Experimental Listeriosis During Pregnancy and the Perinatal Period

by

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Dissertation Abstract

Experimental Listeriosis During Pregnancy and the Perinatal Period

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Listeriosis is a potentially fatal foodborne infection caused by *Listeria monocytogenes* that affects mainly humans and domesticated ruminant species. Pregnant women, infants, and immunocompromised individuals are over represented in foodborne transmission of *L. monocytogenes*. Increased susceptibility during pregnancy has been hypothesized to be due to loss of homeostasis between pro- and anti-inflammatory T cell populations. In this project, we developed a mouse model to study microbial load and inflammation caused by *L. monocytogenes* infection during pregnancy. Intragastric infection of pregnant C57BL/6 and A/J mice caused reproducible fetal infection and abortion. Although the microbial load in fetal tissues was similar for all fetuses within a single uterus, inflammation and necrosis varied among individual fetuses and placentas. *L. monocytogenes* infection of post-parturient A/J

mice, shown by bioluminescence imaging, disseminated to the mammary chain, and the organism was shed in milk.

We used qPCR to measure relative changes in expression of select genes associated with T cell subsets. We detected moderate increases in expression of IL-17a and IL-22 in placental tissue of mice infected with *L. monocytogenes*. Gene knockout mice were used to assess the roles of IL-17a and IL-22 in resistance to listeriosis during pregnancy. Although IL-17a and IL-22 were up-regulated in placental tissue of wild type mice, resistance to listeriosis and the host inflammatory response were not dependent on IL-17a or IL-22 in pregnant mice infected with *L. monocytogenes*.

We then examined how *L. monocytogenes* infection affects the function of trophoblast cells (JEG-3) that are part of the innate immune barrier that protects the fetus. *L. monocytogenes* infection impaired JEG-3 cell migration and invasion. These defects were due in part to decreased cell metabolism and eventual cell death.

The findings presented in this dissertation show that the mouse is a useful animal model for intragastric *L. monocytogenes* infection during pregnancy. Microbial load and inflammation of fetoplacental units can vary within a single pregnant mother and are not dependent on IL-17a or IL-22. Finally, our findings suggest that *L. monocytogenes* infection of extravillous trophoblast cells compromises functions essential to maintain a healthy pregnancy.

Chapter One

Literature Review

***Listeria monocytogenes*: Background and Clinical Signs**

Listeria monocytogenes is a Gram-positive, facultative intracellular rod that is ubiquitous in the environment [1]. *L. monocytogenes* is one of six species in the genus *Listeria*, which also includes *L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi*. Of these six species, only *L. monocytogenes* and *L. ivanovii* are pathogenic [2]. *L. ivanovii* is responsible for few listeriosis cases and is limited to reproductive disorders in ruminant species. *L. monocytogenes* causes disease in animals and people due to contamination of foodstuffs and typically manifests as sepsis, abortion, and central nervous system dysfunction.

The *L. monocytogenes* species can be classified into three different lineages (reviewed in [3] and [4]). Lineage 1 contains serotypes 1/2b, 3b, and 4b. Lineage 2 is made up of serotypes 1/2a, 3a, 1/c, and 3c. Lineage 3 includes serotypes 4a, 4c, and a few 4b strains. The vast majority of human listeriosis outbreaks are attributed to serotypes 1/2a, 1/2b, and 4b [5-8]. In the United States, *L. monocytogenes* strains associated with human outbreaks are identified by molecular sub typing using pulsed-field gel electrophoresis. The resulting band patterns are published on PulseNet, a web-based network managed by the Centers for Disease Control and Prevention [9]. PulseNet from the United States is also shared with PulseNet International, which

encompases Africa, Asia (Pacific), Canada, Europe, Latin America and the Carribean, and the Middle East.

Molecular typing is used to place *L. monocytogenes* strains isolated from human outbreaks into epidemic clone (EC) groups EC1, EC1a, and EC2 [4, 10]. Strains belonging to EC1 and EC1a are from earlier documented human listeriosis outbreaks in the late 1970s and early 1980s associated with coleslaw and Mexican-style cheese, respectively (reviewed in [11]). EC2 strains of *L. monocytogenes* strains were first recognized in a multistate hot-dog associated outbreak in 1998-1999 and are associated with the majority of recent listeriosis outbreaks in the USA [10, 12-14].

L. monocytogenes infection of veterinary species results in clinical disease almost exclusively in ruminant and pseudo-ruminant species [15, 16]. Other monogastric and hind-gut fermenting species can become infected with and shed *L. monocytogenes*, but rarely show signs of clinical disease (reviewed in [2]). One exception is the rabbit, which is used as an infection model and displays similar clinical syndromes to humans and ruminants during experimental and natural *L. monocytogenes* infection [17]. Commercial fresh water fisheries occasionally recover *L. monocytogenes* in product [18, 19]. Poultry operations have also reported encephalitis in broiler chickens and turkey poults [20, 21]. Turkey processing plants commonly have *L. monocytogenes* contamination issues, which suggests that the birds shed the bacteria. However, the source of contamination has yet to be determined and is thought to be multifactorial and involve human, environmental and animal sources (reviewed in

[2]). The most recognizable clinical syndromes in cattle and sheep are abortion storms and “circling disease,” caused by meningioencephalitis. *L. monocytogenes* also is a common cause of Gram-positive neonatal septicemia. *L. monocytogenes* is a rare cause of mastitis on commercial dairies; many lactating animals shed the pathogen without overt clinical signs of mastitis (reviewed in [2, 22]).

Human listeriosis shares two of the common syndromes with veterinary medicine: abortion storms and neonatal septicemia. The latter often leads to neonatal meningitis (reviewed in [17]). Severe clinical disease in humans is almost exclusively found in at risk populations including pregnant women, infants, and immunocompromised adults [23]. Healthy adults who consume contaminated foods typically are asymptomatic or have mild to moderate self-limiting gastroenteritis [8, 24].

L. monocytogenes Virulence Factors

Listeria monocytogenes has been used as a model for studying the pathophysiology of intracellular bacterial pathogens. *L. monocytogenes* can invade and multiply within a variety of cell types including macrophages, dendritic cells, and various types of parenchymal cells (Figure 1) [25]. The most important virulence factors for *L. monocytogenes* include internalin A and B, a hemolysin (listeriolysin O), two phospholipases, and a protein (ActA) responsible for intracellular motility. As *L. monocytogenes* bacterial cells come into contact with host cells they use internalin proteins, internalin A (InlA) and internalin B (InlB), respectively to mediate cell entry via

E-cadherin and c-Met tyrosine kinase on the host cells (Figure 1 (A)) [26, 27]. Recognition of InlA by E-cadherin is specific to primates, guinea pigs, ruminants, and gerbils for invasion of *L. monocytogenes* [28, 29]. There are many more internalin genes in the *L. monocytogenes* genome (currently there are 24 described), but many of these have undetermined roles [10].

Once the listerial cells are internalized, they escape the phagosome by expressing listerolysin O (LLO) and phosphatidylinositol-specific phospholipase C (Figure 1 (B)) [30-33]. *L. monocytogenes* cells multiply in the cytoplasm with a generation time of approximately 1 hour at 37°C (Figure 1 (C)) [34]. As the bacteria multiply, they move to the periphery of the infected cell via action of the bacterial surface protein, ActA, which orchestrates addition of actin monomers to the caudal end of the bacteria. This results in formation of actin filaments that propel the bacterial cells rostrally (Figure 1 (D)) [35-37]. As the bacterial cell is propelled into the cell membrane, it forms an invagination of the adjacent cell membrane resulting in the listerial cell being contained within a double membrane vacuole (Figure 1 (E)). To escape from this double-membraned vacuole, *L. monocytogenes* uses a phosphatidylcholine-dependent phospholipase C (also referred to as lecithinase) and LLO (Figure 1 (F)). The process of intracellular mobility and multiplication is then repeated (Figure 1 (G)) [30, 38].

Nearly all of the *L. monocytogenes* virulence factors are encoded by genes that cluster on a well-defined area on the bacterial chromosome [3]. Their expression is regulated by a transcriptional activator, positive regulatory factor A (PrfA) [39-41]. After

transcriptional activation of the virulence genes, PrfA also acts to enhance transcription, via a variety of promoters in the PrfA box, of InlA, InlB, and LLO [25]. The action of PrfA is temperature-dependent and repressed at temperatures <25°C [42]. However, bacterial growth continues at cold temperatures without expression of virulence genes. PrfA therefore is part of *L. monocytogenes*'s ability to sense different environments, such as soil, vegetation, or refrigerated dairy products, versus the intracellular environment of mammals. Clinical isolates of *L. monocytogenes* are more able than non-pathogenic strains to detect environmental changes and resume log phase growth after moving from a cold to warm environment [43]. PrfA activity is upregulated in low pH environments (pH of 4.5 to 5.5), to which *L. monocytogenes* cells are exposed several times during the infection cycle. Examples include many types of fermented foods (which are acidic), passage through the stomach, and within the acidified phagosome of the infected cell [44].

Immune Response to *L. monocytogenes*

The host immune response to *L. monocytogenes* infection is thought to be quite effective in healthy adults, as listeriosis is a relatively rare cause of foodborne disease (estimated 1600 cases per year in the USA [45]. Because of this, *L. monocytogenes* has been widely used as a model to investigate intracellular pathogen interactions with the host innate and adaptive immune responses.

The first line of defense against *L. monocytogenes* infection is through innate mechanisms including the low pH environment of the stomach, the intestinal microflora, antimicrobial peptides, and several types of leukocytes [2, 46]. Neutrophils, or polymorphonuclear leukocytes (PMNs), respond to bacterial infection. Together with monocytes and macrophages, they ingest and kill a portion of bacterial cells [38, 47, 48]. Cells of the innate immune response recognize *L. monocytogenes* in part via receptors for pathogen-associated molecular patterns (PAMPs) on the bacterial cell surface. These PAMPs include lipoteichoic acid, peptidoglycan, and flagellin [49, 50]. Toll-like receptors (TLR) 2 and TLR5 have been reported to bind listerial PAMPs lipoteichoic acid and flagellin, respectively [51]. After recognition by TLR2 and TLR5, signaling mediated by myeloid differentiation factor 88 (MyD88) prompts macrophages to produce pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α [52, 53]. These cytokines, in turn, activate the arachidonic acid inflammatory cascade to produce leukotrienes (specifically LTB_4), which attract additional neutrophils to the site of infection [54].

