, critical for monitoring and clearing invading microbes. Macrophages are the first cells infected with *L. monocytogenes*, upon entry into the Peyer's patches of the small intestine (Disson, Bleriot et al. 2018), marginal sinus of the spleen (Conlan 1996, Aichele, Zinke et al. 2003), and sinusoids of the liver (Cousens and Wing 2000). However, studies into the specific role of macrophage subsets within the mesenteric lymph nodes (MLN), the last line of defense against bacterial systemic spread, during *L. monocytogenes* infections are limited. Recent research has highlighted a subset of intestinal CX_3CR1^{int} mononuclear phagocytes (MP) that are derived from monocytes and transcriptionally align with macrophages, but express genes related to migration to the lymph node (Koscso, Kurapati et al. 2020). Given that macrophages are thought to poorly migrate out of the tissue, this discovery suggests a potential new role for these cells during a *L. monocytogenes* infection.

We previously showed that there is an unknown critical cell type in the MLN that allows for the systemic spread of *L. monocytogenes* (Tucker, Cho et al. 2023). Since macrophages have been identified as significant reservoirs for *L. monocytogenes* replication at other anatomical sites, we questioned if a specific MLN macrophage subset might serve as the niche allowing the bacteria to escape from the MLN, such as intestinal CX_3CR1^{int} macrophages. Thus, we hypothesized that infected CX_3CR1^{int} subsets could transport the bacteria from the lamina propria to the MLN and potentially other distant systemic organs. In this study, we show that primary CX_3CR1^{int} macrophages isolated from the lamina propria poorly

internalize *L. monocytogenes*. However, primary MLN macrophage and MP subsets produce a significant amount of IFNαβ and IL-18 during *in vitro L. monocytogenes* infection, suggesting these subsets could indeed be a replicative niche for the bacteria.

III. Results

L. monocytogenes does not efficiently invade lamina propria CX_3CR1^+ macrophage subsets.

Macrophages are thought to be the primary growth niche for *L. monocytogenes* during foodborne infection, however, it is unclear if they are essential for bacterial escape from the MLN. A recent study identified two distinct macrophage populations originating from circulating monocytes in the lamina propria: CX_3CR1^{hi} resident macrophages and CX_3CR1^{int} macrophages that migrate from the lamina propria to the MLN (Koscso, Kurapati et al. 2020). We previously demonstrated that both intracellular replication and cell-to-cell spread were crucial for the quick escape from the MLN (Tucker, Cho et al. 2023). However, our results indicated the existence of an alternate, less prominent pathway for systemic spread, that relies on intracellular replication within the MLN but does not depend on the cell-to-cell spread. Taken together, this prompts the question of whether this migratory CX_3CR1^{int} macrophage population could facilitate *L. monocytogenes* growth and contribute to the bacteria's subsequent escape from the MLN, potentially carrying *L. monocytogenes* to distant systemic organs.

To determine if primary small intestine lamina propria macrophages could support *L. monocytogenes* growth, we isolated the two distinct populations defined by cell surface expression of CX_3CR1 (Fig. 4.1A). The two macrophage populations were infected with *L. monocytogenes* at MOI=1 directly *ex vivo* and bacterial invasion efficiency was determined. Surprisingly, both CX_3CR1

macrophage populations inefficiently took up the *L. monocytogenes* at one-hour post-infection (Fig. 4.1B). CX_3CR1^{hi} macrophages internalized only 1% of the *L. monocytogenes* inoculum, while CX_3CR1^{int} macrophages had an invasion rate of less than 0.1%. For comparison, we previously found that bone-marrow-derived monocytes internalized very few bacteria, while a majority of the inoculum invaded bone-marrow-derived macrophages one-hour post-infection (Jones and D'Orazio 2017). Thus, our results suggest that lamina propria CX_3CR1 macrophage subsets have an invasion phenotype more similar to its monocyte progenitor than a bona fide macrophage.

We were concerned that the reduced invasion efficiency might be linked to cell death caused by the technical processing of cells. To investigate this possibility, we evaluated the viability of uninfected sort-purified cells over time. We found a significant decrease in cell viability within 18 hours post-sort, with only 60% of CX_3CR1^{hi} cells and 40% of CX_3CR1^{int} cells remaining viable (Fig. 4.1C). This indicated a potential issue affecting the health of the macrophages. Consequently, we decided to refrain from assessing *L. monocytogenes* growth within these cells until we addressed and resolved this concern.

MLN macrophages and MP cells produce a robust proinflammatory cytokine response to *L. monocytogenes* exposure.

Our previous research indicates that there is an unknown critical growth niche in the MLN that is required for the systemic spread of *L. monocytogenes* (Tucker, Cho et al. 2023). Macrophages have been highlighted as the predominant cell type that associates with *L. monocytogenes* upon bacterial entry into an organ. For instance, Disson et al. showed uptake of *L. monocytogenes* by CX_3CR1^{int} MP cells in Peyer's Patches following intestinal invasion (Disson, Bleriot et al. 2018). Other studies demonstrated that after intravenous infection, *L. monocytogenes* are trapped by marginal sinus macrophages before transport to the T cell zone

(Conlan 1996, Aichele, Zinke et al. 2003). Therefore, given their similarities, we hypothesized a similar process in the MLN, where SCS macrophages would promptly internalize and support *L. monocytogenes* growth upon entry via afferent vessels. Additionally, we speculated that following migration through the node, *L. monocytogenes* might also invade and replicate within medullary sinus (MM) macrophages before exiting the MLN through efferent vessels.

To determine if MLN macrophage subsets internalize *L. monocytogenes,* we assessed cytokine production triggered by cytosolic pattern recognition receptors (e.g., IL-18, Interferon α and β). These cytokines are induced when *Listeria* are present in the cell cytosol and either releases DNA (detected by RIG-I), or secretes c-di-AMP, triggering STING-dependent signaling (Stetson and Medzhitov 2006, Woodward, Iavarone et al. 2010), or activates the inflammasome and caspase-1 (Shi, Gao et al. 2017, Feng, Fox et al. 2018). We also assesed the production of IL-6, TNF-α and IL-12p70, cytokines likely induced due to the relate of lipoteichoic acid from dying bacteria. To isolate MLN SCS and MM macrophages, we initially utilized a gating strategy from a prior publication (Phan, Green et al. 2009), relying on CD169, CD11c, and F4/80 cell surface expression (Fig. 4.2A). However, this approach led to the identification of two distinct $CD169^{hi}$ populations: one expressing an intermediate level of CD11c and the other expressing no CD11c, a population that was not seen in the Phan et. al study. This discrepancy raised confusion, as the gating strategy was intended to isolate $CD169^{hi}CD11c^{lo}$ macrophages. Due to this uncertainty, we decided to adopt an alternative gating strategy outlined in Fig. 4.2B, allowing for the isolation of total macrophages, MP cells, $Ly6C^{hi}$ monocytes, and $CD11c^{hi}$ dendritic cells.

To evaluate cytokine production, we isolated and purified the four myeloid populations from the cMLN, infecting them with *L. monocytogenes* for an hour. Following infection, cells were washed and gentamicin was introduced to kill any extracellular bacteria, and supernatants were collected from both uninfected control cells and infected cells 24 hours later. We first assessed the production of type I interferons (IFN) and IL-18. We observed a significant production of IFNαβ

and IL-18 from cMLN macrophages and MP cells following *in vitro L. monocytogenes* infection (Fig. 4.3 A and B), suggesting the presence of cytosolic bacteria within these cells. CD11c^{hi} dendritic cells and Ly6C^{hi} monocytes did not produce substantial amounts of IFNαβ or IL-18 (Fig. 4.3 C and D). This was not a surprising result, as previous studies showed that both subsets inefficiently internalize and do not support the growth of *L. monocytogenes* (Jones and D'Orazio 2017, Jones, Smith et al. 2017). Additionally, we examined the production of IL-6, TNFα, and IL-12p70. Infected macrophage and MP populations significantly produced all three cytokines, while dendritic cells and monocytes showed minimal response to the *L. monocytogenes* infection. These results indicate that both MLN macrophage and MP populations contain cytosolic bacteria following *L. monocytogenes* infection *in vitro*, further suggesting these cells could be a potential growth niche for the bacteria *in vivo*.

MLN CD11c^{hi} dendritic cells and Ly6C^{hi} monocytes produce a pro-inflammatory response when extracellular *L. monocytogenes* are present throughout *in vitro* infection.

While the minimal production of IFNαβ and IL-18 in dendritic cells and monocyte subsets was not unexpected, the absence of IL-6, TNFα, and IL-12p70 production in these cells raised concerns. Our earlier studies demonstrated that *L. monocytogenes* attach to monocyte surfaces and can survive within dendritic cells for at least 4 hours (Jones and D'Orazio 2017, Jones, Smith et al. 2017), suggesting that the presence of the bacteria should trigger some surface TLRs, leading to cytokine production. We speculated that the immediate washing one hour post-infection before the addition of gentamicin did not provide enough time for the release of lipoteichoic acid from the bacteria and the subsequent stimulation of TLRs on dendritic cells and monocytes, thereby preventing cytokine production. To address this, we repeated the experiment using a “2X gentamicin method”, eliminating the washing step and removal of bacteria after the initial invasion and

allowing subsets to interact with dead/dying bacteria throughout the assay duration. This approach notably boosted cytokine production in *Lm*-infected monocytes and MP populations isolated from the cMLN, except for IFNα and IL-12p70, which were undetected in all cell subsets (Fig. 4.4 A and B). Surprisingly, cMLN dendritic cells and monocytes displayed significant IFNβ production, albeit not at the same level as macrophage and MP subsets (Fig. 4.4 C and D). Additionally, there was a marked increase in IL-6 and TNFα production in these subsets. Taken together, these results suggest that extracellular or endocytic *L. monocytogenes* does indeed trigger TLR responses in dendritic cells and monocytes, reaffirming our prior findings.

IV. Discussion

Macrophages have long been considered the primary growth niche for *L. monocytogenes* during foodborne infection. However, experimental confirmation of whether primary macrophages indeed serve as a replicative niche has never been shown. In our study, we present evidence indicating that both primary MLN macrophages and MP cells from the MLN produce a significant amount of IFNαβ and IL-18 during *in vitro L. monocytogenes* infection, indicating the presence of cytosolic bacteria within both subsets. These findings prompt speculation that the early events shown to occur in the Peyer's patches (Disson, Bleriot et al. 2018) of the intestine and the spleen (Conlan 1996, Aichele, Zinke et al. 2003) also occur within the MLN.

L. monocytogenes are readily taken up by macrophages *in vitro*. However, we found that *L. monocytogenes* inefficiently invaded both subsets of intestinal CX_3CR1 macrophages. This phenotype is the complete opposite from what has previously been shown when macrophages are infected with *L. monocytogenes in vitro*. One reason for this difference could be due to cell viability issues due to cell processing. Koscso et al. were among the first to identify intestinal CX_3CR1^{int} MP cells as macrophages (Koscso, Kurapati et al. 2020). Other recent functional studies have classified these cells as either monocyte-derived dendritic cells

(Zigmond, Varol et al. 2012) or bona fide dendritic cells (Cerovic, Houston et al. 2013, Scott, Bain et al. 2015). This suggests that while these cells show transcriptional similarities to macrophages, they might have a similar phenotype as monocytes or dendritic cells during *L. monocytogenes* infection—two cell types we have previously shown inefficiently internalize *L. monocytogenes*. To further characterize this intermediate population, additional functional studies like phagocytosis assays are warranted.

Our luminex immunoassay revealed that during *in vitro L. monocytogenes* infection, both MLN macrophages and MP cells produce comparable amounts of proinflammatory cytokines. This suggests that transitioning MP cells might function similarly to macrophages in the MLN. Supporting this notion, Jones et al. demonstrated that when bone marrow cells are differentiated into monocytes, macrophages, and transitioning cells (retaining Ly6C), the transitioning cells harbored a notably higher amount of gentamicin-resistant *L. monocytogenes* that contained actin tails compared to monocytes (Jones and D'Orazio 2017). This suggested that these cells could potentially serve as a replicative niche. Considering these findings, one might speculate that if these transitioning MP cells are migratory in the MLN, they could act as a growth niche and aid in *L. monocytogenes* escape into the bloodstream. Future studies should further separate these subsets based on their anatomic positions within the node and investigate whether *L. monocytogenes* can be detected within these subsets *in vivo*.

Figures

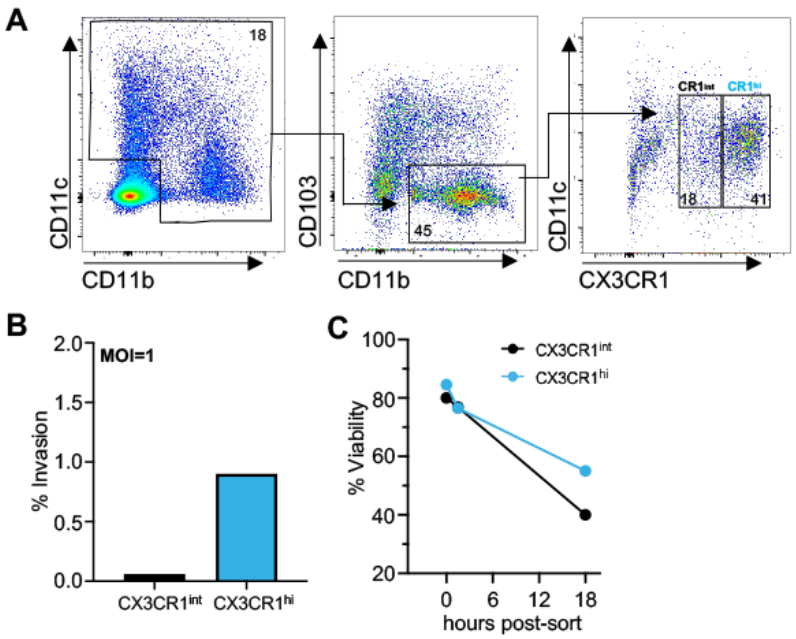

Figure 4.1: *L. monocytogenes* does not efficiently invade intestinal CX$_3$CR1 macrophages. CX$_3$CR1 macrophages were sort-purified from the small intestine lamina propria of uninfected BALB.CX$_3$CR1^{GFP} mice. (A) Gating schemes used to identify CX$_3$CR1^{int} and CX$_3$CR1^{hi} macrophages. (B) Sorted cells were infected *ex vivo* with with *Lm* SD2000 (MOI=1). Percent invasion of the inoculum that was gentamicin resistant 1-hour post-infection is shown. (C) Percent viability of cells post-sort. For panels B and C, data from one of two independent experiments is shown.

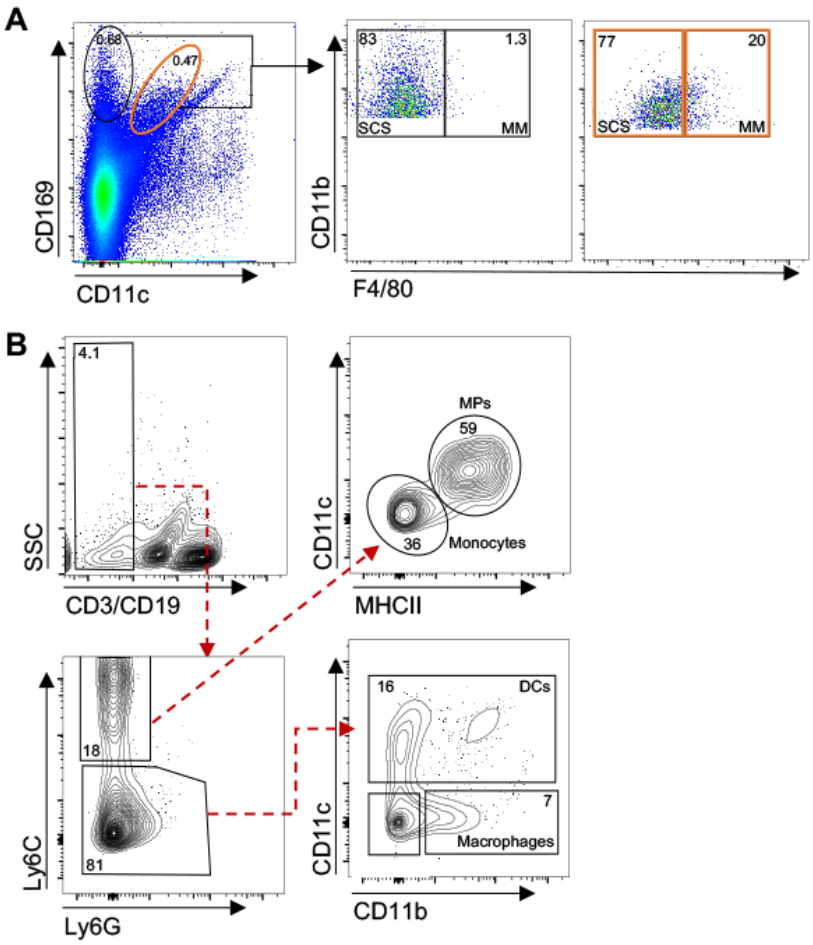

Figure 4.2: Gating scheme to isolate myeloid populations from the MLN. Ly6C^{hi} monocytes, MP cells, CD11c dendritic cells (DC), and macrophages were sort-purified from the MLN of uninfected BALB mice. (A) Gating scheme used to identify subcapsular sinus (SCS) and medullary sinus (MM) macrophages isolated from the small intestine draining lymph node (sMLN). (B) Gating scheme used to identify Ly6C^{hi} monocytes, MP cells, DC, and total macrophages isolated from the sMLN.