The innate immune response sets the stage for an adaptive immune response to *L. monocytogenes*. Protective immunity is dependent on CD8+ T cells and peaks 7-10 days after infection [55]. CD4+ T cells also are activated, which mediate delayed type hypersensitivity but are less important for clearance of listerial cells [56]. Antigen specific CD8+ and CD4+ T cells, as well as natural killer cells, produce interferon-gamma (IFNγ), which is important for activation of macrophages and differentiation of T helper (Th) 1 cells. Products of Th1 cells are responsible for increasing MHCI and

MHCII antigen presentation, continued expansion of CD8+ T cells, and inhibiting CD4+ T cell expansion [56]. Recently, attention has been paid to a potential role of Th17 cells (CD4+) and gamma-delta T cells, which both produce IL-17 early in the course of *L. monocytogenes* infection [57, 58]. The role of Th17 cells and IL-17a are considered in detail in the following section.

Immune Response to *L. monocytogenes* Infection During Pregnancy

The protective immune response to *L. monocytogenes* infection typically seen in healthy adults is often not the case with pregnant women, who have an approximately 20 fold greater risk of acquiring listeriosis [59]. The reasons why a pregnant mother cannot protect her fetus are unclear, but may be partially due to changes in the maternal immune response during pregnancy. Previously it was thought these changes were chiefly due to a shift in the Th1:Th2 cell ratio towards a humoral (Th2), and away from a cell-mediated (Th1), response. The latter is potentially harmful to the fetal allograft [60]. The switch to a Th2 humoral response is thought to be due to in part to local expression of progesterone and progesterone induced blocking factor by placental trophoblast cells [61]. The change in Th cell populations is important, because cytokines associated with the Th1 cell response (e.g. IFNγ and TNFα) are critical for protective cellular immunity to *L. monocytogenes* [56]. However, more recent studies suggest that Th1:Th2 cell ratios may be an over-simplification of the complex systemic and local immune response in the gravid uterus [62].

The recent description of pro-inflammatory Th17 cells may provide additional insight into fetal inflammation due to *L. monocytogenes* infection. Rapid Th17 cell proliferation is more frequently associated with extracellular bacterial infections than intracellular bacteria and viruses [63]. However, intracellular bacteria including *Mycobacterium tuberculosis* and *Staphlococcus aureus* have also been reported to induce Th17 cells in humans and mice, respectively [64-66]. Th17 cells have also been implicated in fetal inflammation and placental necrosis in pregnant humans [67]. The proliferation of Th17 cells during pregnancy led to the hypothesis that Th17 cells are likely involved in the inflammatory response to *L. monocytogenes* infection during pregnancy [68-71]. Orgun et al. showed that *L. monocytogenes* infected mice produce a significant Th17 response when the main Th1 effector cytokine, IFNγ, and the type 1 IFN receptor, were experimentally neutralized in non-pregnant mice [58]. These results suggest that when Th1 cell proliferation is repressed during pregnancy, Th17 cell production could be significant during *L. monocytogenes* infection.

The Th17 cell's main effector cytokine is IL-17a, which is part of the IL-17 family and IL-17a influences the production of chemokines (CXCL1, CXCL2, CXCL5, CXCL8) and cytokines (GM-CSF and G-CSF) that are responsible for recruitment, differentiation, and activation of neutrophils [72, 73]. IL-17a has been linked to many immune-mediated and autoimmune diseases including rheumatoid arthritis, asthma, lupus, allograft rejection and anti-tumor immune responses [74]. These functions highlight potential roles for IL-17a in *L. monocytogenes* infection during pregnancy because the hallmark histopathological lesion in placental tissue during listeriosis is neutrophilic inflammation

[75, 76]. Furthermore, the fetus is essentially an allograft that can be recognized as such by the maternal immune system. IL-17a has been detected in the human placenta during inflammation, and is associated with miscarriage and other pregnancy abnormalities [67]. IL-17a has been found in the mouse placenta during normal pregnancy, but its presence in the placenta during *L. monocytogenes* infection has not been investigated [77].

Another important product of Th17 cells is IL-22, which has been implicated in host defense, inflammation, and tissue repair [78-80]. IL-22 is part of the IL-10 family and regulates function of parenchymal cells, such as epithelial cells and hepatocytes [81]. In certain circumstances IL-22 works synergistically with IL-17a and is reported to have multiple functions that are both pro- and anti-inflammatory [82]. IL-22 has been found in human placenta, and was shown to be significantly elevated in experimental *L. monocytogenes* infection [83, 84]. Intravenous *L. monocytogenes* infection of IL-22$^{(-/-)}$ mice showed that bacterial clearance is not dependent on IL-22 [85, 86]. However, there are no reports of the role of IL-22 in the pathogenesis of oral infection with *L. monocytogenes*.

Clinical Listeriosis During Pregnancy

Listeria monocytogenes tends to cause clinical disease most commonly in humans and domesticated ruminant species. Despite major differences in diet and placental anatomy, pregnancy is a common risk factor in both humans and ruminant species associated with maternal illness, fetal infection, and abortion [22, 87]. “Abortion

storms" occur in people and ruminants. In both cases, outbreaks occur following ingestion of a common contaminated foodstuff. An important difference between human and ruminant outbreaks is that human outbreaks are amplified by intra- and interstate distribution of foods, while ruminant outbreaks tend to remain within a herd [88]. Abortion does not occur in all infected women during pregnancy and is observed rarely in pregnant cattle in which meningioencephalitis is more common [22, 89]. In a review of 191 cases of human listeriosis during pregnancy, 32% had mild flu-like symptoms, 65% reported fever, and 29% were asymptomatic [90].

Listeriosis in early pregnancy, results in abortion or fetal resorption [90]. *L. monocytogenes* infection later in gestation results in abortion, stillbirth, or premature birth [90-92]. Listeriosis in ruminants commonly occurs in the 3rd trimester. It is not known whether *L. monocytogenes* is a risk factor for early embryonic death, because definitive diagnostics are costly and fetuses typically are not submitted to a veterinary diagnostic laboratory [22]. Transmission from mother to fetus is most likely due to fetal inhalation of infected amniotic fluid, transplacental infection (hematogenous), or ascending colonization of the fetoplacental unit from the birth canal [93, 94].

Premature labor is one outcome of *L. monocytogenes* infection during pregnancy. Prematurity for any reason is a high risk factor for short and long term neonatal complications, including death [95]. Infants born prematurely due to maternal listeriosis are at a high risk of neonatal sepsis (reviewed in [87]). One study reported 68% of mothers diagnosed with listeriosis had neonates who also developed listeriosis

[90]. From a review of 222 cases of listeriosis during pregnancy that resulted in neonatal listeriosis, 68.2% of neonates recovered, 12.7% developed long-term neurologic deficits, and 24.5% died [90]. Neonatal listeriosis manifests as either early or late-onset disease. Early onset is apparent within 1-3 days of birth, with an average of 36 hours [96]. Neonates with early onset listeriosis tend to be born prematurely; 44-89% of those mothers had been diagnosed with *L. monocytogenes* and often have suffered recently from a flu-like illness, [88, 97]. The most common clinical presentation of early onset neonatal listeriosis is septicemia with pneumonia being less common [88, 97]. Late onset neonatal listeriosis affects term infants and develops at 5 to 14 days of life [97, 98]. Unlike early onset neonatal listeriosis, infants with late onset neonatal listeriosis usually are born to asymptomatic mothers with negative *L. monocytogenes* diagnostic tests [88, 97]. Meningitis is the most common clinical syndrome associated with late onset neonatal listeriosis [52, 99]. Transmission of *L. monocytogenes* to the neonate can occur in utero, as described above, during passage through the birth canal, or by ingestion of contaminated colostrum and breast milk [100]. Human breast milk currently is not recognized as a risk factor for *L. monocytogenes* transmission from a mother to her nursing infant. However, this is not the case for ruminant species, which are well known to shed *L. monocytogenes* in their milk [22].

Animal Models for *L. monocytogenes* Infection During Pregnancy

Listeria monocytogenes has been associated with human disease since the early 1920s and recognized as a cause of neonatal disease since the mid 1940s [17]. The *L. monocytogenes* epidemics of the 1980s intensified the use of animal models to

investigate the pathophysiology of listeriosis in pregnancy. Laboratory animal models of listeriosis were studied for years prior to recognition of *L. monocytogenes* as a human foodborne pathogen and were responsible for a significant amount of information regarding the pathogenesis of intracellular pathogens and cell mediated immunity against them [101]. Each of the animal infection models currently used has advantages and disadvantages due to microbial variables and the complex series of events that lead to clinical listeriosis.

Models of *L. monocytogenes* infection during pregnancy need to consider the variability of placental structure. Mammals tend to fit into three broad categories of placentation: 1) Epitheliochorial (horse, pig, cow); 2) Endothelialchorial (carnivores); and 3) Hemochorial (rodents and primates) (reviewed in [102]). Epitheliochorial placentation has the most complete separation of fetal and maternal blood, endothelialchorial has moderate separation, and hemochorial has the least. Epitheliochorial placentation and long gestation time limit the use of ruminant species as models for human listeriosis.

Pregnant non-human primates have placental tissue with the greatest similarity to humans [17, 103]. Rhesus macaque monkeys have been used as a model for *L. monocytogenes* fetal infections, as they exhibit similar responses to humans including abortion, stillbirth, and neonatal death (reviewed in [104]). The pathological lesions seen with *L. monocytogenes* infection are essentially the same in humans and non-human primates [105]. However, there are ethical considerations regarding the use of non-

human primates, and their use to study infections during pregnancy is often cost-prohibitive [104].

Guinea pigs have been used to study *L. monocytogenes* since the 1970s and provided one of the first models to characterize virulence of different *L. monocytogenes* strains [104]. More recently, guinea pigs have gained renewed attention after it was discovered that their E-cadherin amino acid sequence is the same as humans [28, 106]. Although guinea pigs are considered an acceptable model for human infection via the g.i. tract, they require a high infective dose and it is difficult to quantify microbial load in their tissues [29, 107]. Furthermore the utility of the guinea pig as a *L. monocytogenes* infection model is limited because the guinea pig Met receptor, responsible for binding InlB, is dissimilar to the human Met receptor. Some have suggested infection of pregnant gerbils as a potential listeriosis model as they have the same E-cadherin site and similar Met receptors as humans [108]. Intravenous infection of pregnant gerbils with *L. monocytogenes* has been shown to cause fetal death, but oral infection has yet to be reported [108].

The mouse has been used for *L. monocytogenes* infection studies since the 1950s [109]. In 1966, the pregnant mouse model was first described to study the effects of listeriosis on the fetus [110]. The advantages of using mice include their short generation time, their relatively small size, and the availability of genetic, immunological, and molecular biological tools (e.g. antibodies, genetic mutant strains, cytokines). The genetic background of inbred mouse strains is an important factor for *L. monocytogenes*

infection studies. Resistance to *L. monocytogenes* is regulated principally by the *Hc* locus on chromosome 2 [111-113]. Mouse strains with the resistant allele (e.g. C57BL/6) at the *Hc* locus are significantly more resistant to intravenous, intraperitoneal, or intragastric *L. monocytogenes* infection than strains with the susceptible allele (e.g. A/J) [112-114]. Interestingly, this genetically-mediated difference in resistance to *L. monocytogenes* infection in the mouse is not so apparent in pregnancy. Recently, our laboratory has shown there is no significant difference in susceptibility of C57BL/6 compared to A/J mice when infected with *L. monocytogenes* at 10-14 days of gestation [115]. The mouse infection model is criticized by some because murine E-cadherin differs from humans at a critical amino acid residue and hence does not interact with *L. monocytogenes* InlA. Specifically, the difference is at the 16^{th} amino acid in the chain of the peptide that is a proline in human and glutamic acid in murine E-cadherin [28]. A transgenic mouse has been developed that expresses human E-cadherin in intestinal tissue, but not other tissues including placenta. Infection of the pregnant mouse results in more severe clinical symptoms than human infection (e.g. meningitis, stillbirth) including high mortality in adult mice and high fetal mortality with severe inflammation and necrosis of the fetus [104]. However, several studies have shown that intragastric infection of mice leads to fetal infection and fetal death in the mouse, and inflammatory lesions resemble those of *L. monocytogenes* infection during multiple human pregnancies (pregnancy of more than one fetus) [115, 116].

In Vitro Models for *L. monocytogenes* Infection of Placental Trophoblasts

During pregnancy, listerial cells have a trophism for placental cells at the maternal-fetal interface [2]. Despite extensive work with *L. monocytogenes* to elucidate its pathogenesis, little is known about the underlying mechanisms of fetal and placental infection. As stated earlier, humans have a hemonochorial villous placenta with minimal barriers between maternal and fetal structures (Figure 2) [102]. Syncytiotrophoblast and extravillous trophoblast cells are located on the villous parts of the fetal placenta. Syncytiotrophoblasts line the villi, which are immersed in maternal blood. At the tip of the villi, extravillous trophoblast cells anchor the fetal placenta to the decidual tissue, and are essential in altering the uterine vasculature to increase maternal blood flow to the fetus (reviewed in [117] and [118]).

Previously, it has been shown that *L. monocytogenes* can infect primary cultures of extravillous trophoblast cells and immortalized lines of these cells (JEG-3, JAR, and BeWo cells) [119, 120]. Entry of listerial cells into the trophoblast cells on placental villi is mediated by E-cadherin – InlA interaction [28]. However, different laboratories have suggested alternative trophoblast target cells for *L. monocytogenes* entry. Lecuit et al. showed syncytiotrophoblast cells to be the point of entry into the placenta to infect the fetus [121]. On the other hand, Robbins et al. have shown that syncytiotrophoblast cells constitute a barrier to transmission of *L. monocytogenes* from the maternal bloodstream to the placenta. These authors also found that primary cultures of extravillous trophoblast cells can become infected, but restrict growth and intracellular spread *L. monocytogenes* [120]. All of the published work on *L. monocytogenes* infection of

placental cells focused on the ability of the bacterial cells to infect trophoblasts and the mechanism by which listerial cells spread to the fetus. The effect of *L. monocytogenes* infection on trophoblast function, and how this might affect the ability of the placenta to support the continued growth of the fetus, has yet to be examined.

Figure 1

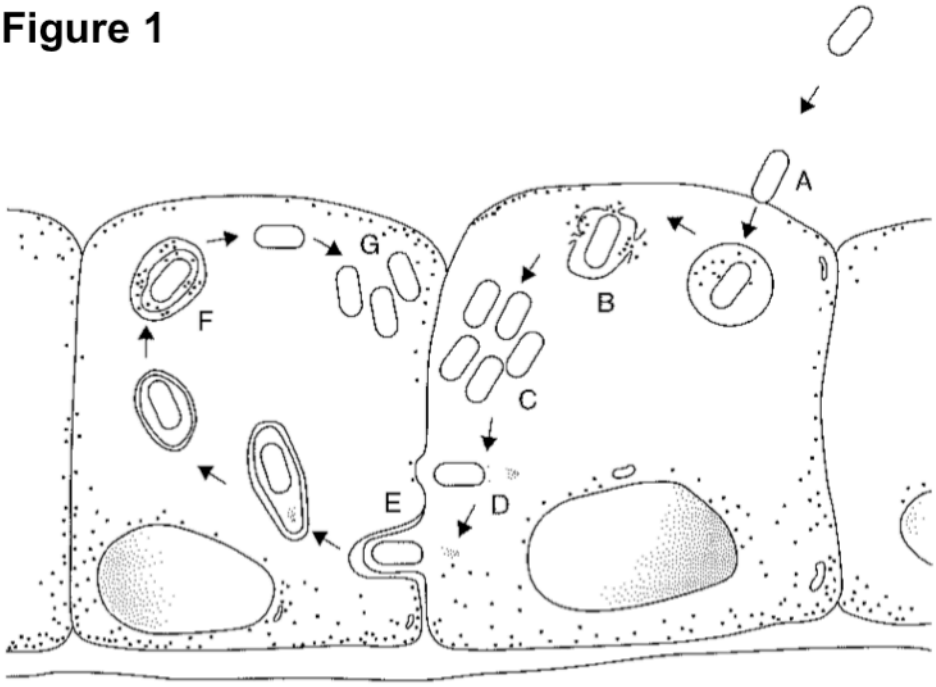

Figure 1. Illustration of the events of listerial cell invasion, intracellular growth, intracellular movement, and cell to sell spread. (A) As *L. monocytogenes* use internalin proteins internalin A (InlA) and internalin B (InlB), respectively to mediate cell entry via E-cadherin and c-Met tyrosine kinase on the host cells. (B) After internalization, the listerial cells escape the phagosome by expressing listerolysin O (LLO) and phosphatidylinositol-specific phospholipase C. (C) In the cytoplasm, *L. monocytogenes* cells multiply with a generation time of approximately 1 hour at 37°C. (D) Listerial cells move to the periphery of the infected cell via action of the bacterial surface protein, ActA, which orchestrates addition of actin monomers to the caudal end of the bacteria forming actin filaments that propel it rostrally. (E) Spread from cell to cell occurs by the bacteria being propelled into the cell membrane, forming an invagination of the adjacent cell membrane resulting in the listerial cell being contained within a double membrane vacuole. (F) To escape the double-membraned vacuole, *L. monocytogenes* uses a phosphatidylcholine-dependent phospholipase C (also referred to as lecithinase) and LLO. (G) The process is repeated. This figure was reproduced from [2].

Figure 2

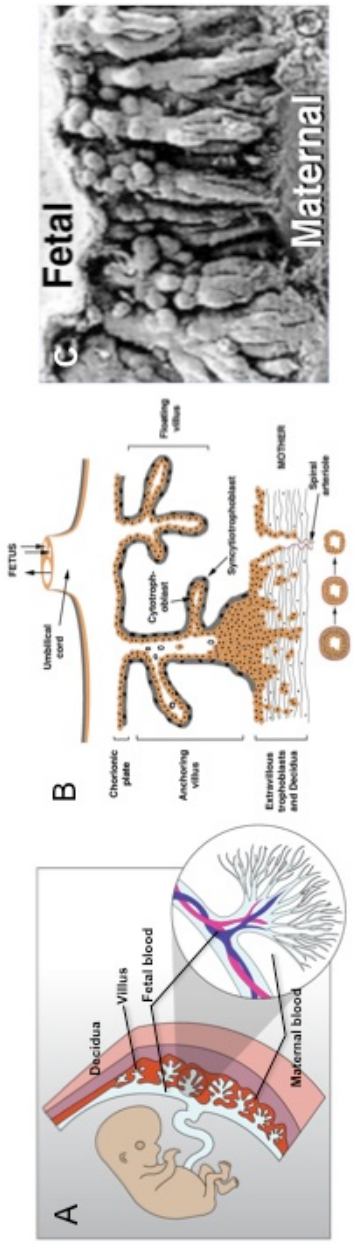
C
Fetal
Maternal
B
FETUS
Umbilical cord
Cytotroph-oblast
Syncytiotrophoblast
Floating villus
MOTHER
Spiral arteriole
Chorionic plate
Anchoring villus
Extravillous trophoblasts and Decidua
A
Decidua
Villus
Fetal blood
Maternal blood

Figure 2. Human placenta structure. (A) Developing fetus with placental villi bathed in maternal blood in the intervillous space. These tissues are attached to the decidual tissue in the uterus. The inset illustrates an enlargement of a fetal villus, which facilitates oxygen exchange and nutrient transport to the fetal circulation via the umbilical arteries (blue) and vein (red). (B) Enlargement of panel A shows different trophoblast cell types. Extravillous trophoblast cells anchor the fetal placenta to the decidual tissue, influence the maternal immune system to allow fetal tolerance, and alter maternal blood flow in the spiral arteries to increase nutrient availability to the fetus. Extravillous trophoblasts arise from the trophoblast cell columns, while villous cytotrophoblast cells differentiate from syncytiotrophoblast layer overlying the villus. The syncytiotrophoblast cell layer is responsible for gas and nutrient exchange from the maternal blood in the intervillous space to the fetal blood in capillaries located in the villous stroma. (C) Scanning electron micrograph of the maternal-fetal interface of a rhesus monkey showing anchoring villi of fetal placenta attached to maternal decidual tissue (reproduced from [122]).