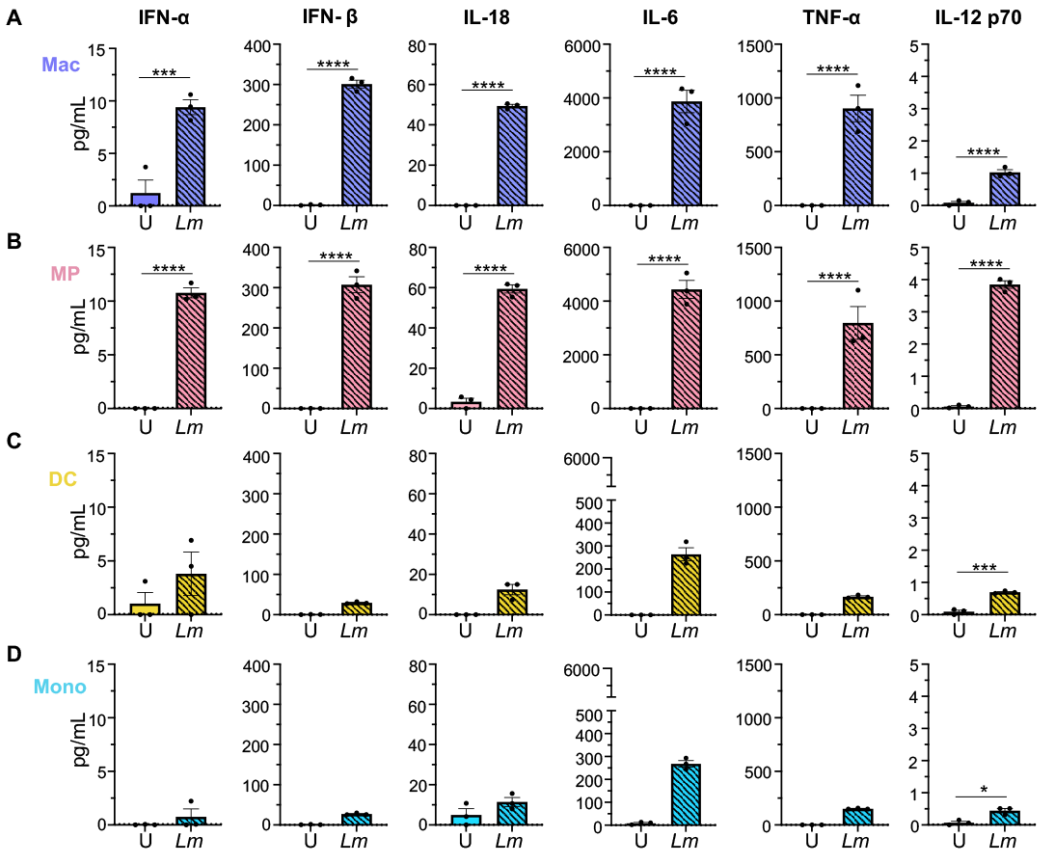

Figure 4.3: MLN macrophages and MP cells produce a robust proinflammatory cytokine response to *L. monocytogenes* exposure. Supernatants were collected from uninfected (U) and *Lm* SD2000-infected (*Lm*) cMLN macrophages (Mac), MP, dendritic cells (DC), and monocytes (Mono) (3 x 10^4 cells/well) at 24 hours for subsequent multiplex immunoassay. Cytokine responses of (A) Mac (B) MP (C) DC and (D) Mono are shown; symbols indicate mean values (+/- SEM) from three technical replicates; dotted lines indicate limits of detection. Bars indicate mean values for the technical replicates. Statistical significance was determined by one-way ANOVA.

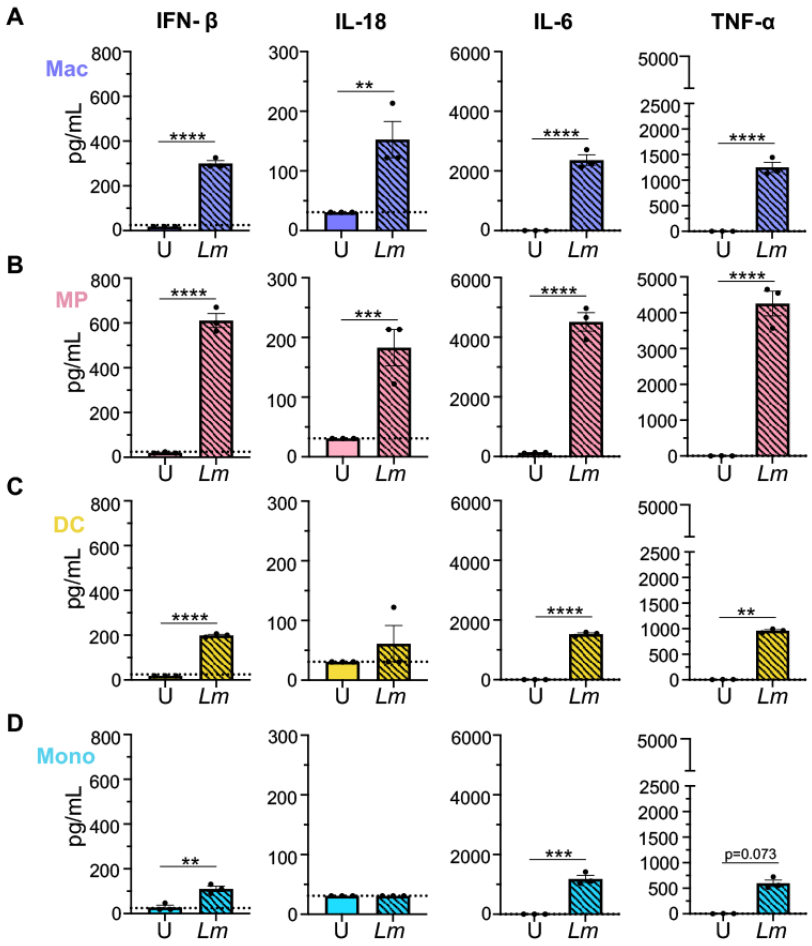

Figure 4.4: MLN dendritic cells and monocytes produce a pro-inflammatory response when extracellular *L. monocytogenes* are present throughout *in vitro* infection. Supernatants were collected from uninfected (U) and *Lm* SD2000-infected (*Lm*) cMLN macrophages (Mac), MP, dendritic cells (DC), and monocytes (Mono) (3 x 10^4 cells/well) at 24 hours for subsequent multiplex immunoassay. Selected cytokine responses of (A) Mac (B) MP (C) DC and (D) Mono are shown; symbols indicate mean values (+/- SEM) from three technical replicates; dotted lines indicate limits of detection. Bars indicate mean values for the technical replicates. Statistical significance was determined by one-way ANOVA. Not shown are 2 cytokines (IFNα and IL-12p70) which were either undetected or unchanged after *Lm* infection.

CHAPTER 5. LYMPH NODE STROMAL CELLS VARY IN SUSCEPTIBILITY TO INFECTION BUT CAN SUPPORT THE INTRACELLULAR GROWTH OF *LISTERIA MONOCYTOGENES*

The following chapter is modified from: Tucker, J. S., Khan, H., & D'Orazio, S. E. F. (2024). Lymph node stromal cells vary in susceptibility to infection but can support the intracellular growth of *Listeria monocytogenes*. *in revision* at Journal of Leukocyte Biology.

I. Summary

Lymph node stromal cells (LNSC) are an often overlooked component of the immune system, but play a crucial role in maintaining tissue homeostasis and orchestrating immune responses. Our understanding of the functions these cells serve in the context of bacterial infections remains limited. We previously showed that *Listeria monocytogenes*, a facultative intracellular foodborne bacterial pathogen, must replicate within an as-yet-unidentified cell type in the mesenteric lymph node (MLN) to spread systemically. Here, we show that *L. monocytogenes* could invade, escape from the vacuole, replicate exponentially, and induce a type I IFN response in the cytosol of two LNSC populations infected in vitro, fibroblastic reticular cells (FRC) and blood endothelial cells (BEC). Infected FRC and BEC also produced a significant chemokine and pro-inflammatory cytokine response after *in vitro* infection. Flow cytometric analysis confirmed that GFP^+ *L. monocytogenes* were associated with a small percentage of MLN stromal cells *in vivo* following foodborne infection of mice. Using fluorescent microscopy, we showed that these cell-associated bacteria were intracellular *L. monocytogenes* and the number of infected FRC and BEC changed over the course of a three-day infection in mice. *Ex vivo* culturing of these infected LNSC populations revealed viable, replicating bacteria that grew on agar plates. These results highlight the unexplored potential of FRC and BEC to serve as suitable growth niches for *L.*

monocytogenes during foodborne infection and to contribute to the pro-inflammatory environment within the MLN that promotes clearance of listeriosis.

II. Introduction

Lymph node stromal cells (LNSC) are non-hematopoietic structural cells responsible for compartmentalizing the lymph node and facilitating homeostasis by regulating immune cell migration and interactions within the node. Four subsets of $CD45^{neg}$ LNSC can be defined using expression of cell surface markers gp38 (podoplanin) and CD31 (platelet endothelial cell adhesion molecule): $gp38^{pos}CD31^{neg}$ fibroblastic reticular cells (FRC), $gp38^{pos}CD31^{pos}$ lymphoid endothelial cells (LEC), $gp38^{neg}CD31^{pos}$ blood endothelial cells (BEC), and $gp38^{neg}CD31^{neg}$ double negative cells (DNC) (Malhotra, Fletcher et al. 2012, Rodda, Lu et al. 2018). FRC are found in multiple compartments within the lymph node and can be further divided into nine subsets that vary in functionality (Rodda, Lu et al. 2018, Kapoor, Muller et al. 2021). For example, marginal FRC that line the cortical area are thought to give rise to follicular dendritic cells and promote B cell follicle formation during infection (Jarjour, Jorquera et al. 2014, Dubey, Lebon et al. 2016). In contrast, T cell zone FRC support naïve T cell homeostasis and facilitate interactions between T cells and dendritic cells (Link, Vogt et al. 2007). LEC compose the floor and ceilings of lymphatic vessels, subcapsular sinuses, and medullary sinuses and function to attract and then guide antigen-bearing immune cells entering the node (Jalkanen and Salmi 2020, Xiang, Grosso et al. 2020). Additionally, naïve lymphocytes enter the lymph node through high endothelial venules, which are specialized areas of blood vessels formed, in part, by BEC that generate a chemokine gradient and express a variety of adhesion molecules (Gowans and Knight 1964, Marchesi and Gowans 1964, Low, Hirakawa et al. 2018). Little is known about the DNC, a potentially heterogenous population of non-hematopoietic cells currently defined only by the lack of cell surface molecules associated with well characterized LNSC.

The ability of *Listeria monocytogenes* to invade a variety of mammalian cells is thought to be its primary virulence strategy. We previously found that the intracellular life cycle of *L. monocytogenes* was essential for bacteria egress from the MLN following foodborne infection; however, the key cell types in the MLN required for intracellular growth and systemic spread remain unknown (Tucker, Cho et al. 2023). Ly6C^{hi} monocytes are the predominant cell type associated with *L. monocytogenes* in the MLN three days post-infection; however, primary Ly6C^{hi} monocytes sort-purified from uninfected animals did not support intracellular growth of *L. monocytogenes* (Jones and D'Orazio 2017). Likewise, *L. monocytogenes* could invade primary conventional dendritic cells sort-purified from naïve mice but survived intracellularly for only a few hours (Jones, Smith et al. 2017). These results suggested that the critical intracellular growth niches in the MLN were likely to be a minor population of cells associated with *L. monocytogenes*. In the present study, we assessed the relative efficiency of *L. monocytogenes* invasion, survival, and intracellular replication in various stromal cell subsets. Using both transformed cell lines and primary cells isolated from the MLN, we show that at least two stromal subsets (FRC and BEC) can support exponential intracellular growth of *L. monocytogenes* and mount a significant inflammatory cytokine/chemokine response during a *L. monocytogenes* infection.

III. Results

L. monocytogenes replicate exponentially in a stromal cell line despite inefficient invasion.

To determine if *L. monocytogenes* could invade and replicate within stromal cells, we first used SVEC4-10, a murine blood endothelial cell line originally isolated from an axillary lymph node (O'Connell and Edidin 1990). The cells were infected with either mid-logarithmic phase or stationary phase *L. monocytogenes* for one hour and then gentamicin was added to kill any extracellular bacteria. This resulted in an invasion rate of less than 0.2% for either inoculum (Fig. 5.1A). For

comparison, non-phagocytic cells such as the human intestinal epithelial cell line Caco-2 or the murine colorectal cell line CT-26 typically internalize 1-3% of *L. monocytogenes* when used at a similar multiplicity of infection (Fig. 5.1B) (Gaillard, Berche et al. 1987, Monk, Casey et al. 2010). Thus, SVEC4-10 cells did not efficiently internalize *L. monocytogenes*. We did find a notable decrease in the invasion efficiency of *L. monocytogenes* grown at mid-log phase relative to stationary phase (Fig. 5.1A); this may suggest that a surface protein which promotes invasion of SVEC4-10 cells could be differentially expressed by the bacteria.

To determine if the few bacteria taken up by SVEC4-10 could replicate inside the cells, we performed an intracellular growth assay using stationary phase *L. monocytogenes*. We found that at 3 hours post-infection, the number of gentamicin-resistant *L. monocytogenes* had not changed relative to the number of bacteria recovered at 1 hour post-infection (Fig. 5.1C). However, by 5 hours post-infection, gentamicin-resistant *L. monocytogenes* had increased exponentially and continued through the duration of the assay (Fig 5.1C). To confirm these results, we performed differential “in/out” staining and found that the majority (~85%) of infected SVEC4-10 cells contained intracellular bacteria 4 hours post-infection (Fig 5.1D and 5.1E). At this early time point, most cells contained on average only 4 bacteria per cell (Fig. 5.1F) further demonstrating that infection of the SVEC4-10 cells was inefficient and proceeded slowly.

Following vacuole escape, *L. monocytogenes* polymerize host actin in the cytoplasm, which creates a “cloud” of actin surrounding the bacteria. Once *L. monocytogenes* begin replicating, polarized expression of the bacterial surface protein ActA recruits the actin to one pole of the bacterium forming a “tail,” and resulting in directional movement of the bacteria (Kocks, Hellio et al. 1993, Tucker, Cho et al. 2023). Therefore, to assess if the intracellular bacteria had escaped from the vacuole in SVEC4-10 cells and were replicating in the cytosol by 4 hours post-infection, we stained for F-actin (Fig. 5.1G). We found that approximately three-quarters of the intracellular bacteria visualized had either actin clouds or actin tails,

suggesting that most of the *L. monocytogenes* were localized to the cytosol by 4 hours post-infection. To determine if *L. monocytogenes* could spread from the cytosol of one SVEC4-10 cell to the cytosol of an adjacent cell using actin-based motility we performed plaque assays. L2 fibroblasts were used as a positive control since it is well established that *L. monocytogenes* form large clear plaques in these cell monolayers (Sun, Camilli et al. 1990, Marquis 2006). We found *L. monocytogenes* could create plaques within SVEC4-10 cells, although they were about half the size of those formed in L2 fibroblasts (Fig. 5.1H). Taken together, these results indicate that *L. monocytogenes* do invade SVEC4-10 cells, albeit inefficiently, and the few internalized bacteria can escape from the vacuole, replicate exponentially in the cytosol, and spread cell-to-cell.

Bulk LNSC isolated from either small or large intestine draining MLN support the exponential growth of intracellular *L. monocytogenes*.

Since LNSC constitute a small proportion of the MLN cellular composition, before attempting to purify the four stromal populations we first assessed if bulk $CD45^{neg}$ stromal cells isolated from the MLN could serve as an intracellular growth niche for *L. monocytogenes*. The small intestine (sMLN) and colon (cMLN) draining lymph nodes are distinct anatomic sites exposed to a varying composition and quantity of gut microbiota (Houston, Cerovic et al. 2016, Kho and Lal 2018) and we previously showed that *Listeria* infection persists longer in the colon than in the ileum following foodborne infection of mice (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015). Although not yet extensively characterized, there is some evidence to suggest that the proportions of immune cell subsets can also differ between these two MLN sites (Houston, Cerovic et al. 2016). Thus, we reasoned that the stromal populations within these two anatomical sites could also differ in their ability to support *L. monocytogenes* growth and opted to analyze the nodes separately.

Primary CD45^{neg} cells were enriched by magnetic bead separation and then sort-purified from the sMLN and cMLN of uninfected BALBcBy/J mice (Fig. 5.2A) with a sort purity consistently above 99% in each experiment (Fig. 5.2B). Cells were infected with stationary phase *L. monocytogenes* directly *ex vivo,* and the amount of intracellular bacteria was determined at multiple time points. At 1 hour post-infection, we recovered similar amounts of gentamicin-resistant *L. monocytogenes* from the sMLN and the cMLN, with CD45^{neg} cells at both sites internalizing approximately 1% of the *L. monocytogenes* inoculum (Fig. 5.2C). Gentamicin-resistant *L. monocytogenes* decreased slightly by 4 hours post-infection, but then increased exponentially in both MLN sites. This suggests that a substantial portion of the internalized bacteria were likely killed, and that the small subset of the bacteria that reached the cytosol required a few hours of replication to restore the bacterial burden. By 16 hours post-infection, a one log increase in bacterial burdens was observed (Fig. 5.2C). Notably, it took 16 hours to achieve these bacterial burdens, a growth rate slower than observed in SVEC4-10 cells where *L. monocytogenes* increased 100-fold over 8 hours (Fig. 5.1B). These results indicated that there was a CD45^{neg} cell type in the MLN that did support exponential growth of *L. monocytogenes* when infected directly *ex vivo*. Since we did not observe any difference in the ability of cells isolated from either sMLN or cMLN to serve as an intracellular growth niche, for the remaining experiments in this study we pooled cells from all the gut draining MLN to increase cell yields from each mouse.