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Chapter Two

Pregnancy reduces the genetic resistance of C57BL/6 mice to *Listeria monocytogenes* infection by intragastric inoculation

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Abstract

In this study, we compared genetically resistant C57BL/6 and susceptible A/J mice for their resistance to *L. monocytogenes* infection model during pregnancy. Intragastric infection with modest numbers of bacterial cells (10^5 CFU) caused reproducible fetal infection and abortion in both mouse strains. Bioluminescence imaging demonstrated dissemination of *L. monocytogenes* cells from maternal to fetal organs within 3 days of intragastric infection. Although non-pregnant C57BL/6 mice were significantly more resistant to infection than non-pregnant A/J mice, C57BL/6 and A/J mice had similar microbial loads (CFU) in maternal and fetal tissues during pregnancy. Inflammation and necrosis, however, were more severe in A/J mice as evaluated by semi-quantitative histopathology. Although the microbial load in fetal tissues was similar for all fetuses within a single uterus, inflammation and necrosis varied among individual fetuses and placentas. We also noted that the uterus is a target for *L. monocytogenes* infection in non-pregnant mice.

Introduction

Listeria monocytogenes continues to be a significant cause of disease, especially in the industrialized nations of the world. Listeriosis results in an estimated 1600 cases (range 600-3200) and 250 deaths (range 0-700) annually in the United States [1]. The infectious dose for humans is uncertain and likely depends on the virulence characteristics of individual *L. monocytogenes* strains. Clinical disease can result from estimated ingestion of as few as 10^4-10^6 CFU/g in contaminated foodstuffs. However, the infectious dose is likely lower in immunocompromised or pregnant individuals [2, 3]. Pregnant women are estimated to be 20 times more likely than the average adult to acquire invasive listeriosis, and represent 16% of reported listeriosis cases in the USA [4, 5]. Most maternal *L. monocytogenes* infections occur in the 3rd trimester and are mild or asymptomatic. However, fetal infection can be severe with stillbirth and spontaneous abortion occurring in 20% of cases, and 68% of the surviving pregnancies resulting in neonatal infection [6]. Despite considerable knowledge of the pathogenesis of listerosis, it is unclear why pregnant women do not successfully protect their fetus against intracellular pathogens such as *L. monocytogenes*.

Several different animal models have been developed to study *L. monocytogenes* infection during pregnancy. These differ in host species and strain of *L. monocytogenes* used, route of infection, dose of inoculum, and gestational length at inoculation, [7, 8]. It has been hypothesized that InlA and InlB mediate internalization of *L. monocytogenes* into non-phagocytic cells of the gastrointestinal system [8-12]. The receptors for InlA and InlB are E-cadherin and Met, respectively [13-15]. Importantly,

InlA interacts with human and guinea pig E-cadherin but not that of mouse or rat [16]. On the other hand, InlB interacts with human and mouse Met but not that of the guinea pig [17]. Despite the poor ability of mouse E-cadherin to bind InlA, several previous studies used intravenous or intragastric infection with various strains and doses of *L. monocytogenes* to cause fetal infection and abortion in the mouse [18-22]. Pregnant mice exhibited increased severity of maternal disease after intragastric infection when compared to non-pregnant mice [18]. These studies suggest that virulence factors other than InlA allow translocation of *L. monocytogenes* cells across the intestinal epithelium in the mouse. In this paper, we sought to develop an intragastric (i.g.) infection model of fetal infection in pregnant mice, using a clinical isolate of *L. monocytogenes* from a human abortion outbreak, to more closely mimic natural infection in humans and domestic animals.

Inbred mouse strains differ in their innate resistance to experimental infection with *L. monocytogenes*. Resistance is regulated principally by the *Hc* locus on chromosome 2 [23-25]. Mouse strains with the resistant allele at the *Hc* locus are significantly more resistant to i.v., i.p. or i.g. challenge with *L. monocytogenes* than strains with the susceptible allele [24-26]. Previous studies identified the C57BL/6 and A/J strains of mice as prototypic resistant and susceptible strains, respectively [24, 25]. Whether these mouse strains differ in resistance to *L. monocytogenes* infection during pregnancy is unknown. In this study, we compared pregnant C57BL/6 and A/J mice for their resistance to i.g. infection with a strain of *L. monocytogenes* isolated from a human listeriosis outbreak associated with abortion and fetal death [27]. To provide additional

insights into the temporal response to *L. monocytogenes* infection in pregnant mice, we used bioluminescence imaging to visualize the progression of infection from maternal to fetal tissues. Our results show that pregnant C57BL/6 and A/J mice do not differ significantly in their microbial load in tissues. We also identify the uterus of non-pregnant mice as a site of *L. monocytogenes* infection and show that not all fetuses suffer the same degree of inflammation and necrosis during *L. monocytogenes* infection of the dam.

Materials and Methods

Strains of L. monocytogenes

L. monocytogenes strain 2203 (serotype 4b) was generously donated by Dr. Sophia Kathariou (Raleigh, NC). This is a clinical isolate from a food-borne disease outbreak that caused disease in 13 people, 11 of whom were pregnant. Five of the pregnant women experienced stillbirth, 3 were induced into premature labor, and 3 births resulted in neonatal infections [27]. Bioluminescent *L. monocytogenes* strain 10403S was originally obtained from Dr. Christopher Contag (Stanford, CA) and made bioluminescent by transformation with the plasmid pAUL-A Tn4001 *luxABCDE* Km^r [28, 29].

Preparation of L. monocytogenes

L. monocytogenes cells were stored at -20°C on Cryobank™ Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of brain heart infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500 X g for 5 minutes), washed three times in phosphate buffered saline and kept on ice prior to inoculating mice. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial dilutions onto tryptic soy agar with 5% sheep blood (BD® Biosciences).

Inoculation of mice

Female inbred A/J and C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) at 6 weeks of age and housed under microisolator caps at the School of Veterinary Medicine animal care facility. For bioluminescence studies, albino C57BL/6 (Jackson Laboratories) were obtained at 6 weeks of age and housed under microisolator caps at the UW-Madison Microbial Sciences animal care facility. Mice were acclimated for 1 week in these facilities prior to being paired with a breeding male. Female mice were allowed to reach 7-10 days of gestation prior to use in an experiment. Mice received food and water ad libitum until 5 hours prior to an intragastric inoculation experiment, at which time food was removed from the cage. This was done to minimize the risk of delivery of the bacterial inoculum into stomachs that were engorged with mouse chow, which could lead to aspiration of the inoculum into the lungs. Mice were anesthetized by i.p. injection of sodium pentobarbital (40 mg/kg). When the mice were sedated, the listerial inoculum was introduced (as a total volume of 0.1 ml) via a 1.5 in.-long, 24 gauge, stainless steel oral esophageal tube attached to a 1-ml syringe.

Bioluminescence imaging

Bioluminescence imaging was performed using an IVIS® 200 Imaging System (Caliper Life Sciences, Hopkinton, MA) as instructed by the manufacturer. Mice were anesthetized with isoflurane and bioluminescence was recorded for 3 minutes at a pixel binning of 8. Bioluminescence was measured as total photon flux (photons/sec/cm^2) by the Living Image® software package (Caliper Life Sciences, Hopkinton, MA).

Recovery of L. monocytogenes from the tissues of infected mice

At the desired time points, mice were humanely euthanized by asphyxiation with CO_2 followed by exsanguination and cervical dislocation. Blood was collected into a syringe containing sodium citrate as an anticoagulant. The blood was then serially diluted in sterile saline, plated (0.1 ml) on blood agar, and the plates incubated at 37°C. The abodominal cavity was then aseptically opened and portions of the spleen, liver, and fetoplacental units (FPU) (3 FPUs per pregnant mouse) were removed. These tissues were weighed in sterile weigh boats and placed into separate sterile tissue grinders that contained 1 ml of cold, sterile saline. The tissues were homogenized with sterilized Teflon tissue grinders, diluted in sterile saline, and plated on blood agar. The plates were allowed to dry and then incubated at 37°C for 48 hours. Colonies were counted and the data expressed as mean ± standard error of the mean (SEM) $\log_{10}$ CFU of *L. monocytogenes* per gram of tissue (wet weight). Mice that died or were euthanized due to systemic disease were assigned a value of 10^8 CFU/g for the spleen, liver, and FPU, based on bacterial burden typically observed in previous experiments [24, 26, 30].

Histopathology

At the time of necropsy, portions of the spleen, liver, and fetoplacental units were removed, placed in plastic cassettes, and fixed in 10% buffered formalin. Following fixation and embedding into paraffin, the tissues were serial sectioned, mounted on glass slides, and stained with hemotoxylin and eosin or a tissue gram stain. The sections were coded and evaluated by a veterinary pathologist who is board certified by

the American College of Veterinary Pathologists (H.S.). Pathological changes in spleen samples were scored on a 0-3 scale with 0 defined as no lesions present, 1 mild to moderate inflammation, 2 moderate to severe inflammation, and 3 severe inflammation and necrosis. Liver sections were scored based on number of focal inflammatory lesions per five -100x fields.

Statistical analysis

Non-parametric analysis was done using Wilcox Rank Sum Test due to bimodal distribution, and variability of intragastric infection (SAS program Version 9.2 Cary, NC). The statistical significance for all comparisons was set at $P < 0.05$.

Results and Discussion

Pregnancy alters the susceptibility of A/J and C57BL/6 mice *to intragastric inoculation with L. monocytogenes*

In our first experiments, we compared the susceptibility of non-pregnant and pregnant C57BL/6 and A/J mice (10-14 days of gestation) to an i.g. challenge with 10^5 CFU of *L. monocytogenes* strain LM2203. Mice were euthanized 72 hours post inoculation, a time point that represents the peak bacillary burden in the spleen and liver [31, 32]. As expected, significantly greater numbers of CFU were recovered from the spleens, livers, and uteri of non-pregnant A/J mice than C57BL/6 mice (Figure 1A) [26]. These data confirm previous reports regarding the greater innate resistance of C57BL/6 vs. A/J mouse strains[26]. It should be noted that *L. monocytogenes* were recovered from the uteri of both strains of non-pregnant mice. It is unknown whether colonizing uterine tissue is a general property of *L. monocytogenes,* or a unique attribute of strain LM2203.

We observed a trend for greater microbial loads in the spleen, liver, and fetoplacental units (FPUs) of pregnant A/J mice when compared to C57BL/6 mice (Figure 1B). However, these differences are not statistically significant (spleen P=0.16, liver P=0.093, FPU P=0.24). We assessed mice at 72 hours of infection because this time produced the most consistent maternal and fetal infections following inoculation. Longer incubation times (96 hours) could not be used because some of the C57BL/6 and all of the A/J mice had developed severe clinical disease requiring euthanasia by that time. We repeated these experiments using challenge inocula of 10^3 and 10^6 CFU

of *L. monocytogenes* LM2203. The lower dose (10^3 CFU) resulted in consistent systemic infection of maternal but not fetal tissues (Figure 1C) and the higher dose (10^6 CFU) produced severe disease, which required euthanasia of all mice (data not shown). These findings suggest there is threshold dose (>10^3 CFU of LM2203) to cause fetal infection. This finding supports a previous report by Robbins et al. (2010), which suggest the placenta has multiple mechanisms to resist bacteremic spread of *L. monocygogenes* from maternal blood. As the challenge dose increases, these defenses are overcome, allowing fetal infection to occur. Transport of listerial cells from maternal to fetal tissues may not be unidirectional, as Bakardjiev et al. [33] reported trafficking of *L. monocytogenes* back to maternal organs, allowing the fetal tissues to become a source for re-infection of maternal tissues. We also examined the effect of *L. monocytogenes* infection in pregnant mice at different times of gestation (7-10 days and 14-17 days) and found that the 10-14 day gestation period resulted in the most reproducible infection of fetal tissues (data not shown). These data show that pregnancy reduces resistance to *L. monocytogenes* infection of C57BL/6 compared to A/J mice in terms of microbial load in maternal liver and spleen. Interestingly, despite similarities in microbial load in maternal and fetal tissues, the pregnant C57BL/6 mice displayed fewer clinical signs associated with septic listeriosis than pregnant A/J mice.

The above results are somewhat contrary to our previous report of the greater resistance to listeriosis in C57BL/6 vs. A/J non-pregnant mice [26]. The data suggest that the immunosuppressive effect of pregnancy diminishes the heritable resistance of C57BL/6 mice compared to A/J mice, which is linked to the *Hc* locus of chromosome 2

[23-25]. Reduced maternal resistance *to L. monocytogenes* in pregnant mice is consistent with listeriosis in pregnant women, who have a greater likelihood of contracting *L. monocytogenes* after ingesting contaminated food. There is an earlier report of decreased maternal resistance to i.g. *L. monocytogenes* infection in pregnant mice [18]. Those authors used two strains of *L. monocytogenes*, serotypes (1/2a and 4nonb), and found significant differences in microbial load in maternal livers and spleens of non-pregnant vs. pregnant mice at 120 hrs after inoculation. Hamrick et al.[18] also reported colonization of the conceptus 48 hours post inoculation, although the microbial load in fetal tissues was not quantified. Our data differ from those of Hamrick et al. by reporting a greater microbial load following infection with a serotype 4b strain of *L. monocytogenes,* and by quantifying microbial load in fetal tissues. The mechanism by which pregnancy increases susceptibility to listeriosis is unknown. Possibilities include pregnancy-related changes in cell-mediated immunity [34, 35], changing hormonal status in the peri-parturient period [36], and alterations in cytokines and chemokines present in amniotic and allantoic fluids [22]. Perhaps these and other changes during pregnancy influence resistance to *L. monocytogenes* infection.

Histopathological examination of lesions in maternal and fetal tissues from A/J and C57BL/6 mice

Severity of inflammation and necrosis in the spleen was scored on a 0-3 scale, with 0 defined as normal and 3 denoting severe inflammation (Figure 2a, 2c). All inflammation noted was of mixed cellular populations. Histopathology scores were significantly greater in pregnant A/J than C57BL/6 mice ($p=0.030$). Non-pregnant A/J

mice also tended to have greater histopathology scores than non-pregnant C57BL/6 mice, however, in both strains the histopathological changes were less severe and the difference between strains did not achieve statistical significance (p=0.08). The clinical significance of semi-quantitative scoring of splenic tissue is difficult to interpret because of the heterogenous architecture of the spleen, especially during inflammation. Interpretation of liver pathology was more straightforward, as we quantified the number of focal inflammatory lesions per five 100X magnification fields (Figure 2b). Both pregnant and non-pregnant A/J mice had significantly more liver lesions than pregnant (p=0.02) or non-pregnant (p=0.02) C57BL/6 mice (Figure 3). The reason for greater histopathological changes in A/J than C57BL/6 mice, despite recovery of similar numbers of CFU from the livers of the two mouse strains, is unclear. We also examined Gram-stained liver sections from C57BL/6 and A/J mice. Gram-positive rods were commonly observed in liver sections from A/J mice (pregnant and non-pregnant). However, bacteria were not seen in non-pregnant C57BL/6 mice and were rarely observed in liver sections from pregnant C57BL/6 mice. The former is likely due to the bacterial burden in non-pregnant C57BL/6 mice being near the minimum threshold for microscopic visualization of bacterial cells. The Gram stain and liver histopathology data are curious because they contradict somewhat the microbial load data for A/J and C57BL/6 mice illustrated in Figure 1. The histopathology data resemble more closely the previously reported greater resistance of C57BL/6 vs. A/J in non-pregnant mice [26]. The mechanism underlying the relatively high microbial load but lesser histopathologic lesions in pregnant C57BL/6 mice is unknown and warrants further study.

Pregnant uterine horns were serially sectioned to visualize each fetus and placenta. Not all feti within a single uterus displayed the same degree of inflammation. In both A/J and C57BL/6 mice, histopathology was variable within each pregnant uterus, ranging from normal viable feti to severe inflammation and autolysis of dead feti (Figure 2e, 2f). These findings are consistent with reports in the human clinical literature of twin human pregnancies in which culture positive *L. monocytogenes* infection was observed in only one fetus [37-39]. Human listeriosis infections also have variable inflammation and fetal mortality rates [37-39]. What is curious in the present study is that the CFU of *L. monocytogenes* recovered from the FPUs of *L. monocytogenes*-infected pregnant mice are consistent, whereas the inflammation and necrosis in fetal tissues from a single pregnant mouse uterus were variable. We do not know whether the normal appearing fetuses would have resulted in normal deliveries and healthy pups. Our findings are contrary to reports in sheep and cattle, in which *L. monocytogenes* is a common cause of abortion. Similar to ruminants, which often have 2-3 developing fetuses per gestation, mice have multiple fetuses. However, published reports indicate that all fetal lambs and calves become infected and are aborted when a dam is infected with *L. monocytogenes* [40-42].

Bioluminescence imaging shows real-time progression of L. monocytogenes 10403S from maternal to fetal tissues in vivo

We next used bioluminescence imaging to visualize and quantify *L. monocytogenes* infection of pregnant mice. To do so we used a different strain of *L. monocytogenes* (LM10403S), which contains bacterial luciferase [28, 29]. The parent

strain of *L. monocytogenes* is a serotype 1/2a strain, first isolated in 1968 from a human skin infection,[43] that is widely used to study the pathogenesis of listeriosis. This strain contains a luciferase construct that emits light only when the bacterial cells are multiplying. It is important to note that this strain is a different serotype than LM2203 (serotype 4b) and requires a larger dose (10^6 CFU vs. 10^5 CFU) to cause fetal infection. To quantify the infection in pregnant mice, the signal was imaged and quantified with an IVIS® 200 Imaging System (Caliper Life Sciences, Hopkinton, MA).

A/J and C57BL/6 mice were inoculated i.g. with 10^6 CFU (based on pilot experiments with strain LM10403S) and imaged every 24 hours during a 72-hour infection period. This allowed us to monitor progression of the *L. monocytogenes* infection from maternal to fetal tissues (Figures 4a-c). Bioluminescence was quantified as photons/sec/cm^2 in regions of interest (ROI). After the last luminescence reading at 72 hours post infection, the mice were euthanized and tissues collected for microbial culture (Figure 4d). The CFU recovered from maternal and fetal tissues at the 72 hour timepoint correlated with the bioluminescent signals (ROI) calculated for the same tissues (r^2 = 0.768 and p = 0.02). These experiments show that following an i.g. infection, the maternal tissues are infected first, which leads to sepsis, fetal infection and abortion. Bioluminescence technology has been previously reported to image fetoplacental listeriosis following intravenous injection of *L. monocytogenes* into gerbils [44]. Here, we report use of bioluminescence imaging to visualize fetal infection following the physiologically relevant, intragastric route of inoculation. This more closely models how contaminated foodstuffs infect humans and their fetuses. We also show

that infection can be quantified *in vivo* using ROI measurements. This imaging technology could be used in future studies to test microbial growth in tissues *in vivo* with respect to novel therapeutic or prophylactic regimens.

One disadvantage of this bioluminescence model is that *L. monocytogenes* strain LM10403S emits bacterial luciferase only during log phase growth. Thus, tissue can contain viable *L. monocytogenes* (as confirmed by CFU) but yield little or no luminescence signal at a given timepoint. An example of this is illustrated in Figure 4d, in which only one FPU is luminescent at 48 hours post i.g. infection despite the recovery of similar CFU/g from feti. However, these *in vivo* imaging data are consistent with our histopathology finding that not all fetuses within a single uterus suffer the same degree of inflammation and necrosis during *L. monocytogenes* infection.

Conclusions

Taken together, our infection model and bioluminescent imaging technology show that i.g. infection of pregnant mice with *L. monocytogenes* causes reproducible fetal infection and abortion that mimics several aspects of clinical listeriosis in human beings. In addition, we find that the superior resistance of C57BL/6 mice is muted when mice are infected during pregnancy. We also report that infection with *L. monocytogenes* strain LM2203 results in bacterial colonization of the uterus, even in non-pregnant mice. Perhaps this affinity for the uterus explains in part the predilection of *L. monocytogenes* to cause fetal infection and abortion.