Primary fibroblastic reticular cells and blood endothelial cells support exponential growth of *L. monocytogenes.*

To determine which primary LNSC subsets could support *L. monocytogenes* growth, we isolated four distinct stromal populations defined by cell surface expression of gp38 and CD31 (Fig. 5.3A and 5.4A). Although we could readily isolate sufficient FRC and DNC to perform *in vitro* infection experiments,

we found that the two endothelial populations constituted less than 20% of MLN stromal cells (~7-8 x 10^3 cells/mouse), a cell yield insufficient for *in vitro* experiments. Pezoldt et al. found that the transcriptome of LEC and BEC isolated from naïve peripheral lymph nodes (PLN) such as the inguinal and axial nodes did not differ significantly from the transcriptional profile of LEC and BEC isolated from the MLN (Pezoldt, Pasztoi et al. 2018). Therefore, to further increase the yield of these smaller populations, we also separately sorted cells harvested from PLN, and combined the isolated cells with the MLN populations only for the analysis of LEC and BEC.

$CD45^{neg}$ cells from the lymph nodes of uninfected mice were enriched using magnetic bead separation and FACS-purified, with a sort purity consistently above 98% for each experiment (Fig. 5.4B). The four stromal populations were infected with *L. monocytogenes* directly *ex vivo* and the bacterial invasion efficiency was determined. As shown in Fig. 5.4C, primary FRC, LEC, and DNC populations internalized approximately 1% of the inoculum, a level similar to that seen for internalin-mediated invasion of *L. monocytogenes* in many transformed epithelial cell lines (Gaillard, Berche et al. 1987, Monk, Casey et al. 2010). However, BEC were significantly different from the rest of the cells, internalizing less than 0.2% of the *L. monocytogenes,* an invasion rate similar to SVEC4-10 cells (Fig. 5.1A and Fig. 5.4C).

To determine if the bacteria that were taken up by the stromal populations could replicate intracellularly, we assessed the amount of gentamicin-resistant bacteria at multiple time points. In FRC, there was a 10-fold increase in bacteria over time (Fig. 5.5A). In contrast, gentamicin-resistant *L. monocytogenes* increased almost 100-fold in BEC during the 16 hour time period. To verify the intracellular localization of *L. monocytogenes* within FRC and BEC, we performed differential “in/out” staining at 8 hours post-infection (Fig. 5.5B). FRC contained ten *L. monocytogenes* per cell on average (Fig. 5.5C). For BEC, we observed a bimodal distribution with half of the cells containing 25-35 intracellular bacteria per cell and the other half harboring less than 15 per cell (Fig. 5.5C). To confirm that

L. monocytogenes were located within the cytoplasm of FRC and BEC, we also stained for F-actin at 8 hours post-infection (Fig. 5.5D). We found that 50-60% of *L. monocytogenes* were associated with either actin clouds or tails, suggesting that most of the internalized bacteria had localized to the cytosol by 8 hours post-infection in both FRC and BEC (Fig. 5.5E).

Sort-purified primary LEC and DNC that were infected *in vitro* displayed a different pattern. The number of gentamicin-resistant *L. monocytogenes* recovered from LEC stayed relatively consistent over a 16-hour period (Fig. 5.5A), suggesting that the bacteria were surviving, but not replicating extensively in this cell type. LEC contained, on average, only five *L. monocytogenes* per cell at 8 hours post-infection (Fig. 5.5B and 5.5C). None of the bacteria observed in LEC were associated with F-actin (Fig. 5.5D), suggesting that *L. monocytogenes* did not escape the vacuole. Although DNC readily internalized *L. monocytogenes*, gentamicin-resistant bacteria decreased significantly over time (Fig. 5.5A). We found that DNC only harbored 1-2 bacteria per cell and did not contain any *L. monocytogenes* associated with F-actin at 8 hours post-infection (Fig. 5.5B, 5.5C and 5.5D). Thus, DNC did not support the growth or survival of intracellular *L. monocytogenes*.

To confirm that our results for LEC and BEC were not altered pooling cells isolated from different lymph nodes, we performed a single experiment comparing MLN cells to PLN cells. As expected, BEC isolated from both type of nodes supported exponential growth of *Listeria* over 16 hours while LEC showed no change in bacterial burdens (Fig. 5.3B). For the more plentiful FRC and DNC we were able to demonstrate that cells isolated from PLN were nearly identical to those isolated from MLN. FRC and DNC internalized nearly 1% of the *L. monocytogenes* inoculum (Fig. 5.3C), and gentamicin-resistant bacteria increased over time in FRC, and decreased in DNC (Fig. 5.3D). Thus, the observed growth phenotypes of *L. monocytogenes* in LNSC appeared to be consistent irrespective of whether sMLN, cMLN, or PLN cells were used, indicating a broader applicability of these findings across different lymph nodes.

Pro-inflammatory cytokine responses of LNSC correlate with susceptibility to infection.

An important function of stromal cells is the secretion of chemokines and cytokines to maintain homeostasis and cell migration within lymph nodes, but it is not currently known if these cells produce pro-inflammatory mediators in response to *L. monocytogenes* that could promote clearance of the infection. To assess this, the four distinct stromal populations were FACS-sorted, infected with *L. monocytogenes*, and supernatants were collected from both uninfected control cells and infected cells 16 hours later. Given the small number of cells seeded in each sample well, we expected to find relatively low cytokine output, but detectable using a multiplex immunoassay. We first assessed the production of type I interferons, cytokines that are induced when *Listeria* localized to the cell cytosol either release DNA detected by RIG-I or secrete c-di-AMP to trigger STING-dependent signaling (Stetson and Medzhitov 2006, Woodward, Iavarone et al. 2010). Both FRC and BEC produced detectable IFNβ in response to *L. monocytogenes* (Fig. 5.5F), further confirming that these cell types contained cytosolic bacteria. Neither LEC or DNC contained cytosolic *Listeria* at 8 hours post-infection (Fig. 5.5D), so the lack of type I IFN secretion in these cell types was expected (Fig. 5.5F). Even bacteria that cannot escape from endocytic vacuoles can trigger a TLR-dependent cytokine responses, so we also assessed the production of IL-6, TNFα, and GM-CSF. We found that infected FRC and BEC displayed a proinflammatory phenotype, while the infected LEC and DNC remained relatively unchanged compared to cells at steady state (Fig. 5.5G and Fig. 5.6A-D). None of the stromal populations produced IL-10, suggesting that the infection did not illicit an anti-inflammatory response.

FRC and BEC have robust chemokine responses in response to *L. monocytogenes*.

Stromal cell-derived homeostatic chemokines play a crucial role in compartmentalizing the lymph node by guiding the precise localization of immune cells to distinct anatomical regions. In their absence, the lymph node architecture is disrupted, resulting in a dysfunctional immune response (Muller, Hopken et al. 2003). Using the same multiplex immunoassay as described above, we found that production of the gut-homing chemokines CCL19 and CCL22 did not change significantly in any of the four stromal populations, suggesting that *L. monocytogenes* does not alter the homeostatic function of these cells in the MLN (Fig. 5.7).

We next assessed the ability of these stromal cells to produce recruitment chemokines in response to *L. monocytogenes* infection (Fig. 5.8). We found that FRC produced the most robust chemokine response during *in vitro* infection of the FACS-sorted cells. Chemokines involved in neutrophil recruitment (CXCL1 and CXCL5), dendritic cell and monocyte migration (CCL2 and CCL7), and both T and B cell recruitment (CXCL13 and CXCL11), were all significantly increased in *L. monocytogenes*-infected FRC (Fig. 5.8A). Infected BEC also significantly increased production of a smaller subset of chemokines that included CXCL5, CCL2 and CXCL11 (Fig. 5.8B). On the contrary, uninfected LEC secreted low, but detectable amounts of CCL11 and CXCL13, but there was no induction following exposure to *L. monocytogenes* (Fig. 5.8C). Surprisingly the DNC did produce slightly more chemokines following infection, despite there being no evidence of replicating *L. monocytogenes* within these cells (Fig. 5.8D). This may reflect the fact that microbial ligands that can trigger TLR mediated chemokine production are more likely to be present when the bacteria are degraded. Together, these results suggest that both FRC and BEC can contribute significantly in attracting immune cells to the MLN following foodborne *L. monocytogenes* infection.

L. monocytogenes associate with stromal cells during foodborne infection of mice.

The results described above indicated that FRC and BEC could serve as an intracellular growth niche for *L. monocytogenes* when the bacteria were added directly to purified cells in a controlled *in vitro* environment. We next questioned whether infection of either of these stromal subsets occurred *in vivo* following foodborne transmission of *L. monocytogenes*. To begin to address this, we first confirmed that gp38 and CD31 could effectively be used to distinguish the four LNSC subsets in the context of a bacterial infection since the surface levels of some proteins do change in inflammatory environments. . MLN were harvested at 48- and 72- hours post-infection, timepoints when *L. monocytogenes* are consistently recovered from the lymph nodes of all orally infected mice (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015). As shown in Fig. 5.9A, expression profiles of gp38 and CD31 did not change in the MLN of infected mice, but the relative abundance of each stromal cell population was altered following foodborne infection (Fig. 5.9B). Notably, the two cell types found to support intracellular growth of *L. monocytogenes ex vivo*, FRC and BEC, increased in both percentage (Fig. 5.9C) and absolute number (Fig. 5.9B) of cells in the MLN at 48- and 72-. In contrast, the DNC population decreased significantly following foodborne infection, while the LEC population remained unchanged.

To determine if stromal cells were associated with *L. monocytogenes* during foodborne infection, we infected mice with *L. monocytogenes* that constitutively expressed GFP and harvested the MLN at 48 and 72 hours post-infection. The percentage of GFP-positive LNSC was determined by comparison to LNSC isolated from mice fed *L. monocytogenes* that lacked GFP. As shown in Fig. 5.9D, all of the GFP-positive LNSC had only low levels of fluorescence. As a positive control, we infected SVEC4-10 cells *in vitro* with the same *Listeria* strains and assessed their fluorescence by flow cytometry at 2- and 4-hours post-infection. At 2 hours post-infection, we expected to find a very small number of *Listeria*

associated with each cell (see Fig. 5.1C) and in fact, the GFP signal was very weak (Fig. 5.9E). In contrast, by 4 hours post-infection when the number of intracellular bacteria was expected to have increased exponentially (Fig. 5.1C), the GFP signal was much stronger. Thus, we interpret a weakly GFP-positive cell as identified by flow cytometry to be a cell that is infected with only one or perhaps a few *Listeria.*

Overall, we found that less than 5% of the stromal cells were infected with *L. monocytogenes* following foodborne infection. FRC were associated with *L. monocytogenes* at both time points during infection, however there were significantly fewer bacteria associated with FRC at 72 hours post-infection, suggesting that FRC infection peaks early and then begins to decline (Fig. 5.9F). The inverse was observed for BEC, with more GFP+ cells detected at 72 hours, although the difference was not statistically significant (Fig. 5.9F and 5.9G). Approximately 1% of the LEC were associated with *Listeria* at both time points (Fig. 5.9G). DNC gave variable results at 48 hours post-infection, with a relatively high level of GFP+ cells in two animals, but no GFP+ cells in the other three (Fig. 5.9F and 5.9G). These results suggest that the bacteria may associate with DNC for only a short period of time early during infection, which would be consistent with the ex vivo infections showing a continual decrease in the number of CFU detected.

We next questioned whether the *L. monocytogenes* associated with these cells were intracellular or extracellular bacteria. To test this, we infected animals with GFP-expressing *L. monocytogenes* and harvested MLN at 48, 60, and 72 hours post-infection. We performed differential ‘in/out’ staining and analyzed the sorted populations by immunofluorescence microscopy. As expected, FRC, LEC, and BEC all contained intracellular bacteria (Fig. 5.10A). When we assessed how many *L. monocytogenes* were found within each cell, FRC and BEC had significantly more intracellular bacteria (8-12) per cell at 72 hours post-infection than at earlier timepoints (Fig. 5.10B). Additionally, we found that that the percentage of GFP positive FRC significantly decreased by 72 hours post-infection, while the opposite phenotype was observed in BEC (Fig. 5.10C). Thus,

these results confirmed the infection patterns we observed using the flow cytometric approach shown Fig. 6E. In contrast, the amount of intracellular *L. monocytogenes* in LEC and the percentage of GFP^+ cells stayed relatively consistent throughout infection. Unsurprisingly, we did not observe any extracellular or intracellular *L. monocytogenes* associated with DNC, indicating that these cells were not well infected *in vivo* (Fig. 5.10A).

To determine if these intracellular *L. monocytogenes* were viable and capable of replicating, we sort-purified infected cells from mice 48 hours after foodborne challenge and then incubated them at 37˚C in the presence of gentamicin to allow for the growth of any intracellular bacteria. One-half of each population was incubated in gentamicin for 20 minutes and then lysed to determine how many intracellular *L. monocytogenes* were within the cells immediately post-sort. The remaining cells were incubated in gentamicin for an additional 8 hours to assess intracellular replication *ex vivo*. Notably, gentamicin-resistant *L. monocytogenes* significantly increased in the FRC population within 8 hours compared to the bacteria recovered immediately post-sort (Fig. 5.10D), a result similar to FRC exposed to *L. monocytogenes ex vivo* (Fig. 5.5A*)*. We found that only half of the mice harbored BEC that contained gentamicin-resistant bacteria, however, the bacteria within this subset increased 100-fold within 8 hours. LEC recovered from infected mice also harbored intracellular *L. monocytogenes*, and surprisingly, the number of gentamicin-resistant bacteria in LEC increased significantly during ex vivo incubation. This was in stark contrast to our findings using sort purified cells that were infected *in vitro*, suggesting that there may be a signal *in vivo* that alters these cells into a growth niche for *L. monocytogenes*. Consistent with our previous findings, few animals had DNC that contained gentamicin-resistant *L. monocytogenes* (Fig. 5.10D). Together, these results suggest that viable, replicating intracellular *L. monocytogenes* can be found within MLN stromal cells following foodborne infection.

IV. Discussion

In this study, we demonstrated that two LNSC subsets, FRC and BEC, could support exponential growth of *L. monocytogenes* and that replicating intracellular bacteria were found within these cells following foodborne infection of mice. Prior to this report, it was long thought that macrophages were the primary cell type in the MLN that served as a growth niche for *L. monocytogenes*. Macrophages are very likely to be a major site of intracellular growth in lymphoid tissues until they become activated by IFNg (Biroum 1977, Shaughnessy and Swanson 2007, Jones and D'Orazio 2017), but our data indicate that other cells within the MLN also need to be considered. Intracellular replication and subsequent cell-to-cell spread of *L. monocytogenes* is essential for rapid egress from the MLN (Tucker, Cho et al. 2023), but the critical cell type(s) in the MLN that are required for bacterial access to the bloodstream are still unresolved. Although *L. monocytogenes* can replicate in a wide variety of transformed cell lines, we previously showed that neither Ly6C^{hi} monocytes (Jones and D'Orazio 2017) nor conventional dendritic cells (Jones, Smith et al. 2017) isolated from the MLN were permissive for the intracellular replication. Identifying all the possible intracellular growth niches for *L. monocytogenes* in the MLN is essential to gain insights into the *in vivo* progression and systemic spread of these facultative intracellular pathogens.

Studies investigating the involvement of LNSC in the clearance of bacterial infections are limited. LNSC are difficult to work with and are often overlooked since they comprise only about 1% of the cells found in lymph nodes (Malhotra, Fletcher et al. 2012). However, LNSC are perfectly positioned to interact with infiltrating pathogens as they traffic through the lymph node, and there is some evidence to suggest that these cells can be important for bacterial virulence. For example, St John and Abraham showed that interactions between *Salmonella*-derived LPS and BEC in the mesenteric lymph node (MLN) resulted in the upregulation of suppressor cytokine signaling-3 expression, which in turn, disrupted secretion of homeostatic chemokines CCL21 and CXCL13 produced by

BEC (St John and Abraham 2009). The absence of these chemokines disrupted immune cell trafficking, which allowed the bacteria to persist. In another study, RNA-Seq analysis revealed that LNSC can upregulate MHC-II machinery in response to LPS (Malhotra, Fletcher et al. 2012). Although this typically results in the generation of T regulatory cells or T cell apoptosis during steady state conditions (Malhotra, Fletcher et al. 2012), it is not yet clear how the presentation of antigen via MHC-II by LNSC would influence the inflammatory environment during *L. monocytogenes* infection. Both these studies suggest that the role of LNSC can shift significantly during infection.

The primary virulence strategy of *L. monocytogenes* is to spread from cell-to-cell to avoid extracellular immune defenses. However, the lymph node contains an abundance of circulating immune cells that lack permanent cellular junctions and thus, do not readily allow for this mode of spread. FRC, which are present in nearly every lymph node compartment, may provide a three-dimensional network for spread of *L. monocytogenes* (Katakai, Hara et al. 2004, Link, Vogt et al. 2007). Given that FRC are in close proximity to one another and other cells within the lymph node, one could speculate that *L. monocytogenes* may preferentially target FRC early during the infection, allowing the bacteria to spread to different regions of the node while avoiding extracellular immune recognition. In fact, FRC have been shown to be a known key target within lymphoid tissues for several different viral infections (Davis, Anderson et al. 1997, Mueller, Matloubian et al. 2007, Steele, Anderson et al. 2009, Ng, Nayak et al. 2012, Twenhafel, Mattix et al. 2013). This idea is also consistent with our previous observation that cell-to-cell spread is required for rapid egress of *L. monocytogenes* from the MLN (Tucker, Cho et al. 2023), indicating that the critical cellular niche must be closely situated to other cells.

We found that *L. monocytogenes* replicated with a rapid growth rate within BEC, but the bacteria did not invade this subset very efficiently. Surprisingly, there was a notable bimodal distribution in the amount of intracellular *L. monocytogenes* found within BEC at 8 hpi. One possibility for this result could be that specific

subsets of BEC exhibit varying susceptibilities to *L. monocytogenes* infections. Alternatively, there might be a slight delay in bacterial vacuole escape, resulting in delayed exponential replication in some cells. BEC are positioned directly underneath FRC, and together, they create the high endothelial venules located in the T cell zone of the lymph node. Thus, the escape of bacteria from an infected BEC could provide direct access to the bloodstream, and we show here that the BEC are more heavily infected later during the course of the infection when the bacteria begin to spread systemically. We speculate that *in vivo L. monocytogenes* may initially invade the perivascular FRC and then use actin-based motility to spread into the underlying BEC rather than directly invade BEC. Supporting this idea, a study by Davis et al. showed a similar phenomenon in the MLN of Ebola virus-infected African green monkeys (Davis, Anderson et al. 1997). Electron microscopy images revealed that virally infected FRC surrounding high endothelial venules contained inclusions and budding virions that were later found in the underlying endothelium and bloodstream. Spreading from infected FRC into BEC could serve as an effective strategy for *L. monocytogenes* to also spread systemically.

Our data suggest that FRC and BEC may play a significant role in enhancing both the innate and adaptive immune response against *L. monocytogenes*. In addition to producing a variety of chemokines for the recruitment of immune cells into the node, we found that FRC produced a significant amount of IL-6 in response to *L. monocytogenes*. Brown et al. showed that FRC-derived IL-6 enhanced IL-2 and TNF-α production, as well as chromatin remodeling in activated $CD8^+$ T cells (Brown, Sen et al. 2019). *L. monocytogenes* is known to elicit a robust CD8 T cell response, and these cells play a pivotal role in resolving the infection. Outside the lymph node, cytokine production by stromal cells was shown to block reinfection of intestinal epithelial cells. Disson et al. found that *L. monocytogenes* that invaded Peyer's patches in gut triggered the production of IL-11 by $gp38^+$ stromal cells in the underlying lamina propria (Disson, Bleriot et al. 2018). This activated STAT3-dependent secretion of IFNg, resulting in increased epithelial proliferation, decreased goblet cell maturation, and

importantly, decreased accessibility to E-cadherin which blocked further InlA-mediated invasion of the intestinal villi. IL-11 was not included in our multiplex immunoassay, however, the idea that critical inflammatory mediators may be produced by infected stromal cells and that this can influence the course of *L. monocytogenes* survival and dissemination is likely to apply to other sites in the body such as the lymph node.

We observed a noteworthy difference in the ability of LEC to support intracellular growth of *L. monocytogenes* when comparing cells infected during foodborne challenge of mice and cells purified from naïve mice and then infected *ex vivo*. *L. monocytogenes* are auxotrophic for several nutrients that can be provided by the host. For example, we previously demonstrated that exogenous lipoate was severely limited in the extracellular environment of the MLN and *Listeria* could only obtain this nutrient within the cytosol of a mammalian cell (Tucker, Cho et al. 2023). It is possible that LEC have limited quantities of an essential nutrient at steady state that *L. monocytogenes* require for growth. The metabolic profile of LEC changed significantly when the cells were co-cultured with a breast cancer cell line (Acevedo-Acevedo, Millar et al. 2020), so it is possible that soluble factors produced during the inflammatory response to infection alter the cells in a way that promotes cytosolic replication of *L. monocytogenes*. Alternatively, the absence of *L. monocytogenes* growth noted *in vitro* might stem from a stress response triggered during the processing of the tissue and manipulation of the cells to achieve highly purified populations. However, it is worth noting that this was not observed in the case of FRC and BEC.

During *L. monocytogenes* foodborne infection, there was a significant shift in the proportions of FRC and BEC in the mesenteric lymph nodes. Since these cells have a critical structural role it makes sense that they would need to expand to accommodate the increased number of phagocytes and lymphocytes in the node. Likewise, other studies have shown that FRC and BEC both increase in response to viral (Abe, Shichino et al. 2014, Gregory, Walter et al. 2017) and parasitic infections (Dubey et al., 2016). However, Pezoldt et al. found that during

a *Y. pseudotuberculosis* bacterial infection, FRC decreased in number by 3 days post-infection (Pezoldt, Pasztoi et al. 2018). This might be attributed to the fact that *Y. pseudotuberculosis* has been shown to cause caseous necrosis of the MLN (El-Maraghi and Mair 1979). Interestingly, LEC proportions were unchanged following *L. monocytogenes* infection. Lucas et al. showed that both type I and type II interferons can inhibit LEC division following various inflammatory stimuli (Lucas, Finlon et al. 2018). Thus, the lack of change in the LEC population in our study could be due to interferon release in response to *L. monocytogenes*.

DNC are certainly the most understudied of all the stromal populations, but this study suggests that they may play a role during *L. monocytogenes* infection. Although DNC did not support the intracellular growth of *L. monocytogenes ex vivo*, they still produced cytokines and chemokines in response to the bacteria. These cells upregulated expression of IL-6, CXCL5 and CCL11, indicating that the DNC also contribute to the pro-inflammatory environment during *L. monocytogenes* infection. The DNC population decreased significantly during foodborne infection of mice. We speculate that DNC may consist of immature FRC or hematopoietic cells that have downregulated CD45 expression, and that the DNC may further differentiate in the setting of an inflammatory response such as occurs during *L. monocytogenes* infection. Identifying distinguishable surface markers on DNC in the future would significantly improve investigations into this particular cell subset.

The migration of *L. monocytogenes* through the spleen has been well-studied using the intravenous model of inoculation. It was previously shown that *L. monocytogenes* is quickly filtered from the bloodstream into the marginal sinus of the spleen, a compartment that resembles the subcapsular sinus in the MLN, in that it is organized by stromal cells and resident macrophages that quickly phagocytize *L. monocytogenes* (Conlan 1996, Aichele, Zinke et al. 2003, Neuenhahn, Kerksiek et al. 2006, Hardy, Chu et al. 2009, Edelson, Bradstreet et al. 2011, Mitchell, Brzoza-Lewis et al. 2011, den Haan, Mebius et al. 2012). Within 24 hours, the bacteria are localized within the periarteriolar lymphoid sheaths

surrounded by T cells (Berg, Crossley et al. 2005, Aoshi, Zinselmeyer et al. 2008). Although dendritic cells are implicated in the process, the exact mechanism is not well understood (Perez, Yeung et al. 2017). Only recently have research efforts shifted towards exploring the mechanism *L. monocytogenes* uses to spread systemically following foodborne transmission and the critical cellular niche in the MLN required for this escape (Jones and D'Orazio 2017, Jones, Smith et al. 2017, Imperato, Xu et al. 2020, Tucker, Cho et al. 2023). It is now clear from this study that both FRC and BEC can support the growth of *L. monocytogenes*, and any model of how *L. monocytogenes* overcomes the MLN barrier should take these potential growth niches into account. We predict that *L. monocytogenes* readily invade perivascular FRC, replicate in the cytosol, and use actin-based motility to spread into adjacent BEC. The rapid exponential intracellular growth we observed in BEC could lead to cellular lysis or membrane damage that would allow for bacteria to escape into the bloodstream. However, many unanswered questions remain that are essential for refining this model. Future studies should focus on determining how *L. monocytogenes* migrate through the lymph node from the afferent lymphatics to the T cell zone and what role, if any, FRC play in this process.

Figures

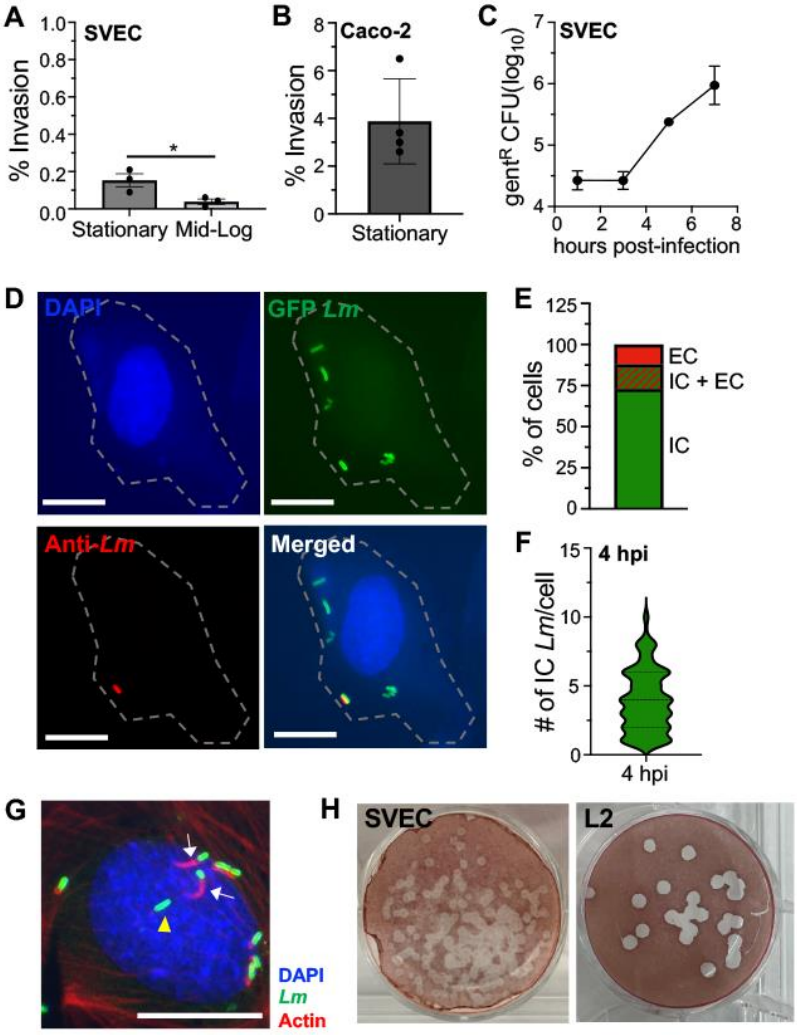

Figure 5.1: *L. monocytogenes* replicate exponentially in SVEC4-10 cells despite inefficient invasion. SVEC4-10 cells were infected with *L. monocytogenes (Lm)* at MOI=10. (A-B) Mean percentage (analyzed by Mann-Whitney) of *Lm* SD2000 inoculum that was gentamicin-resistant 1 hr post-infection in (A) SVEC4-10 cells or (B) Caco-2 cells. (C) Intracellular growth assay using stationary phase *Lm* SD2000. For panels A and C, mean values (±SEM) for triplicate samples from one of two experiments is shown. For panel B, mean values (±SEM) for quadruplet samples from one experiments is shown. (D) Representative images for differential "in/out" staining of cells infected with stationary phase *Lm* SD2710 (constitutive GFP), fixed at 4 hpi, and stained with DAPI (nucleus/blue), and anti-*Listeria* antibody (Lm/red). Scale bar, 5 µm. Dashed

line indicates the outline of infected cell from the brightfield image. (E) Stacked bar indicates the percentage of *Lm*-infected cells (n=300) with only intracellular bacteria (IC), both intracellular and extracellular (IC+ EC), or only extracellular bacteria (EC). (F) Violin plot indicates the number of intracellular *Lm* observed per cell (n=300). For panels C, D and E data from one of two independent experiments are shown. (G) Representative image for phalloidin staining of SVEC4-10 cells infected with *Lm* SD2710 at 4 hpi; white arrows indicate *Lm* associated with actin; yellow arrowhead indicates *Lm* not associated with actin. Scale bar, 10 µm. Two independent experiments (n=100 cells analyzed in each) were performed; in Exp. 1, 72% of Lm were associated with actin and in Exp. 2 80% were. (H) Representative images from one of three independent plaque assays using SVEC4-10 cells or L2 fibroblasts infected with *Lm* SD2000 and visualized 4 dpi.

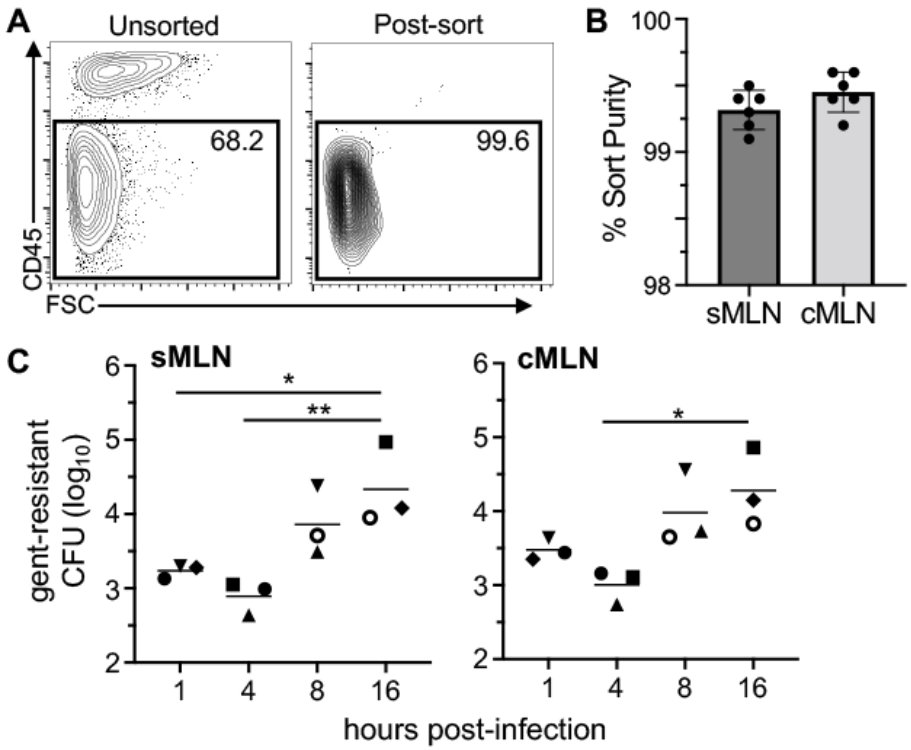

Figure 5.2: Bulk LNSC isolated from both small and large intestine draining MLN support exponential growth of intracellular *L. monocytogenes*. $CD45^{neg}$ cells were enriched from the MLN of uninfected BALBc/ByJ mice using magnetic bead separation and then sort-purified. (A) Representative dot plots indicate the purity of $CD45^{neg}$ cells after sorting. (B) Mean percent sort- purity (±SEM) of cells from the small intestine draining (sMLN) or colon draining MLN (cMLN). Each symbol represents the sort purity for pooled cells harvested from three animals that were used in a single experiment. (C) Sorted cells were infected with *L. monocytogenes* SD2000 (MOI=10) directly *ex vivo* and gentamicin-resistant *L. monocytogenes* were quantified. Pooled data from six separate experiments (each designated by a different symbol type) are shown; in each experiment, two time points were assessed. Horizontal lines indicate mean values for each time point. Statistical significance was determined by ANOVA with Tukeys multiple comparison test.

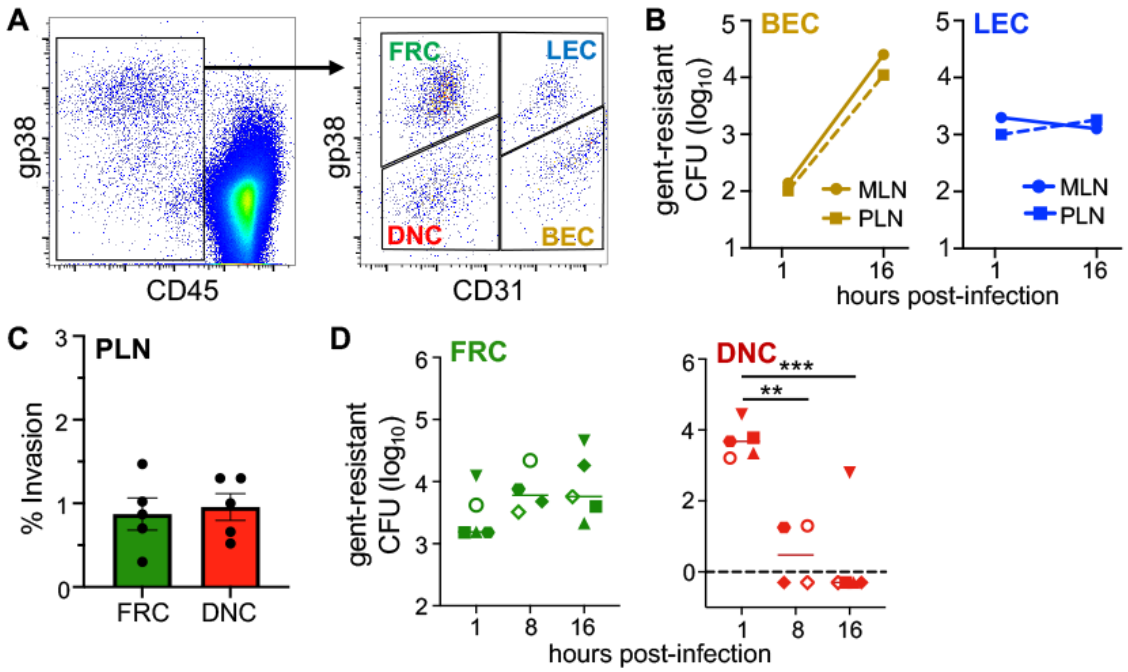

Figure 5.3: Primary fibroblastic reticular cells and blood endothelial cells isolated from murine PLN support the growth of *L. monocytogenes in vitro*. (A) Representative dot plot indicate the gating scheme used to identify stromal populations. (B) Sorted stromal subsets isolated from PLN (peripheral lymph nodes) or pooled sMLN and cMLN were infected directly *ex vivo* with *Lm* SD2000 (MOI=10). Intracellular growth assay comparing cells isolated from MLN (solid lines) and PLN (dashed lines). Symbols represent average values for duplicate samples from a single experiment. (C) Percent invasion (mean +/- SEM) represents the fraction of the inoculum that was resistant to gentamicin after 1 hour. Pooled data from five experiments is shown. (D) Intracellular growth assay for FRC and DNC sort purified from PLN and infected directly *ex vivo*. Pooled data from six separate experiments (each designated by a different symbol) are shown; in each experiment, cells sorted from three mice were used at two time points. Horizontal lines indicate median values; Dashed line indicates the limit of detection.