Acknowledgements

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Figure 1

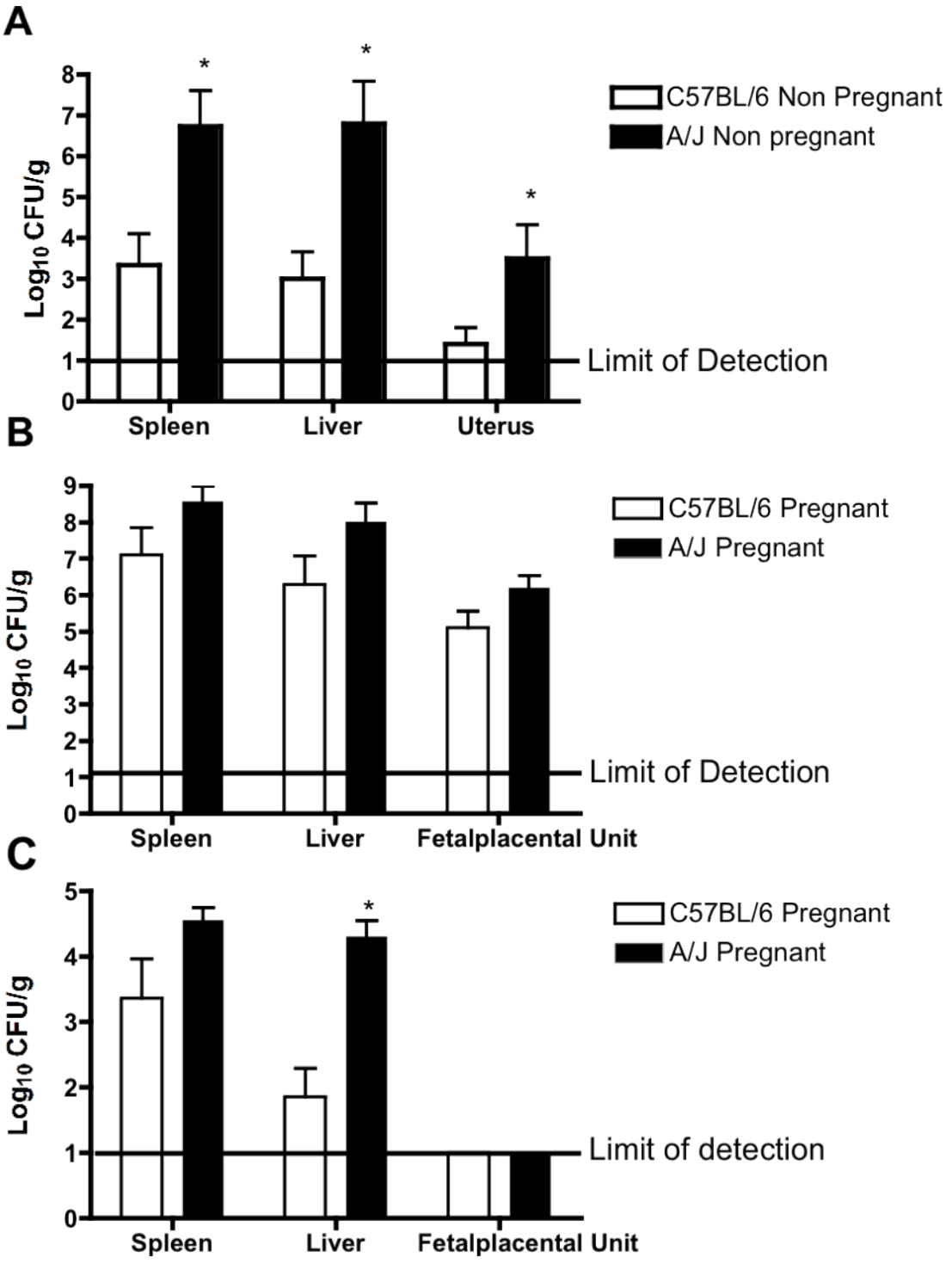
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Figure 1. *Listeria monocytogenes* (CFU/g) recovered from maternal and fetal tissues. Non-pregnant (A) and pregnant (B) C57BL/6 and A/J mice were anesthetized with 40 mg/kg of sodium pentobarbital (i.p.) and inoculated i.g. with 10^5 CFU (A and B) or 10^3 CFU (C) of *L. monocytogenes* strain LM2203. Seventy-two hours later, the mice were euthanized and $\log_{10}$ CFU/g determined for spleen, liver, uterus, and fetoplacental units as described in the Materials and Methods. Data are presented as the mean ± SEM $\log_{10}$CFU/g of tissue. The experimental groups in (A) consisted of 13 C57BL/6 and 11 A/J non-pregnant mice, in (B) of 15 C57BL/6 and 14 A/J pregnant mice, and in (C) of 8 C57BL/6 and 6 A/J pregnant mice. (*) $P<0.05$ A/J vs. C57BL/6 mice.

Figure 2

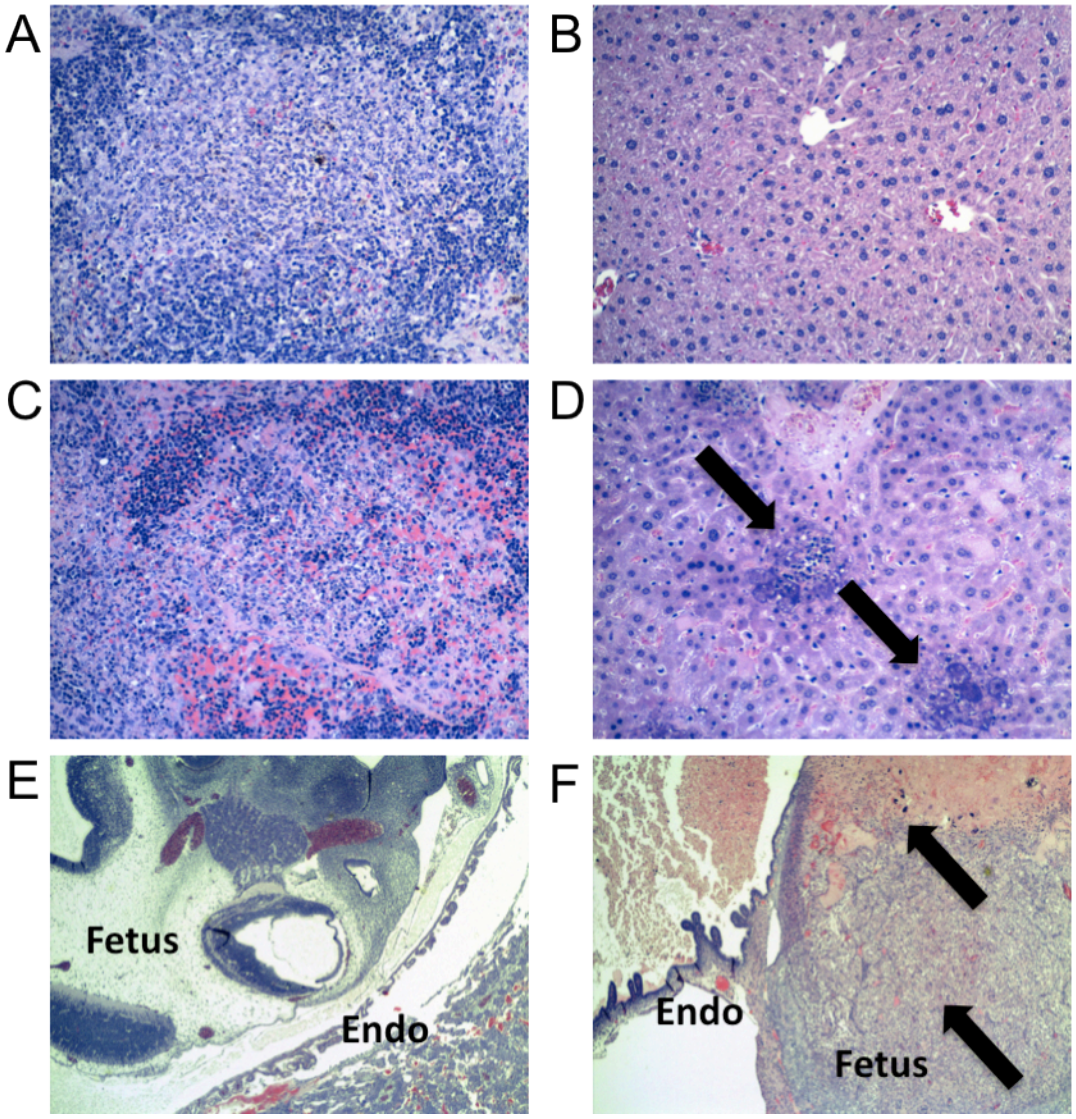
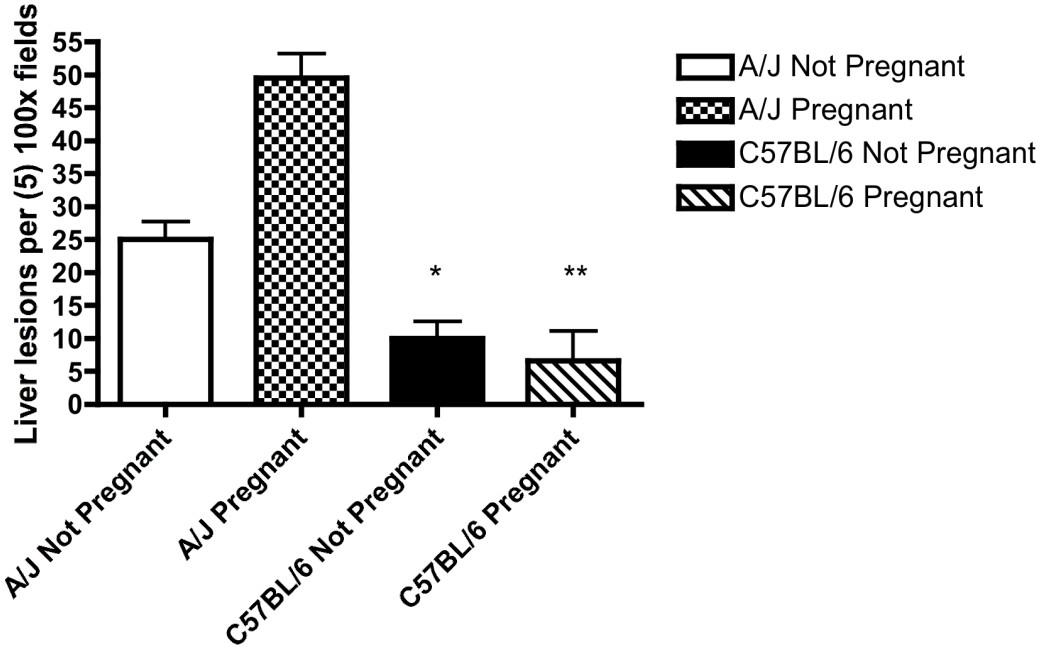