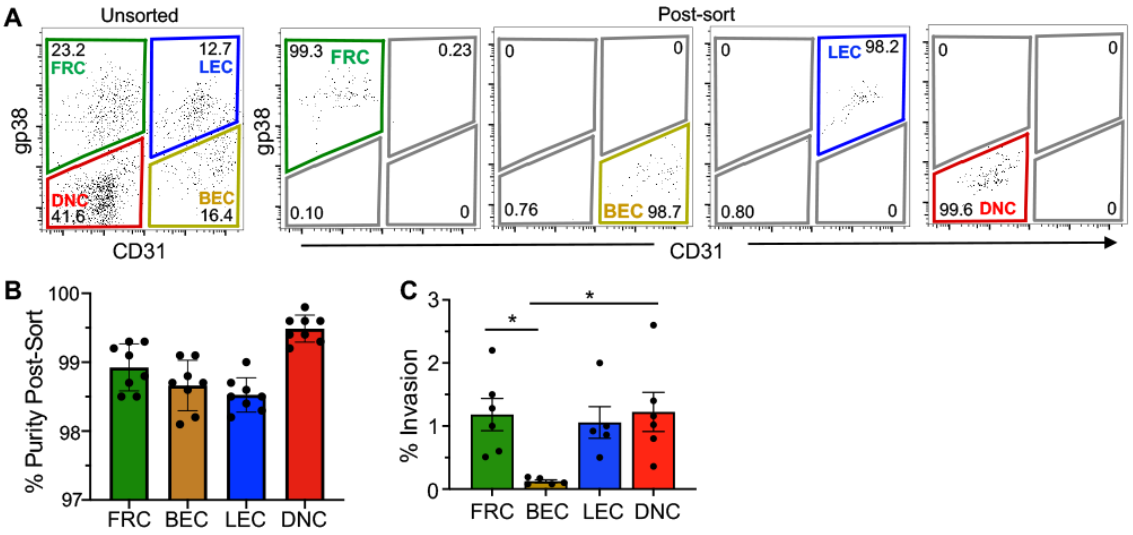

Figure 5.4: *L. monocytogenes* efficiently invade all stromal populations except primary blood endothelial cells when infected directly *ex vivo*. LNSC were sort- purified from the MLN and peripheral lymph nodes (PLN) of uninfected BALBc/ByJ mice. (A) Representative dot plots indicate the purity of stromal populations after sorting. (B) Mean percent sort-purity (±SEM) of each LNSC population; symbols represent individual experiments. (C) Sorted cells were infected *ex vivo* with with *Lm* SD2000 (MOI=10). Mean percent (±SEM) of the inoculum that was gentamicin resistant 1 hpi is shown; Pooled data from six separate experiments were analyzed by ANOVA with Tukeys multiple comparison test.

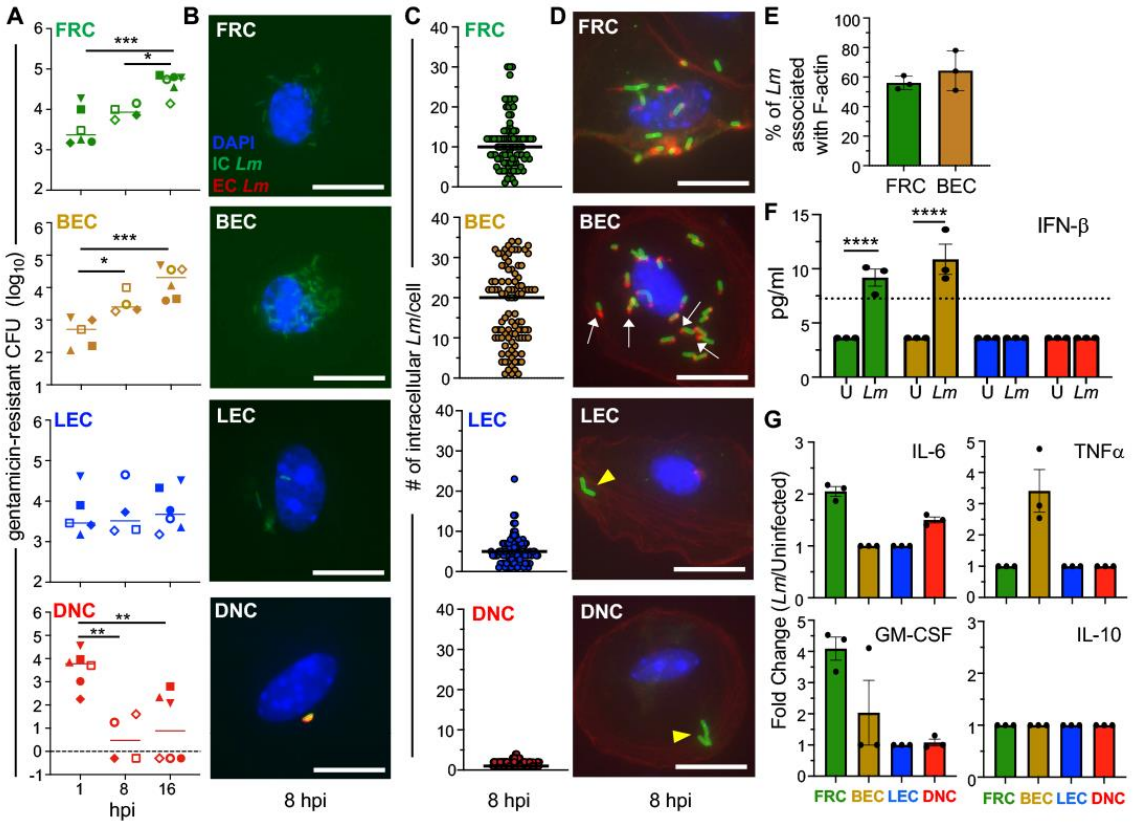

Figure 5.5: Primary FRC and BEC infected *ex vivo* support exponential growth of intracellular *L. monocytogenes.* LNSC were sort-purified from the MLN and PLN of uninfected BALBc/ByJ mice and infected with *Lm* at an MOI of 10. (A) Gentamicin protection assay for cells infected with *Lm* SD2000; pooled data from eight separate experiments (each designated by a different symbol type) were analyzed by ANOVA with Tukeys multiple comparison test. In each experiment, total cells sorted from three mice were used at two time points. (B) Representative images for differential ”in/out” staining of sort-purified LNSC infected with *Lm* SD2710 (GFP+), fixed at 8 hpi and stained with DAPI (nuclei; blue), and anti-*Lm* antibody (red); scale bar, 5 μm. (C) Symbols indicate the number of intracellular *Lm* observed in a single infected cell; horizontal lines indicate median values for n=100 cells. For panels B and C, data from one of two independent experiments are shown. (D) Representative images for phalloidin staining of cells infected with *Lm* SD2710. White arrows indicate actin tails associated with *Lm*; yellow arrowheads indicate *Lm* not associated with actin. Scale bar, 5 μm. (E) Mean percent (±SEM) of intracellular *Lm* associated with

actin. Each symbol represents results from a single experiment. (F) IFNβ present in supernatants of uninfected (U) and *Lm* SD2000-infected (*Lm*) cells (3 x 10^4 cells/well) at 16 hours was measured by multiplex immunoassay; symbols indicate mean values (+/- SEM) for duplicate samples from three separate experiments and were analyzed by one-way ANOVA. (G) Fold change of cytokine responses measured by multiplex immunoassay in *Lm*-infected cells relative to uninfected cells. Dashed lines indicate limits of detection.

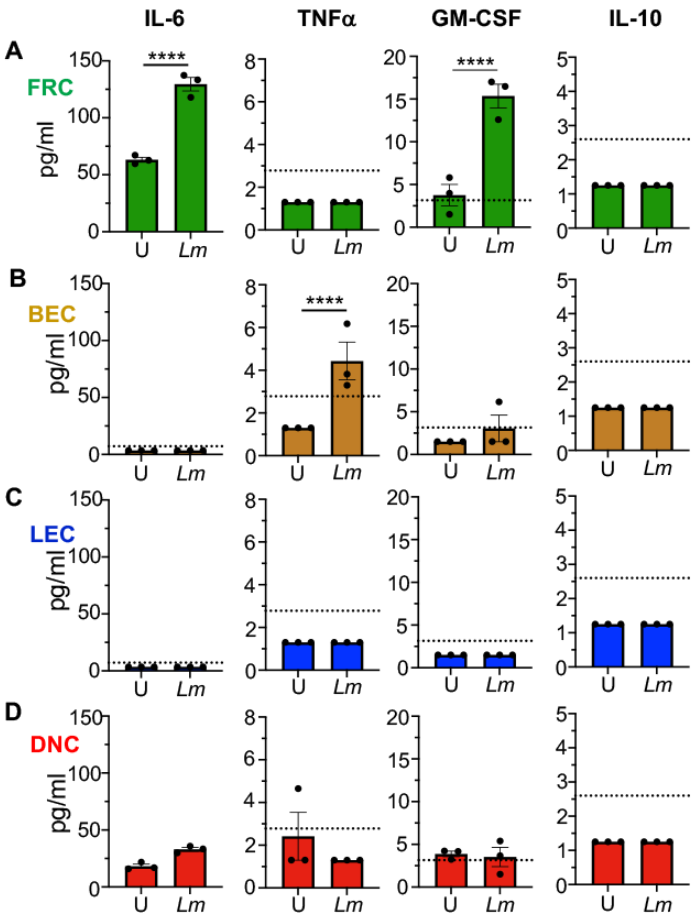

Figure 5.6: Primary data to support data shown in Fig. 5.5. Supernatants were collected from uninfected (U) and *Lm*-infected (*Lm*) stromal populations (3 x 10^4 cells/well) at 16 hours for subsequent multiplex immunoassay. Raw data used to determine the fold change values depicted in Fig. 5.5G are shown here. Symbols represent mean values for duplicate samples collected in three independent experiments; dotted lines indicate limits of detection. Bars indicate mean values for the three biological replicates; statistical significance was determined by one-way ANOVA.

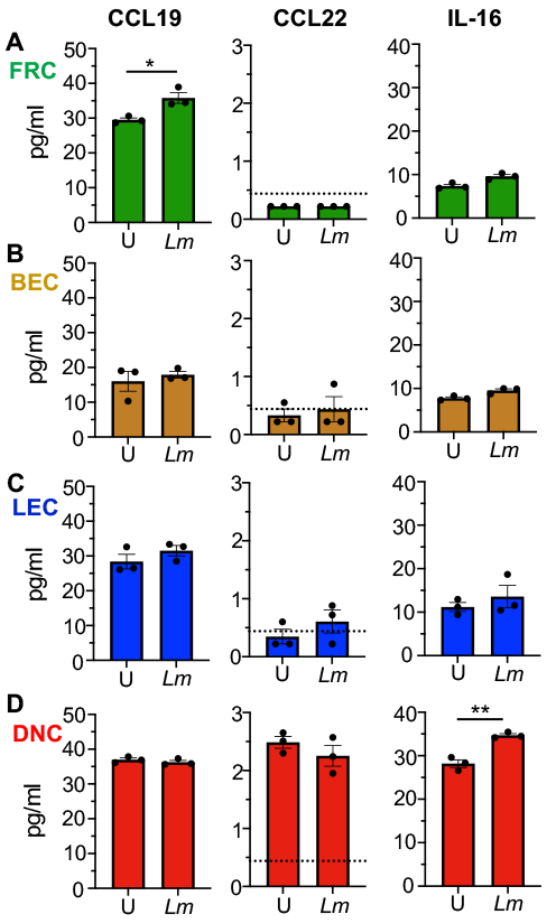

Figure 5.7: Stromal populations continually produce chemokines and cytokines important for homing of immune cells to the gut. Supernatants were collected from uninfected (U) and *Lm*-infected (Lm) stromal populations (3 x 10^4 cells/well) at 16 hours for subsequent multiplex immunoassay. Symbols indicate mean values for duplicate samples collected in three independent experiments; dotted lines indicate limits of detection. Bars indicate mean values (+/- SEM) for biological replicates; statistical significance was determined by one-way ANOVA.

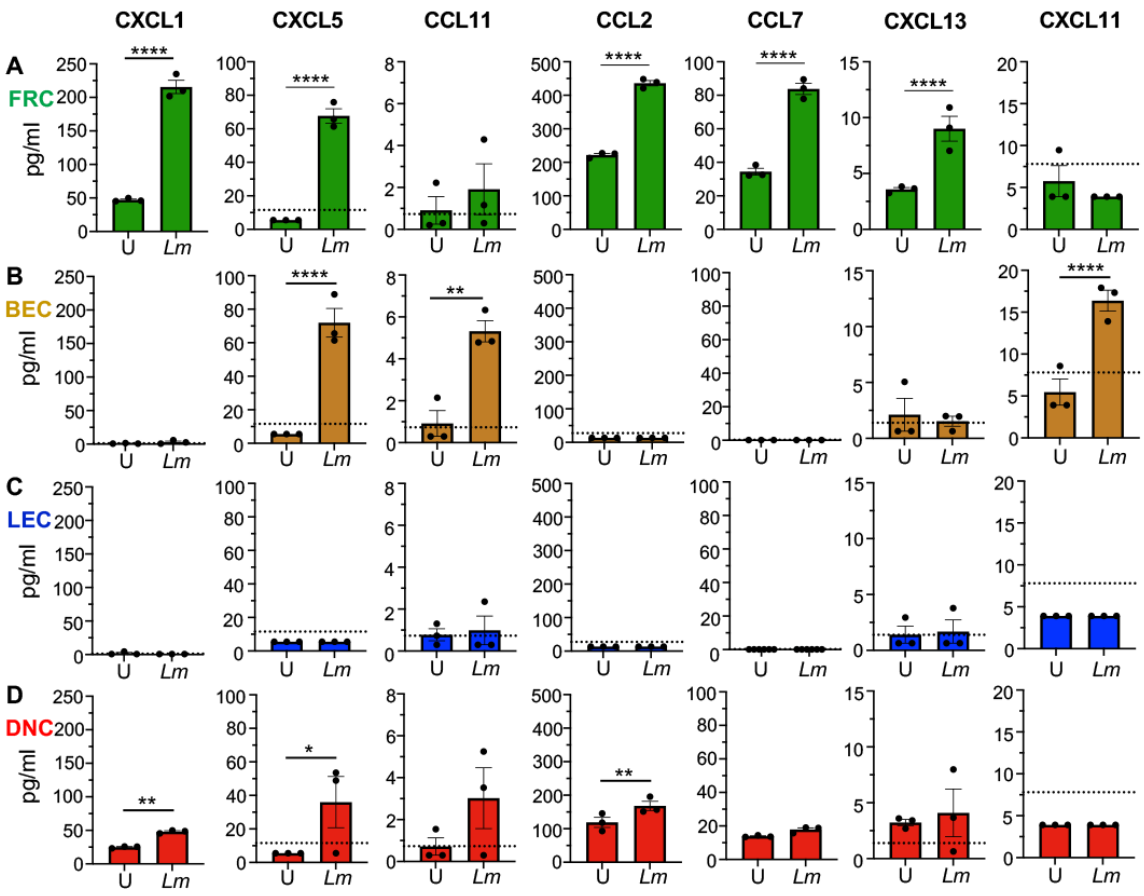

Figure 5.8: FRC, BEC and DNC produce chemokines in response to *L. monocytogenes* exposure. Supernatants were collected from uninfected (U) and *Lm* SD2000-infected (*Lm*) stromal cells (3 x 10^4 cells/well) at 16 hours for subsequent multiplex immunoassay. Selected chemokine responses of (A) FRC (B) BEC (C) LEC and (D) DNC are shown; symbols indicate mean values (+/- SEM) for duplicate samples from three separate experiments; dotted lines indicate limits of detection. Bars indicate mean values for the three biological replicates. Statistical significance was determined by one-way ANOVA. Not shown are 7 chemokines (CCL27, CCL3, CCL4, CXCL16, CXCL10, CCL12, and CCL17) and 9 cytokines (IFNα, IFNγ, IL-1β, IL-2, IL-4, IL-7, RANK-L, M-CSF, and VEGF-A) which were either undetected or unchanged after *Lm* infection.