Figure 2. Inflammation and necrosis of maternal and fetal tissues assessed by histopathology. H&E staining (100X) of maternal spleen (A) and liver (B) from uninfected mice show normal tissue. Spleen inflammation was scored on a 0-3 scale, as described in the Materials and Methods. Panel (C) is an example of severe spleen inflammation (Score=3) from an infected pregnant mouse. Liver sections were evaluated by counting focal inflammatory lesions per five 100x magnification fields. Panel (D) is an example of multiple focal inflammatory lesions (arrows) in a liver section from an infected mouse. H&E staining (100X) of endometrium (Endo) and fetuses from different FPUs from the same uterine horn of a C57BL/6 mouse are labeled in (E) and (F). The FPU in (E) is histopathologically normal, whereas the FPU in (F) shows severe inflammation and necrosis (arrows). Bacterial evaluation of these same tissues yielded an average of 4.1 $\log_{10}$ CFU/g.

Figure 3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A/J Not Pregnant
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C57BL/6 Not Pregnant
C57BL/6 Pregnant

Figure 3. Liver inflammation is more severe in *L. monocytogenes* infected A/J than C57BL/6 mice. Tissue sections were stained with H&E and the number of focal inflammatory lesions per five 100x magnification fields quantified. All lesions contained mixed cellular populations. Pregnant and non-pregnant A/J mice had significantly more liver lesions than pregnant (*p = 0.02) or non-pregnant (**p = 0.02) C57BL/6 mice.

Figure 4

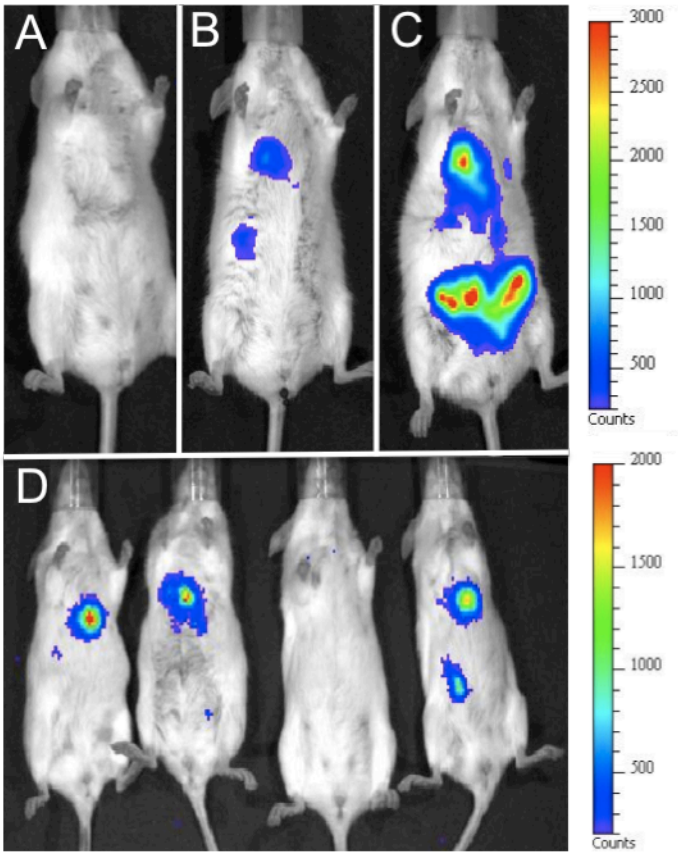
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Figure 4. Bioluminescence imaging of *L. monocytogenes* infection in living mice. All mice were inoculated i.g. with 10^6 CFU of LM10403S at 10-14 days of gestation. Pregnant mice infected with LM10403S demonstrate progression of infection from maternal to fetal tissues at (A) 24 hours, (B) 48 hours, and (C) 72 hours post infection. Panel B illustrates bioluminescence in the liver and one fetus. Panel C illustrates more severe maternal infection with intense bioluminescence in maternal liver and throughout the pregnant uterus. Panel D illustrates 4 pregnant mice at 72 hours after inoculation. Bioluminescence quantification (ROI) of actively growing *Listeria* cells (quantified as total photon flux in photons/sec/cm^2) correlated with $\log_{10}$ CFU/g for the same tissues in these mice (r^2=0.768 and p=0.02).

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Chapter Three

Post-parturient shedding of *Listeria monocytogenes* in breast milk of infected mice

Abstract

Listeria monocytogenes infection of the fetus or neonate during the peri-partum period is associated with high levels of morbidity and mortality. The objectives of this study were to develop an animal model to study *Listeria monocytogenes* infection during the peri-parturient period and identify sources of maternal shedding of the pathogen. Peri-parturient mice were infected intragastrically with *L. monocytogenes* that expressed bacterial luciferase. Bioluminescence imaging technology was able to detect light emitting *L. monocytogenes* in vaginal secretions and maternal and fetal organs at 72 and 96 hrs post infection in mice infected prior to, or just after, parturition. This approach indicates infection of the mammary chain in the dam. Enrichment culture of milk expressed from mouse mammary glands yielded growth of *L. monocytogenes.* The results from this study clearly show that *L. monocytogenes* is shed in vaginal secretions and disseminates to the mammary chain, which is shed in the milk of peri-parturient mice.

Introduction

Listeria monocytogenes is an important foodborne pathogen, with a predilection to infect the fetus and neonate in both human and veterinary species. Twenty-two percent of human perinatal infections result in stillbirth or neonatal death [1]. *L. monocytogenes* is also one of the three major worldwide causes of human neonatal meningitis. Neonatal listeriosis manifests in two forms: early or late onset [2]. Infants with early onset neonatal listeriosis are thought to be infected either in utero during the bacteremic phase of infection in the mother, who may present with a flu-like illness, or by ascending infection from the birth canal [3] . Average time for onset of clinical illness is 1.5 days of age. Presenting symptoms are consistent with sepsis including acute respiratory distress, fever, and lethargy. In addition, infants can develop pneumonia, and rarely meningitis or myocarditis [2]. Late onset neonatal listeriosis is less common, and occurs more frequently in infants, from uncomplicated pregnancies, with normal maternal health, who are healthy at birth [4]. Transmission in late onset listeriosis is assumed to occur during passage through the birth canal, or via nosocomial infection of the infant [5]. Average age for onset of clinical disease is 14.3 days and is more commonly associated with meningitis than sepsis. The late onset form of neonatal listeriosis can also present with mild to moderate clinical signs including fever, anorexia, diarrhea, and lethargy or irritability [6].

Although infants infected with *L. monocytogenes* are at high risk for severe disease, infected adults are often asymptomatic or present with mild flu-like symptoms [7]. However, in people with any degree of immunosuppression, which is seen in

neonates, the elderly, pregnant women, patients suffering immune compromising conditions, and people taking immunosuppressive drugs, listeriosis can result in severe septic disease syndromes [7, 8]. The mouse has been used to study *L. monocytogenes* infection because similar syndromes of sepsis are seen in experimental mouse infection models [9-12]. Specific to pregnant women, *L. monocytogenes* infection carries a high risk of fetal infection and abortion. This is true in pregnant mice as well and the mouse has emerged as a model to study pathogenesis of listeriosis during pregnancy [13-15].

We recently reported a novel murine model of *L. monocytogenes* infection during pregnancy that uses bioluminescence technology. The latter allowed us to perform real-time *in vivo* imaging and semi-quantify the outcome of intragastric (ig) infection, which included maternal sepsis, which subsequently led to fetal infection and abortion [13]. In this study, we use bioluminescence technology to visualize potential routes of transmission of *L. monocytogenes* to infants during parturition and lactation.

Breast milk feeding is the standard of care for infants because of the short and long term medical and neurodevelopmental advantages for the infant [16]. Because of the distinct health benefits, breastfeeding is now considered a public health issue, not solely a lifestyle choice by the American Association of Pediatrics [16]. Historically, breast milk is not considered a significant risk factor for transmission of *L. monocytogenes*, or other bacterial pathogens, to nursing infants. This is surprising considering the high awareness of human breast milk transmission of viral pathogens such as hepatitis B, cytomegalovirus, herpes simplex, Epstein Barr virus, and human

immunodeficiency virus (HIV) [17-21]. There are rare reports that *Listeria* and *Salmonella* contamination of human breast milk can cause neonatal illness [22-25]. Furthermore, transmission of bacterial pathogens via milk is widely recognized as causing infection of offspring in domesticated mammalian species. For example, *L. monocytogenes* contaminated milk and colostrum can cause neonatal sepsis in foals, calves, and crias [26-28]. It is also widely known that sheep and cattle intermittently shed *L. monocytogenes* in their milk, which is a major concern for zoonotic transmission to people via consumption of unpasteurized dairy products [29, 30].

Listeria monocytogenes is well adapted to survive in and on human breast milk collection and storage equipment, as the organism tolerates high and low temperatures, as well as high salt and low pH concentrations [reviewed in 31]. It is common for nursing mothers to collect and store milk in suboptimal storage conditions (e.g. small coolers), when away from home, where thorough cleaning with sanitizers is not available. Considering that exposure and transient colonization of the GI tract is relatively common (44% of healthy pregnant women shed *L. monocytogenes* in their feces when repeatedly cultured, 12% culture positive when a single sample is taken), contamination of breast milk with the pathogen is a distinct possibility [32]. The study presented here uses bioluminescence to test the hypothesis that intragastric *L. monocytogenes* infection of peri-parturient mice results in shedding of bacteria in vaginal and mammary secretions.