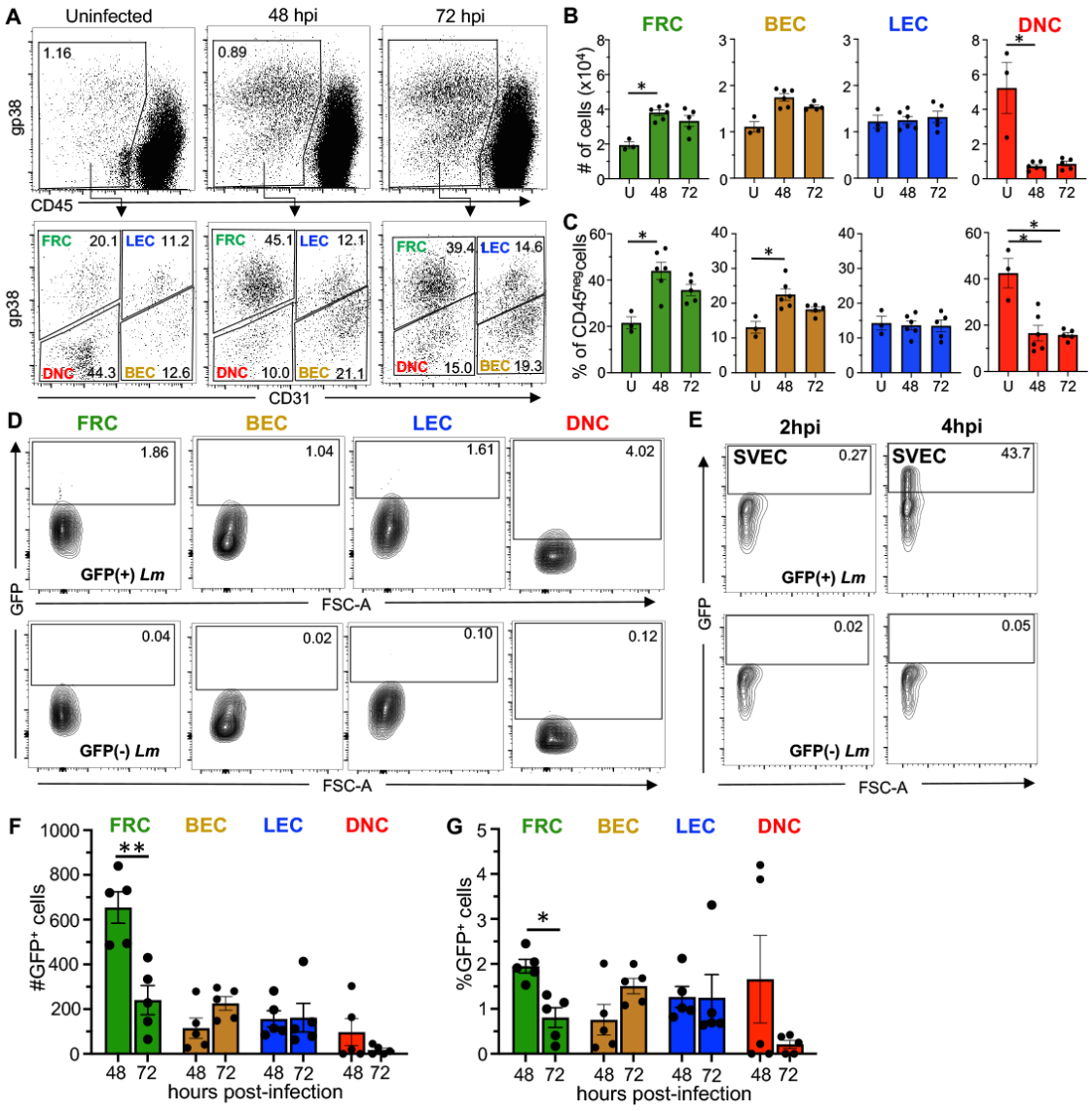

Figure 5.9: *L. monocytogenes* associate with stromal cells following foodborne infection. BALBc/ByJ mice were infected with 5 x 10^8 CFU of *Lm* SD2710 (GFP+) and MLN were harvested either 48 or 72 hpi. (A) Representative dot plots comparing stromal populations in the MLN of uninfected versus infected mice. (B) Total number and (C) Percentage of stromal cells (mean±SEM) in the MLN (n=3 uninfected; n=6 48 hpi) analyzed by Mann-Whitney test. (D) Representative dot plots showing gating strategy to identify GFP positive (*Lm*-associated) cells. All plots were pre-gated as shown in panel A. (E) Average percentage (±SEM) of GFP+ cells in each stromal population. Each symbol

represents results from a single experiment (one infected and one uninfected mouse per experiment). Statistical significance was determined by Mann-Whitney test.

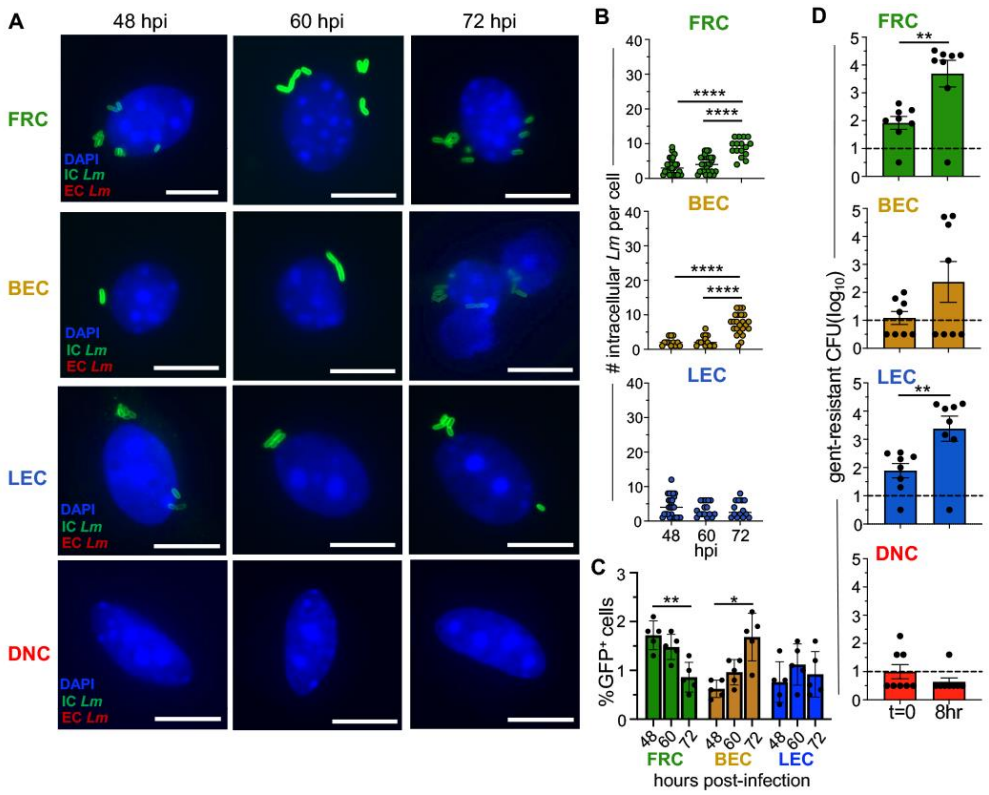

Figure 5.10: Live intracellular *L. monocytogenes* can be found within stromal populations following foodborne infection. BALBc/ByJ mice were infected with 5 x 10^8 CFU of *Lm* SD2710 and stromal populations were sort-purified from pooled MLN at 48, 60 and 72 hpi. (A) Representative images for differential "in/out" staining of stromal populations infected with *Lm* SD2710 at 48- 60- and 72-hours post-infection. Scale bar, 5 µm (B) Symbols represent individual cells in five experiments. Horizontal lines indicate mean values. Statistical significance was determined by ANOVA with Tukeys multiple comparison. At least 1500 cells of each population were visualized. (C) Average percentage (±SEM) of GFP+ cells in each stromal population. Each symbol represents results from a single experiment. Statistical significance was determined by Two-way ANOVA with Tukey's multiple comparisons. (D) Symbols indicate the number of gentamicin-resistant *L. monocytogenes* found within stromal populations at 48 hpi immediately post-sort (t=0) or after an 8 h incubation. Bars indicate mean value (±SEM); Dashed line indicate the limit of detection. Statistical significance was determined by Mann-Whitney analysis.

CHAPTER 6. PREDICTED MODEL OF SYSTEMIC SPREAD OF LISTERIA MONOCYTOGENES

**This work was completed with the help of Alondra Ali, a high school student, and Taylor Albrecht, an undergraduate. Under my mentorship and guidance, Alondra Ali performed invasion assays in SVEC4-10 cells (Fig. 5.1), and Taylor Albrecht performed LDH assays in SVEC4-10 cells (Fig. 5.3). All other experiments, figure generation, and writing were completed by me.

I. Summary

Listeria monocytogenes readily escapes the mesenteric lymph nodes (MLN), the final barrier preventing the systemic spread of both commensal and pathogenic bacteria. However, the events that occur in the MLN during a *L. monocytogenes* infection are understudied. Our previous research identified that there was a critical cellular growth niche within the MLN that facilitates *L. monocytogenes* escape into the bloodstream. We identified two stromal subsets, fibroblastic reticular cells (FRC) and blood endothelial cells (BEC), capable of supporting the exponential growth of *L. monocytogenes in vitro*, and found viable, replicating bacteria within these cells following foodborne infection. Given these novel findings, we show here a model of systemic spread in which *L. monocytogenes* must invade, escape the phagocytic vacuole, replicate, and undergo actin-based motility in the cytosol, in a critical cell type in the MLN that provides access to the bloodstream. Additionally, we show that the exponential growth of *L. monocytogenes* induces damage to BEC *in vitro* and that killing extracellular *L. monocytogenes* at the onset of their spread into the bloodstream effectively prevents spleen colonization.

II. Introduction

Listeria monocytogenes, a Gram-positive foodborne pathogen, must overcome numerous bottlenecks within the gut before spreading into the

bloodstream. Bacteria that withstand the harsh conditions of the stomach and duodenum are thought to undergo genetic modifications favoring gut adaptation, potentially aiding in intestinal invasion (O'Driscoll, Gahan et al. 1996, Saklani-Jusforgues, Fontan et al. 2000, Wemekamp-Kamphuis, Wouters et al. 2002, Toledo-Arana, Dussurget et al. 2009). However, despite these adaptations, their ability to invade gut epithelial cells remains infrequent. Following intestinal invasion, the remaining bacteria encounter yet another barrier: the lamina propria. Within this environment, *L. monocytogenes* may re-invade epithelial cells (Gaillard and Finlay 1996) or interact with infiltrating immune cells (Jones and D'Orazio 2017, Jones, Smith et al. 2017, Tucker, Cho et al. 2023). Alternatively, a few bacteria will disseminate to the mesenteric lymph nodes (MLN), the final physical barrier preventing both commensal and pathogenic bacteria access to the bloodstream.

We previously demonstrated that *L. monocytogenes* requires both intracellular replication and cell-to-cell spread for rapid egress from the MLN (Tucker, Cho et al. 2023), indicating the involvement of a critical cell type within the MLN that facilitates *L. monocytogenes* escape. However, to date, the early events and mechanisms that occur within the MLN during a *L. monocytogenes* infection have yet to be explored. Insights into these potential events can be gleaned from extensive intravenous studies conducted in the spleen during *Listeria* infection, which is similar in structure and function to lymph nodes, and could offer a comparative basis. For example, during intravenous infection, *L. monocytogenes* are quickly filtered from the bloodstream into the splenic marginal sinus, a compartment that resembles the subcapsular sinus in lymph nodes (Hardy, Francis et al. 2004, Neuenhahn, Kerksiek et al. 2006, Hardy, Chu et al. 2009, Edelson, Bradstreet et al. 2011, Mitchell, Brzoza-Lewis et al. 2011). Within this compartment, resident macrophages trap *L. monocytogenes* upon their splenic entry (Conlan 1996, Aichele, Zinke et al. 2003), followed by dendritic cell transport of *L. monocytogenes* to the periarteriolar lymphoid sheaths for T cell antigen presentation (Berg, Crossley et al. 2005, Aoshi, Zinselmeyer et al. 2008)—a plausible scenario that could also unfold in the MLN.

This chapter focuses on the later stages of *L. monocytogenes* infection within the lymph node. Building upon earlier findings, we present our working model of systemic spread. Through a combination of *in vitro* and *in vivo* preliminary studies, we refine our understanding of how *L. monocytogenes* escapes into the bloodstream. We show that the exponential replication *of L. monocytogenes* results in the lysis of a blood endothelial cell line and primary blood endothelial cells (BEC). Additionally, we demonstrate that eliminating extracellular *L. monocytogenes* at 48 hours post-infection, a time when the bacteria begin spreading into the blood, effectively prevents colonization of the spleen.

III. Results

Predicted model of systemic spread of *L. monocytogenes* from the MLN.

L. monocytogenes can readily overcome the MLN barrier, resulting in life-threatening systemic infections. We previously showed that both intracellular replication and cell-to-cell spread were required for the rapid dissemination of *L. monocytogenes* from the MLN, suggesting that there is a critical cellular niche that is required for escape from the lymph nodes (Tucker, Cho et al. 2023). A subsequent study showed that this cell type could be a stromal subset, as we showed that *L. monocytogenes* could escape the phagocytic vacuole, replicate exponentially in the cytosol, and form actin tails within two stromal subsets, fibroblastic reticular cells (FRC) and BEC. Given these findings, we have developed a working model of systemic spread that describes a predominant route of *L. monocytogenes* escape through high endothelial venules (HEV) in the T cell zone of the MLN (Fig. 6.1). We predict that *L. monocytogenes* invade perivascular FRC, use actin-based motility to spread into the underlying BEC, and replicate exponentially within the cytosol, resulting in lysis or membrane damage that would allow for extracellular bacteria to escape into the blood. Additionally, we also predict a minor route of systemic spread that is dependent on intracellular replication in the MLN, but independent of cell-to-cell spread (Fig. 6.1). In this case,

L. monocytogenes either attaches to or invades a migratory cell, and exits the MLN through the efferent vessel, entering circulation via the thoracic duct.

InlA and InlB surface proteins are required for efficient invasion of stromal cells.

We previously showed that *L. monocytogenes* inefficiently invaded SVEC4-10 cells, a murine blood endothelial cell line originally isolated from an axillary lymph node (O'Connell and Edidin 1990), and sort-purified BEC compared to the other stromal subsets, suggesting the bacteria likely employ different surface proteins to facilitate their uptake. *L. monocytogenes* possess a family of internalins (Inl) that are thought to play a role in the attachment (Bierne, Sabet et al. 2007), invasion of host cells (Mengaud, Ohayon et al. 1996, Bierne, Sabet et al. 2007, Ghosh, Halvorsen et al. 2018), and cell-to-cell spread (Polle, Rigano et al. 2014, Dowd, Mortuza et al. 2020). However, InlA and InlB, among these proteins, are considered the primary surface invasins responsible for uptake by nonphagocytic cells (Gaillard, Berche et al. 1991, Dramsi, Biswas et al. 1995, Gaillard and Finlay 1996). Therefore, we hypothesized that the necessity of either InlA or InlB for invasion might differ based on the specific stromal subset.

To determine if either protein was required for invasion, SVEC4-10 cells and sort-purified stromal subsets were infected with either stationary phase SD2000, *ΔinlA*, or *ΔinlA/ΔinlB L. monocytogenes* at an MOI of 10 for one hour and then gentamicin was added to kill any extracellular bacteria. We observed a 50% reduction in the ability of *ΔinlA L. monocytogenes* to invade SVEC4-10 cells and a further decrease in invasion efficiency of *ΔinlA/ΔinlB L. monocytogenes* compared to wildtype bacteria (Fig. 6.2A), indicating that both InlA and InlB are involved in invasion of this cell line. Additionally, we found notable differences in the requirement for InlA and InlB in primary sort-purified stromal cells. Similar to SVEC4-10 cells, both InlA and InlB contributed to *L. monocytogenes* invasion of FRC (Fig. 6.2B). However, only *ΔinlA/ΔinlB L. monocytogenes* had a reduction in the ability to invade primary BEC, LEC and DNC, while *ΔinlA L. monocytogenes*

showed an invasion rate similar to wildtype, suggesting that entry into these cells is primarily mediated by the InlB protein. Though preliminary, these findings suggest that both surface proteins, InlA and InlB, contribute to *L. monocytogenes* entry into stromal cells, with InlB exerting a more significant role than InlA in most subsets.

Exponential replication of *L. monocytogenes* results in lysis of SVEC4-10 and primary blood endothelial cells.

We predict that the egress of *L. monocytogenes* from the MLN is dependent on the exponential replication of the bacteria within BEC and subsequent lysis of the cell, leading to extracellular escape into the bloodstream (Fig. 6.1). Thus, to determine if replication of *L. monocytogenes* within BEC does, in fact, result in cell damage we performed a cytotoxicity assay. SVEC4-10 cells and primary stromal subsets were infected with either *ΔlplA1, lplA1(+), ΔactA, actA(+),* or SD2000 *L. monocytogenes* for 16 or 24 hours and supernatants were collected to measure lactate dehydrogenase release. As expected, *ΔlplA1 L. monocytogenes,* a strain unable to replicate intracellularly, caused significantly less cytotoxicity to SVEC4-10 cells by 24 hours post-infection (Fig. 6.3A). However, both the complemented strain, *lplA1(+),* and SD2000 resulted in cytotoxicity for about 60-80% of the cells, indicating *L. monocytogenes* can cause lysis of this cell line. In contrast, the *actA* mutant caused very little cell death (Fig. 6.3B), a surprising result since this strain is known to achieve high intracellular loads over time (Domann, Wehland et al. 1992, Kocks, Gouin et al. 1992). These results suggested that the intracellular replication of wildtype *L. monocytogenes* does indeed result in lysis of SVEC4-10 cells.

Given the low cellular yield of primary cells, only the capacity of SD2000 to induce lysis of sort-purified stromal subsets at 16 hours was evaluated. A preliminary experiment found that surprisingly, wildtype *L. monocytogenes* caused relatively low cytotoxicity to FRC at 16 hours post-infection, despite our previous finding that *L. monocytogenes* can replicate exponentially in this cell type (Fig

6.3D). In contrast, we found that SD2000 resulted in cytotoxicity of about 40% of BEC, suggesting that replication of the bacteria did result in the cell death of these cells, albeit not to the level of SVEC4-10 cells. As anticipated, there was minimal cytotoxicity in infected LEC and DNC cells, both of which are known to not support the growth of *L. monocytogenes*. These findings collectively indicate that the intracellular growth of *L. monocytogenes* may lead to the subsequent damage of BEC, suggesting that the lysis of this cell type might be a mechanism through which the bacteria escape the MLN.

Killing extracellular *L. monocytogenes* 72 hours post-infection blocks colonization of the spleen.

We previously showed that cell-to-cell spread was required for rapid dissemination of *L. monocytogenes* from the MLN (Tucker, Cho et al. 2023). We hypothesize in our model of systemic spread that this cell-to-cell spread is critical only for *L. monocytogenes* to spread from FRC into the underlying BEC that form HEV, before ultimately escaping into the blood extracellularly. Thus, to address this hypothesis, we used an *in vivo* approach. We knew from our previous studies that colonization of the spleen and liver begins to occur about 48 hours after foodborne transmission (Bou Ghanem, Jones et al. 2012, Jones, Bussell et al. 2015). Therefore, mice were co-infected with *ΔactA L. monocytogenes* and the complemented strain and the infection was allowed to proceed until 45 hours post-infection and then gentamicin was administered every 8 hours until tissues were harvested. If the actA mutant required membrane damage in a cell type inaccessible without cell-to-cell spread, such as BEC, and extracellular release into the blood, then we expected to see no colonization of the spleen and liver in the gentamicin-treated mice. In fact, killing extracellular bacteria completely blocked the dissemination of *ΔactA L. monocytogenes* to the spleen and also reduced dissemination of wildtype bacteria by about ninety percent (Fig. 6.4). Similar results were seen in the liver, although a small number of *ΔactA L. monocytogenes* were able to spread to the livers of a few of gentamicin-treated

mice. Likewise, gentamicin treatment significantly decreased bacterial burdens in the cMLN, but not the sMLN, suggesting that *L. monocytogenes* are predominantly intracellular in the sMLN at 45 hours post-infection. Together, these results indicate that killing extracellular bacteria at a critical time point when *L. monocytogenes* exits the MLN and spreads systemically blocks most of the colonization of the spleen and liver.

IV. Discussion

Very few studies have examined the events that occur in the MLN following *L. monocytogenes* foodborne infection, despite its pivotal role in blocking systemic dissemination. This model stands as the first depiction describing the systemic spread of *L. monocytogenes* from the MLN. Here, we present preliminary evidence indicating that *L. monocytogenes* can lyse primary blood endothelial cells and show that extracellular dissemination is the primary route from the MLN. Overall, this research emphasizes how *L. monocytogenes* has adapted to surpass the gut's primary defense mechanism intended to limit systemic spread.

We found that InlB, and not InlA, play a key role in facilitating entry into primary BEC, LEC, and DNC. However, our previous findings indicated that *L. monocytogenes* inefficiently invaded BEC compared to other stromal subsets. This could be linked to a potential lower expression of Met, a receptor tyrosine kinase that binds InlB, in BEC compared to LEC and DNC. However, further investigation is required to confirm Met expression levels across stromal populations. Nevertheless, these results collectively suggest that direct invasion of BEC *in vivo* is unlikely, supporting our model that ActA-dependent cell-to-cell spread within the MLN could easily lead to intracellular localization within BEC. Consistent with this idea, earlier *in vitro* studies showed that *L. monocytogenes* could readily spread from infected phagocytes directly into cultured human umbilical vein endothelial cells or human dermal microvascular endothelial cells (Drevets, Sawyer et al. 1995, Drevets, Jelinek et al. 2001). Thus, despite the abundant cellularity present in the lymph nodes, most lack the close cellular junctions necessary for cell-to-cell

spread of the bacteria. This highlights that the critical cell types important for escaping the MLN are positioned in close proximity, such as FRC and BEC, forming the HEV.

Direct access to the bloodstream could occur if exponential growth of *Listeria* within the cytosol of BEC resulted in a loss of membrane integrity, as demonstrated here in an in vitro assay. Surprisingly, we found that *ΔactA L. monocytogenes* did not lyse SVEC4-10 cells efficiently. This was unexpected given that *ΔactA L. monocytogenes* did replicate extensively in the endothelial cell line and were previously shown to replicate exponentially in J774 macrophages (Brundage, Smith et al. 1993). Additionally, Neiman-Zenevich et al. showed that intracellular growth of an *ΔactA* variant of strain 10403s led to the same amount of LDH release from the human fibroblast cell line HT1080 as wildtype *L. monocytogenes* (Neiman-Zenevich, Stuart et al. 2017). Though the *ΔactA* and *ΔlplA1 L. monocytogenes* mutants used here were both constructed an *L. monocytogenes EGDe* background, the two strains originated from different labs. When comparing the whole genome sequences of these two EGDe variants, we found a single nucleotide polymorphism in the *plcB* gene of *ΔactA L. monocytogenes* that was not present in *ΔlplA1 L. monocytogenes*. PlcB is a secreted phospholipase that plays a key role in lysing the double membrane vacuole after *L. monocytogenes* spreads into an adjacent cell (Schluter, Domann et al. 1998). However, *plcB* has also been implicated in the lysis of primary vacuoles in some cell types (Marquis, Doshi et al. 1995, Smith, Marquis et al. 1995). Thus, it may be that PlcB has another function in endothelial cells.

A minor pathway for *L. monocytogenes* to exit the MLN could be attached to or inside a migratory phagocyte such as a monocyte or dendritic cell (Jones and D'Orazio 2017, Jones, Smith et al. 2017). The data presented here using *in vivo* gentamicin treatment suggest that this is not a major route of dissemination from the MLN for either wildtype *L. monocytogenes* or the *ΔactA* mutant. Given gentamicin would be expected to kill all extracellular or attached bacteria at the concentration given, we presume that the *L. monocytogenes* that reached the liver

in a few animals were intracellular. However, it is also possible that all egress from the MLN does in fact involve the escape of extracellular bacteria if the systemic gentamicin concentration in our mice decreased below the calculated threshold in certain parts of the body between injections. Thus, more in-depth studies are needed to determine if extracellular escape is the sole mechanism for *L. monocytogenes* egress from the MLN.

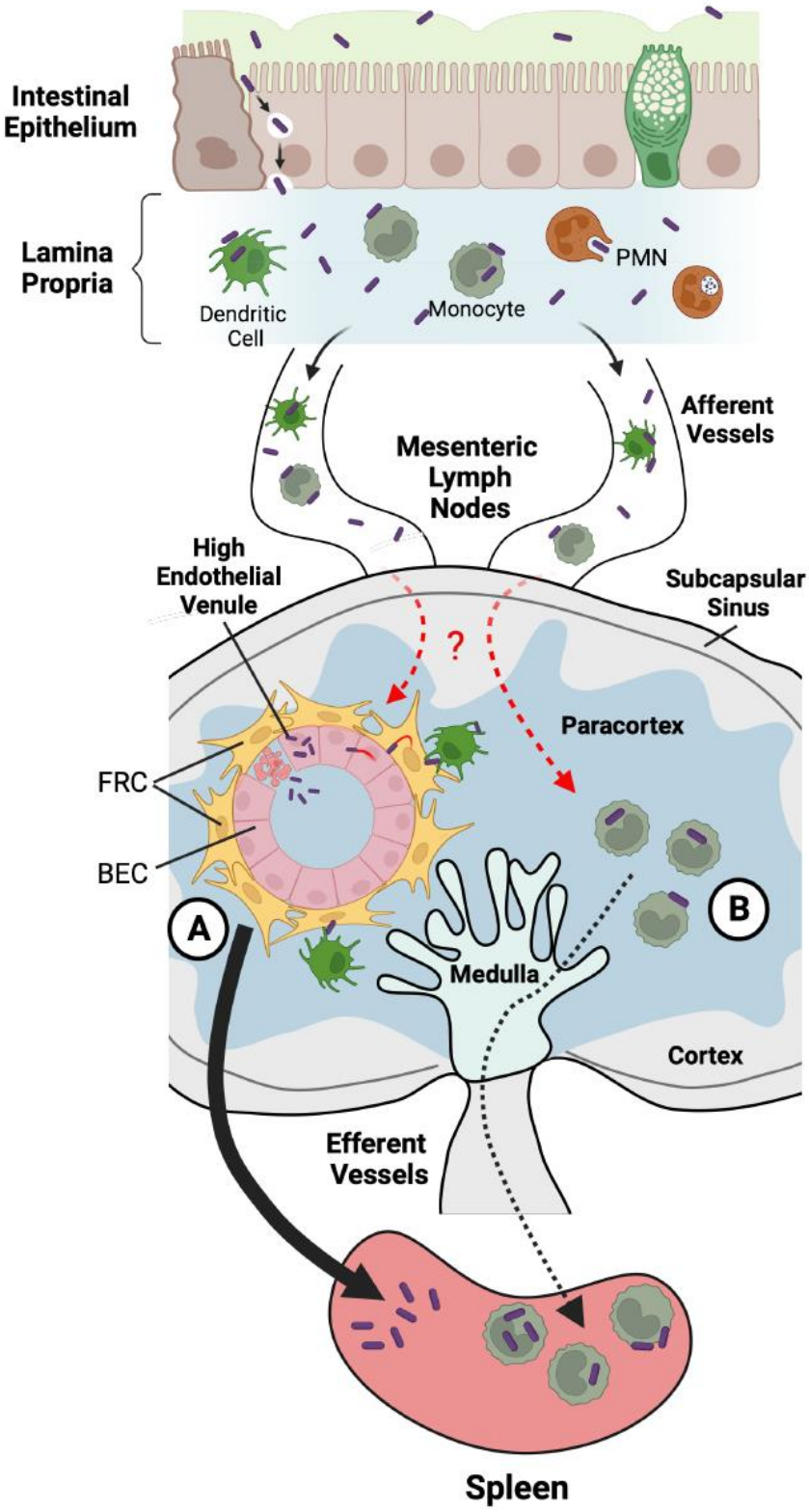
Intestinal Epithelium
Lamina Propria
Dendritic Cell
Monocyte
PMN
Afferent Vessels
Mesenteric Lymph Nodes
High Endothelial Venule
Subcapsular Sinus
?
Paracortex
FRC
BEC
A
B
Medulla
Cortex
Efferent Vessels
Spleen

Figure 6.1: Predicted model of systemic spread of *L. monocytogenes* from the MLN. Following intestinal invasion, *L. monocytogenes* enters the lamina propria on the basolateral side of the epithelium. Within the lamina propria, *Listeria* interacts with innate cells such as monocytes, dendritic cells, and neutrophils (PMN) before disseminating through the afferent lymphatics to the MLN either extracellularly, inside, or attached to a migratory cell. Following migration through the lymph node*, L. monocytogenes* escapes the MLN through two possible routes: (A) The predominant route: *L. monocytogenes* invade perivascular FRC, use actin-based motility to spread into the underlying BEC, and replicate exponentially within the cytosol, resulting in lysis or membrane damage that would allow for extracellular bacteria to escape into the blood. (B) The alternative (rare) route: *L. monocytogenes* escapes the MLN via a migratory cell through the efferent vessel of the MLN.

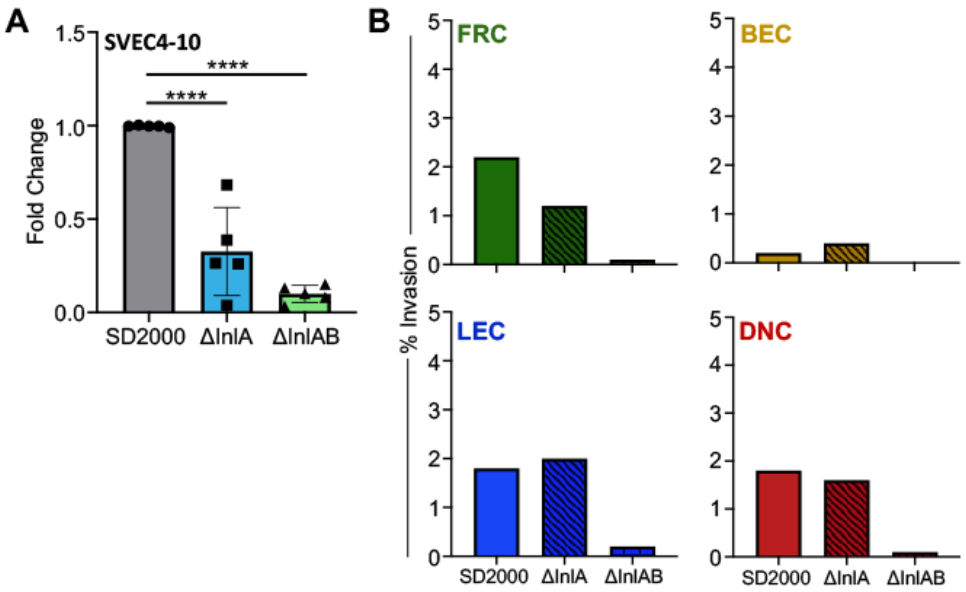

Figure 6.2: InlA and InlB surface proteins are required for efficient invasion of stromal cells. (A) SVEC4-10 cells (B) and sort-purified stromal subsets were infected with stationary phase *SD2000*, *ΔInlA*, or *ΔInlA/ΔInlB (ΔInlAB) L. monocytogenes* at MOI=10. (A) Relative fold change on the invasion of gentamicin-resistant *ΔInlA* and *ΔInlAB L. monocytogenes* inoculum compared to *SD2000 L. monocytogenes* at 1 hr post-infection. Symbols indicate one independent experiment. Pooled data from five separate experiments is shown. Statistical significance was determined by a one-way ANOVA. (B) Percentage of *Lm* inoculum that was gentamicin-resistant 1 hr post-infection. Data from one independent experiment is shown.

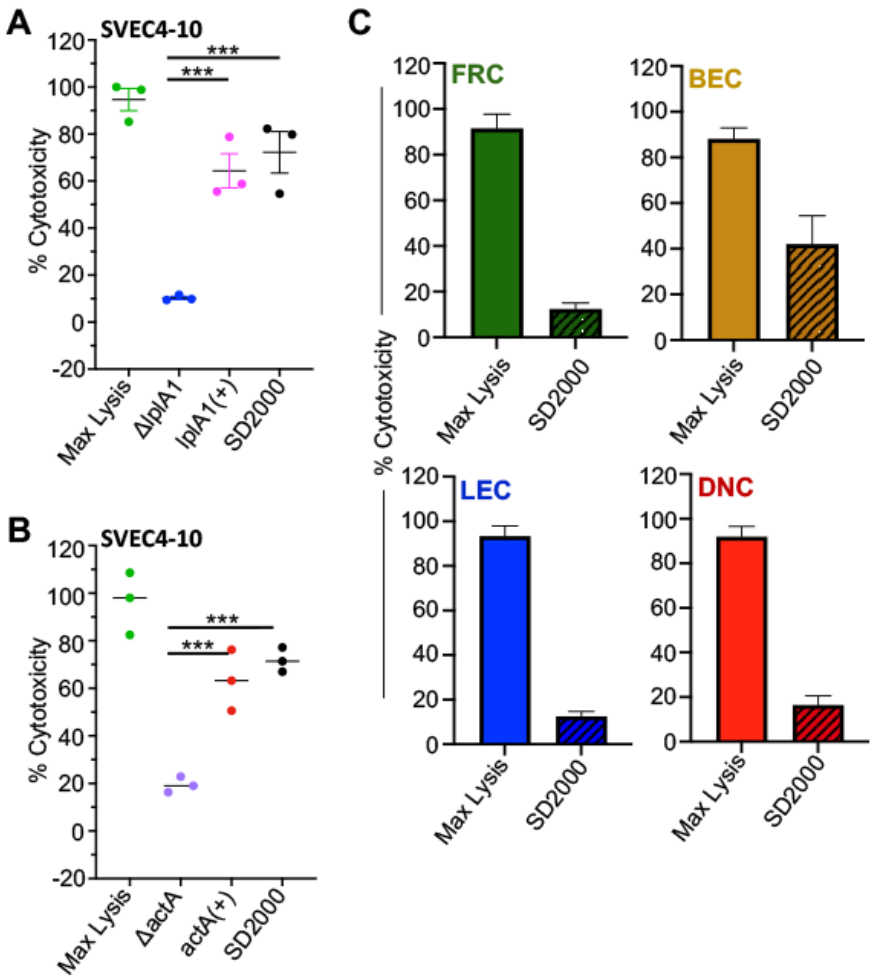

Figure 6.3: Exponential replication of *L. monocytogenes* results in lysis of SVEC4-10 and primary blood endothelial cells. (A) SVEC4-10 cells (B) and sort-purified stromal subsets were infected with *ΔlplA1*, *lplA1(+), ΔactA*, *actA(+),* or SD2000 *L. monocytogenes* at MOI=10. (A-B) Percent cytotoxicity in SVEC4-10 cells at 24 hr post-infection. Symbols indicate technical replicates (+/- SEM) from one of two independent experiments. Statistical significance was determined by a two-way ANOVA with Tukey's multiple comparisons. (C) Percent cytotoxicity in sort-purified stromal cells at 16 hr post-infection. Data from one independent experiment is shown.

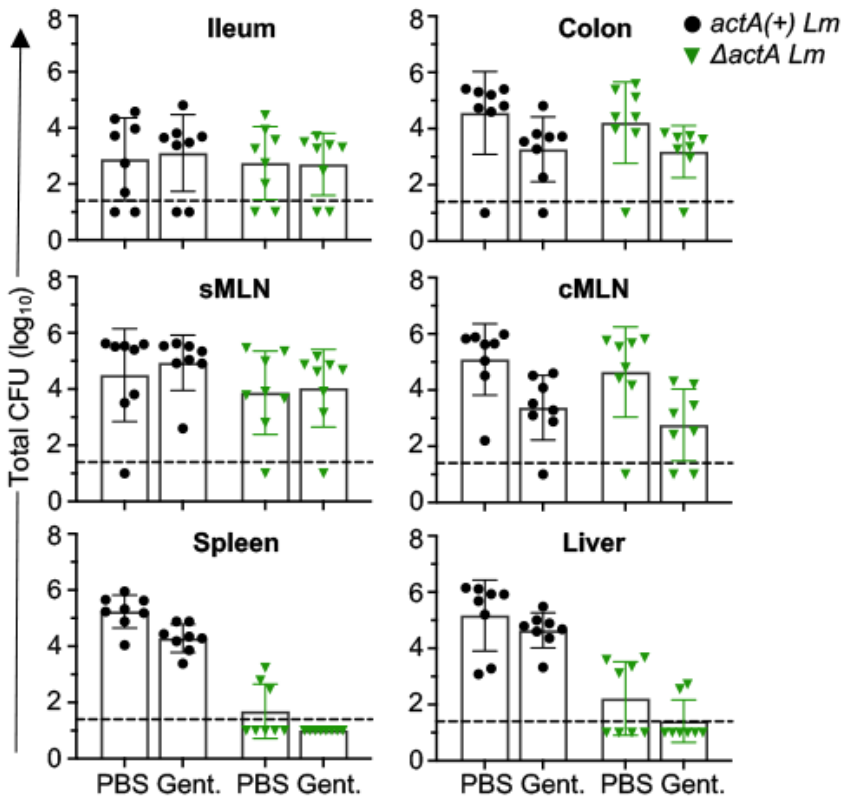

Figure 6.4: Killing extracellular *L. monocytogenes* 72 hours post-infection blocks colonization of the spleen and liver. BALB mice were co-infected with a 1:1 mixture of mouse-adapted *L. monocytogenes* SD2154 (*actA(+))* and SD2152 (*ΔactA),* totaling ~5 x 10^8 CFU. Mice were treated with PBS or gentamicin (1.0 mg) every 8 hours and total CFU were recovered from tissues three days post-infection. Data is pooled from two (n=8) separate experiments. Bars indicate mean values (± SEM). Dashed lines indicate limit of detection.

CHAPTER 7. DISCUSSION

Summary

This study establishes a foundation for understanding the events that occur in the mesenteric lymph nodes (MLN) during *L. monocytogenes* foodborne infection. It shows the key mechanisms, such as intracellular replication and cell-to-cell spread, that are required for bacterial escape from the nodes. Additionally, my work highlights a novel role for lymph node stromal cells (LNSC) as a growth niche for the *L. monocytogenes* in the MLN. The following chapter will discuss the implications of my findings by focusing on (I) the necessity of intracellular growth of *L. monocytogenes* to colonize the MLN; (II) the proposed events in the MLN during a *L. monocytogenes* infection; and (III) the hypothesized role for stromal cells during a *L. monocytogenes* infection.

I. The necessity of intracellular growth of *L. monocytogenes* to colonize the MLN.

We showed that intracellular replication was not required but promoted colonization of the MLN (Tucker, Cho et al. 2023). However, we noted an interesting dichotomy between the small intestine and colon-draining lymph node (sMLN and cMLN) in our studies. We found that while intracellular replication promoted colonization of the sMLN by protecting the bacteria from phagocyte clearance, it was not required for dissemination to the cMLN, suggesting that the extracellular bacteria might persist longer in the cMLN. Consistent with this idea, our *in vivo* gentamicin studies revealed that the majority of *L. monocytogenes* in the cMLN were gentamicin sensitive at 45 hours post-infection. However, we showed that the free lipoate concentration within the cMLN is not sufficient to support the extracellular growth of *L. monocytogenes*. Therefore, why would the majority of the bacterial burden be extracellular in the cMLN if the extracellular environment is not conducive to growth?

One possible explanation behind this result may be attributed to the different environments the bacteria encounter in the lamina propria of the small intestine and colon prior to migrating to the MLN. *L. monocytogenes* have been shown to cross the intestinal epithelium barrier via two mechanisms: InlA-mediated uptake at the villus tip, where E-cadherin is readily accessible (Pentecost, Otto et al. 2006, Nikitas, Deschamps et al. 2011) or through the uptake of M cells that overly the Peyer's patches, a specialized lymphoid follicle containing phagocytes and lymphocytes (Jensen, Harty et al. 1998, Daniels, Autenrieth et al. 2000, Corr, Hill et al. 2006). Given that Peyer's patches are only located within the small intestine, one may speculate that being intracellular would be more advantageous as it would allow the bacteria to avoid immune detection and clearance. Consistent with this idea, we previously found that when mice were coinfected with Δ*lplA1* and *lplA1(+) L. monocytogenes,* there was a notable defect in the ability of the mutant to establish an infection in the ileum at one day post-infection, a phenotype that was less pronounced in the colon (Jones, Bussell et al. 2015). This indicates that being intracellular is critical for survival in the ileum. Therefore, if the bacteria are mostly intracellular within the small intestine, it is likely that migration of *L. monocytogenes* to the sMLN would to be within a cell. Conversely, in the colon, where intracellular replication is not vital for survival, it is possible that *L. monocytogenes* could migrate extracellularly, attached to cell surfaces, or intracellularly.

II. The proposed events in the MLN during a *L. monocytogenes* infection.

Little work has been done to identify the events that occur within the MLN during *L. monocytogenes* infection, rendering it a black box. Though my research focuses on the cell type the bacteria use to escape the MLN, my model is dependent on an unidentified cell type that I predict transports *L. monocytogenes* to the T cell zone where the high endothelial venules (HEV) are located. Many studies have shown that CD8α dendritic cells in the spleen, transport *L. monocytogenes* from the marginal sinus to the periarteriolar lymphoid sheaths,

which are largely populated by T cells (Berg, Crossley et al. 2005, Aoshi, Zinselmeyer et al. 2008). Therefore, given their similarity in structure, a comparable scenario could be occurring in the MLN. This would place the *Lm*-carrying dendritic cells near HEV, and I speculate that *L. monocytogenes* attached to the surface of these cells could then invade perivascular FRC, as depicted in my model (Fig. 6.1). Alternatively, the unidentified cell type could be a proinflammatory monocyte migrating from the lamina propria. We previously showed that Ly6C^{hi} monocytes were the predominant cell type associated with *L. monocytogenes* in the MLN at 48- 60- and 72- hours post-infection (Jones and D'Orazio 2017). Thus, one could speculate that these monocytes, while traversing the T cell zone, could allow for the infection of HEV. Subsequent studies should focus on elucidating the roles of both monocytes and dendritic cells in the lymph node during *L. monocytogenes* infection, specifically focusing on how these cells migrate through the MLN.

My data suggests that FRC support the exponential growth of *L. monocytogenes*. However, this population can be further divided based on location within the nodes. Though we speculate that these cells may not differ in their ability to support *L. monocytogenes* growth, my model only depicts the role of one subset, perivascular FRC. Yet, I hypothesize that each of these subsets may serve as a hub for *L. monocytogenes* replication throughout the MLN. For example, marginal reticular cells (MRC) help form a boundary between the subcapsular sinus (SCS) and cortex within the node (Katakai 2012). Thus, a possible scenario could be that *L. monocytogenes* entering the MLN via afferent vessels could be readily taken up by SCS macrophages and spread into lymphoid endothelial cells and the underlying MRC. These cells, in turn, could then function as a niche for bacterial replication until a migratory cell (e.g., a dendritic cell) transports the *L. monocytogenes* to the T cell zone. The idea is supported by our previous finding that the MLN lacks sufficient free lipoate to sustain the extracellular growth of *L. monocytogenes*, indicating the necessity of an intracellular niche within the node. Furthermore, considering FRC subsets are perfectly positioned throughout the node, it is possible that *L. monocytogenes* could exploit this cell at any point during

the MLN infection. Future research should focus on identifying which FRC subsets *L. monocytogenes* associate with following foodborne infection. These findings could significantly contribute to our understanding of how *L. monocytogenes* survives and migrates through the lymph node.

III. The hypothesized role for stromal cells during a *L. monocytogenes* infection.

Stromal cells are responsible for both the architecture and the creation of microenvironments that regulate our immune responses (Krishnamurty and Turley 2020). Despite this pivotal role, they are often overlooked during infections. Here, we show that two subsets, fibroblastic reticular cells (FRC) and blood endothelial cells (BEC), support the exponential growth of *L. monocytogenes in vitro* and that intracellular bacteria are found within these subsets following foodborne infection. Stromal cells, however, form the backbone of every organ (Manetti 2021), raising the possibility that they might serve as a pivotal growth niche for *L. monocytogenes* at other sites of infection.

For instance, *L. monocytogenes* are thought to colonize the brain through three different mechanisms. One model of this colonization proposes that certain hypervirulent *L. monocytogenes* strains create a protective niche within monocytes, aiding in their survival in the bloodstream and transport to the brain (Maudet, Kheloufi et al. 2022). These infected monocytes were shown to adhere to brain blood vessel endothelium, allowing for bacterial spread. While this study did not directly implicate these endothelial cells (a subset of stromal cells) in supporting *L. monocytogenes* growth, based on our data, it is plausible that these cells could be the replicative niche required for subsequent brain colonization. This possibility becomes even more likely given our previous finding that for *L. monocytogenes* to form actin tails and spread cell-to-cell the bacteria must be able to replicate intracellularly (Tucker, Cho et al. 2023), thus for their model to be true, *L. monocytogenes* would have to replicate within the blood endothelium to invade the brain.

In contrast, our prior work suggested an alternative route of neurotropic *L. monocytogenes* reaching the brain from the gut (Senay, Ferrell et al. 2020). Genome sequencing of these specific neurotrophic strains revealed unique internalin-like proteins absent in reference strains (Albrecht, Kucerova et al. 2021). This led to the hypothesis that these strains might contain an internalin that enables *L. monocytogenes* to target cells in the lamina propria near neurons, potentially utilizing cell-to-cell spread to access the neuron and employ actin-based motility to ascend the axon and reach the brain stem. Mantani et al. previously showed that nerve fibers, particularly below the crypts in the lamina propria, are in close proximity to stromal cells (Mantani, Nishida et al. 2018), previously shown to be a subset of FRC (Brugger and Basler 2023). This is a stromal subset I have shown can support the growth of *L. monocytogenes*. Hence, these neurotropic strains might express an internalin favoring efficient invasion of this FRC subset in the lamina propria that then results in infection of neuronal cells via cell-to-cell spread and colonization of the brain.

These examples serve as a glimpse into how intracellular replication within stromal cells might impact the *L. monocytogenes* infection. Yet, the broader role of stromal cells during bacterial infections remains significantly unexplored. These stromal subsets can be found throughout the body, positioned near a variety of cell types, indicating their potential as a substantial niche for various intracellular bacterial pathogens. My research highlights the necessity to investigate these overlooked cells, not only for their potential as a growth niche for pathogens like *Listeria* but also for their ability to produce numerous cytokines and chemokines, which could potentially alter the infection in different ways. For instance, Disson et al. demonstrated that IL-23 production from $gp38^{+}$ stromal cells in the lamina propria blocks further intestinal invasion by *L. monocytogenes* (Disson, Bleriot et al. 2018). This prompts the question of how the cytokine/chemokine production by stromal cells in other anatomical locations might either promote or impede infection. Taken with the results of my research, these studies emphasize the multifaceted roles of these cells beyond structural support and homeostasis.

Age is a significant risk factor for the development of severe life-threatening listeriosis. While it is assumed this is due to a weakened immune system in these individuals, the reason behind the delayed or reduced immune response remains uncertain. However, given that stromal cells have been shown to play a role during *L. monocytogenes* infection, I propose that the increased susceptibility of elderly individuals to *L. monocytogenes* might stem from changes in their stromal cell composition. This idea is supported by a recent study completed by Bennett et al., revealing notable differences between aged and adult murine lymph node stromal cells (LNSC) (Bennett, Richner et al. 2023). This study showed that aged LNSC have delayed proliferation and alterations in stromal composition which leads to a reduction in the accumulation of leukocytes in the nodes. Additionally, their research also found differing gene expressions in response to the West Nile virus between aged and adult LNSC. Given these findings, it is easy to speculate that similar mechanisms might occur during *L. monocytogenes* infections of aged individuals. A reduced number of leukocytes in the lymph node of would allow for the persistence of the bacteria and potentially lead to life-threatening systemic infections. In fact, our findings support this idea, as we observed a notable increase in bacterial burdens in multiple organs when monocytes and neutrophils were depleted. Hence, future investigations should emphasize age-related deficiencies in LNSC during *L. monocytogenes* infections.

APPENDIX: LIST OF ABBREVIATIONS

BALB	BALBcBy/J mice
BEC	Blood endothelial cells
BHI	Brain heart infusion
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CCL	C-C motif chemokine ligand
CFU	Colony forming unit
CI	Competitive Index
cMLN	Colon-draining mesenteric lymph node
CXCL	X-X-C motif chemokine ligand
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNC	Double negative cells
dpi	Days post infection
Ery	Erythromicin
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSC	Forward-scattered light in FACS
FRC	Fibroblastic Reticular Cells
HEV	High endothelial venules
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hanks' balanced salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	Hours post-infection
IFN-α	Interferon alpha

IFN-β	Interferon beta
IFN-γ	Interferon gamma
IL	Interleukin
$InlA^m$	Mouse-adapted InlA
i.p	Intraperitoneal
i.v	Intravenous
iLSM	Listeria Synthetic Medium
IMM	Improved minimal medium
Kan	Kanamycin
LEC	Lymphoid endothelial cells
LLO	Listeriolysin O
LNSC	Lymph node stromal cells
LplA	Lipoate ligase like protein
LPS	lipopolysaccharide
MLN	Mesenteric Lymph Node
MM	Medullary Sinus
M cell	Microfold cell
MP	Mononuclear Phagocyte
PBS	Phasophate buffered saline
PE	phycoerythrin
PMN	Polymorphonuclear Cell
qRT-PCR	quanitative Revese Transcription Polymerase Chain Reaction
RNA	Ribonucleic acid
RP-10	Complete RPMI based media with 10% FBS
SCS	Subcapsular Sinus

SD	Standard deviation
SEM	Standard error of the mean
sMLN	Small intestine-draining mesenteric lymph node
SSC	Side scattered light in FACS
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WT	Wildtype

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VITA

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BIOGRAPHICAL SKETCH

Jamila Shanta Tucker completed her B.S. at the University of Tennessee-Chattanooga in 2016. She then moved to Nashville, TN, in 2017 to attend Middle Tennessee State University, receiving her M.S. in Microbiology in 2019. Jamila entered the Integrated Biomedical Sciences program in the Fall of 2019 and joined the lab of Dr. Sarah D'Orazio in the Department of Microbiology, Immunology, and Molecular Genetics in July 2020. Jamila has presented her work at the FASEB Gastrointestinal Tract Conference, where she received a poster award, the American Society for Microbiology KY-TN Branch Meetings, where she received awards for a 10-minute talk, poster, and 3-minute thesis presentations, the Autumn Immunology Conference, where she was awarded the Wallace Fellowship, and the Mid-Atlantic Microbial Pathogenesis Meeting where she was awarded a travel award. In 2023, Jamila was named the Lyman T. Johnson Torch Bearer for the College of Medicine and received the Outstanding Graduate Student award for the College of Medicine. Jamila also served on the Diversity and Inclusion Committee for the department and was the president of the American Society for Microbiology UKY Student Chapter for two years. Jamila has one first-author publication (*Infection and Immunity*, 2023) and one first-author manuscript in revision (*Journal of Leukocyte Biology*, 2024). Jamila has started a position at the Centers for Disease Control and Prevention and will be starting a Microbiology research position in the COVID and Other Respiratory Viruses Division in February 2024.

PUBLICATIONS

1. **Tucker, JS**., Guess, TE., & McClelland, EE. (2020). The Role of Testosterone and Gibberellic Acid in the Melanization of Cryptococcus neoformans. Frontiers in Microbiology,11. doi:10.3389/fmicb.2020.01921
2. Subramani, A., Griggs, P., Frantzen, N., Mendez, J., **Tucker, JS**., Murriel, J., Nelson, DE. (2020). Intracellular Cryptococcus neoformans disrupts the

transcriptome profile of M1- and M2-polarized host macrophages. PLoS ONE, 15(8). doi:10.1101/2020.05.14.095752

3. Krusenstjerna, A., Saylor, T., Arnold, W., **Tucker, JS.,** Stevenson, B. (2023). Borrelia burgdorferi DnaA and the Nucleoid-Associated Protein EbfC Coordinate Expression of the dnaX-ebfC Operon. Journal of Bacteriology, 205(1). doi:10.1128/jb.00396-22
4. **Tucker, JS.,** Cho, J., Albrecht, TM., Ferrell, JL., and. D'Orazio, SEF., (2023) "Egress of Listeria monocytogenes from the Mesenteric Lymph Nodes Depends on Intracellular Replication and Cell-to-Cell Spread" Infection and Immunity, doi.org/10.1128/iai.00064-23.
5. **Tucker, JS.,** Khan, H., and. D'Orazio, SEF., (2024) "Lymph Node Stromal Cells Vary in Susceptibility to Infection but Can Support the Intracellular Growth of Listeria monocytogenes" Journal of Leukocyte Biology, doi.org/10.1093/jleuko/qiae040.

TEACHING EXPERIENCE

Jan 2023-May 2023	Graduate Teaching Assistant, University of Kentucky
Aug 2017-May 2019	Graduate Teaching Assistant, Middle Tennessee State University

CERTIFICATIONS

March 2017- current	CPT- Certified Phlebotomy Technician

LEADERSHIP POSITIONS

Aug 2021-Jul 2023	Public Relations Officer, Thrombocyte, Leukocyte, & Cancer Graduate Student Organization, UK College of Medicine, Lexington KY
Jul 2021- Jul 2023	President (Re-Elected), UK Student Chapter, American Society of Microbiology
Aug 2020-Aug 2022	Diversity, Equity, and Inclusion Committee; Dept. of Microbiology, Immunology & Molecular Genetics, UK College of Medicine
Jan 2020-Jul 2021	Diversity Representative, American Society of Microbiology-Student Chapter

Oct 2022-Nov 2023	Graduate Representative, Executive Council Autumn Immunology Conference