Materials and Methods

Strains of L. monocytogenes

Bioluminescent *L. monocytogenes* strain 10403SLUX, serotype 1/2a, was originally obtained from Dr. Christopher Contag (Stanford, CA). This construct was made bioluminescent by transformation with the plasmid pAUL-A Tn4001 *luxABCDE* Km^r, optimized for expression in Gram-positive bacteria [33, 34]. The addition of the *lux-kan* transposon cassette decreased virulence, evidenced by increased LD50 compared to the parent strain. This is theorized to be due to ectopic expression of novel proteins, to which *L. monocytogenes* is known to be sensitive [35].

Preparation of L. monocytogenes

L. monocytogenes cells were stored at -20°C on Cryobank™ Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of brain heart infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500 X g for 5 minutes), washed three times in phosphate buffered saline and kept on ice prior to inoculating mice. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial dilutions onto tryptic soy agar with 5% sheep blood (BD® Biosciences).

Infection of mice

All animal experiments were done under an approved protocol from the Research Animal Care Committee at the University of Wisconsin-Madison. Female inbred A/J mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) at 6

weeks of age and housed under microisolator caps at the UW-Madison Microbial Sciences animal care facility. The inbred A/J mouse strain was used because it is a prototypical susceptible strain of mouse regulated, in part, by the *Hc* locus on chromosome 2 [36-39]. Mice were acclimated for 1 week in this facility prior to being paired with a breeding male. Female mice were infected at various times during pregnancy and after parturition. Pre-partum infections were done at 10-14 days gestation (start of 3rd trimester) or 18-20 days gestation (just prior to whelping). For post-parturition infection experiments, lactating mice were infected 5 days post-whelping. Mice received food and water ad libitum until 5 hours prior to an intragastric infection experiment, at which time food was removed from the cage. This was done to minimize the risk of delivering the bacterial inoculum into stomachs engorged with mouse chow, which could lead to aspiration of the inoculum into the lungs. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) [40]. When the mice were sedated, the listerial inoculum (10^6 CFU) was introduced (in a total volume of 0.1 ml 0.9% NaCl) via a 1.5 in.-long, 24 gauge, stainless steel oral esophageal tube attached to a 1-ml syringe.

Bioluminescence imaging of mammary glands and vaginal secretions

Bioluminescence imaging was performed using an IVIS® 200 Imaging System (Caliper Life Sciences, Hopkinton, MA) as instructed by the manufacturer. Two hours prior to imaging, dams were removed from the pups to allow mammary engorgement. Five minutes prior to imaging, the damns were injected with 10 USP units of oxytocin (Bimeda) to stimulate milk let down to the gland cistern. Imaging the vaginal secretions

of mice experiencing abortion after infection was performed without prior preparation. All imaged mice were anesthetized with isoflurane and bioluminescence was recorded for 1 to 3 minutes at a pixel binning of 8. Blood agar plates were imaged for 10 seconds at a pixel binning of 8. Bioluminescence was measured as total photon flux (photons/sec/cm^2) by the Living Image® software package (Caliper Life Sciences, Hopkinton, MA).

Recovery of L. monocytogenes from luminescent mammary glands

Milk was collected from luminescent mammary glands immediately after imaging while mice remained unconscious from isoflurane anesthesia. The gland was expressed by hand onto a sterile, 5 mm diameter piece of filter paper. The filter paper was immediately placed into 5 ml of BHI broth and incubated overnight with shaking at 37°C. Following 18 hours of incubation, the culture was streaked (100 μl) onto tryptic soy agar with 5% sheep blood (BD® Biosciences). Plates were imaged for luminescent signal 24 and 48 hours later using the IVIS 200 system with Living Image Software.

Results

Pre-partum infection causes contaminated vaginal secretions and luminescent mammary glands.

Bioluminescence imaging revealed infection of maternal and fetal tissues in pregnant mice inoculated ig with 10^6 CFU of *L. monocytogenes* at 10-14 days of gestation [13]. Repeated imaging for 5 days after ig infection revealed luminescent vaginal discharge consistent with actively multiplying *L. monocytogenes* cells (Figure 1A). No live pups were born in mice infected at 10-14 days of gestation. It is unknown if pups were born alive and died, were stillborn, or if they were liquefied by necrosis and discharged. Serosanguinous discharge was noted in the cage but we could not rule out the dam eating sick or dead pups at birth (whelping tends to occur at night). When mice were infected later in gestation (18-20 days) and imaged post-parturition every 24 hrs for 5 days, luminescent vaginal discharge was evident, as were luminescent foci in the axillary mammary glands (Figure 1B). Live pups were whelped in these experiments, but no pup lived for more than 5 days. We do not know whether pups died from sepsis, or because the dams were systemically ill and did not nurse pups effectively. Bacterial culture of dead pups was not performed because they were autolyzed or destroyed by the dam. Clinically, all pregnant mice infected during pregnancy showed signs of systemic illness including decreased grooming, feeding, and hunched posture. Any mice that showed signs consistent with severe sepsis were humanely euthanized as per our approved animal use protocol.

Post-partum infection causes luminescent mammary glands and contaminated breast milk.

The preceding experiments suggested that *L. monocytogenes* disseminated to the mammary glands in pregnant mice. We next asked whether this occurred in mice infected after parturition. Bioluminescent imaging showed active *L. monocytogenes* infection in mammary tissue of mice infected ig with 10^6 CFU *L. monocytogenes* at 5 days after parturition. This time point was chosen to allow mammary development and increased milk production. Seventy-two and ninety-six hours after ig infection, mice were imaged and milk samples taken from luminescent glands (Figures 2A, 2C). To achieve the latter, mammary glands were expressed by hand onto 5 mm diameter, sterile filter paper discs. The discs were then placed in tubes that contained 5 ml BHI broth and incubated overnight at 37°C to enrich for *L. monocytogenes*. Samples from the broth culture were plated on blood agar and incubated at 37°C for 24 hours. When the plates were imaged they exhibited luminescent *L. monocytogenes* colonies intermixed with non-luminescent colonies (presumably normal skin microflora) (Figures 2B, 2D). Unlike mice infected during pregnancy, mice infected post-partum showed few, if any, clinical signs associated with systemic disease. Nor did any of the dams exhibit clinical signs that would have required euthanasia during any experiment.

Discussion

We have previously reported the use of bioluminescent bacteria to study hematogenous spread of *L. monocytogenes* to the uterus, *in vivo* [13]. Here, we use *in vivo* imaging to demonstrate that ig *L. monocytogenes* infection results in infection of the birth canal and contaminated vaginal discharge, as well as infection of mammary tissue and milk secretions. Post-parturient mice remain susceptible to infection and can shed *L. monocytogenes* in breast milk for at least 96 hours after infection without clinical signs of systemic illness. A weakness of our study is that we cannot quantitate the bacterial load in murine breast milk because we enriched milk secretions by incubation overnight in BHI broth. We directly inoculated tryptic soy agar plates with milk secretions but were not able to isolate *L. monocytogenes*, presumably due to competitive inhibition from normal milk and skin flora. This is consistent with other species, including ruminants, that are known to shed *L. monocytogenes*. Recovery of *L. monocytogenes* from cerebrospinal fluid or milk is uncommon without the use of enrichment or molecular biology techniques [41, 42]. This is not the case with human *L. monocytogenes* infection, in which the organism is easily cultured from cerebrospinal fluid, blood, and placenta without the need for enrichment or selective media [43]. We cannot rule out contamination of the teat by skin, fecal, or vaginal secretions because sterile preparation of the teats is not possible. Collection of human breast milk for culture is commonly contaminated with skin flora due to anatomy and culture method. This has been reported with methicillin-resistant *Staphylococcus aureus* (MRSA) and Group B streptococci, which are both known to be skin flora and shed in human breast milk [44-46].

Here we make the interesting observation that mice can become infected after parturition, and shed *L. monocytogenes* in breast milk. Non-pregnant humans and animals are considered to be at a relatively low risk for becoming infected with *L. monocytogenes*. The typical at risk human populations have some degree of immune suppression. These include adults treated with immuno-suppressive drugs, neonates with a developing immune system, and the fetus during pregnancy. Reasons for fetal susceptibility are not completely understood. Our results show that post-parturient mice are susceptible to infection and shed listerial cells without overt signs of septic illness, thus continually exposing their nursing pups to *L. monocytogenes*. These findings raise the question of whether some cases of late onset neonatal listeriosis are transmitted via contaminated breast milk from infected mothers. Further study would be required to more completely assess the clinical status of infected compared to uninfected lactating mice including food and water intake, body weight, pup growth, and pup survival rates. Future studies should also attempt transmission from post-partum infected lactating mice to pups. However, this will be difficult considering we do not have evidence that mice shed large numbers of *L. monocytogenes* in breast milk.

Transmission of bacterial pathogens via contaminated breast milk is commonly encountered in veterinary cases of neonatal listeriosis, but is generally not considered a risk for human neonatal listeriosis. This is not the case for viruses, such as HIV [47-49], for which breastfeeding is a known risk factor for transmission to neonates. Transmission of bacterial pathogens via breast milk has been reported in human neonatal cases of *L. monocytogenes* and *Salmonella enterica*, including *S.*

Typhimurium DT104 [22-25, 50]. If lactating humans shed *L. monocytogenes* in breast milk, it could be a factor in the pathogenesis of late onset neonatal listeriosis (several days to weeks after birth), which is typically associated with uncomplicated pregnancies, normal maternal history, and healthy babies at birth [2].

Effort is needed to define the risk of *L. monocytogenes* contamination of breast milk of nursing mothers with listeriosis, or mothers with infants suffering from neonatal listeriosis. The standard of care is to feed neonates fresh or fresh-frozen breast milk without testing or processing in neonatal intensive care units. Furthermore, maternal shedding of *L. monocytogenes* in the hospital environment could increase nosocomial infections [51-53]. This is particularly important with this bacterium because it is able to survive readily in the environment. In extreme examples, *L. monocytogenes* can persist in a food processing plant for years despite vigorous sanitation and disinfection efforts to eliminate it [54-60]. Bacteriological analysis of breast milk as a potential source of *L. monocytogenes* in infants diagnosed with listeriosis is relatively simple. It only requires a small amount of milk, which is easily collected and stored for qualitative analysis via culture or PCR. Clinical intervention for positive samples would then decrease exposure of the nursing infant and the potential for environmental contamination.

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Figure 1

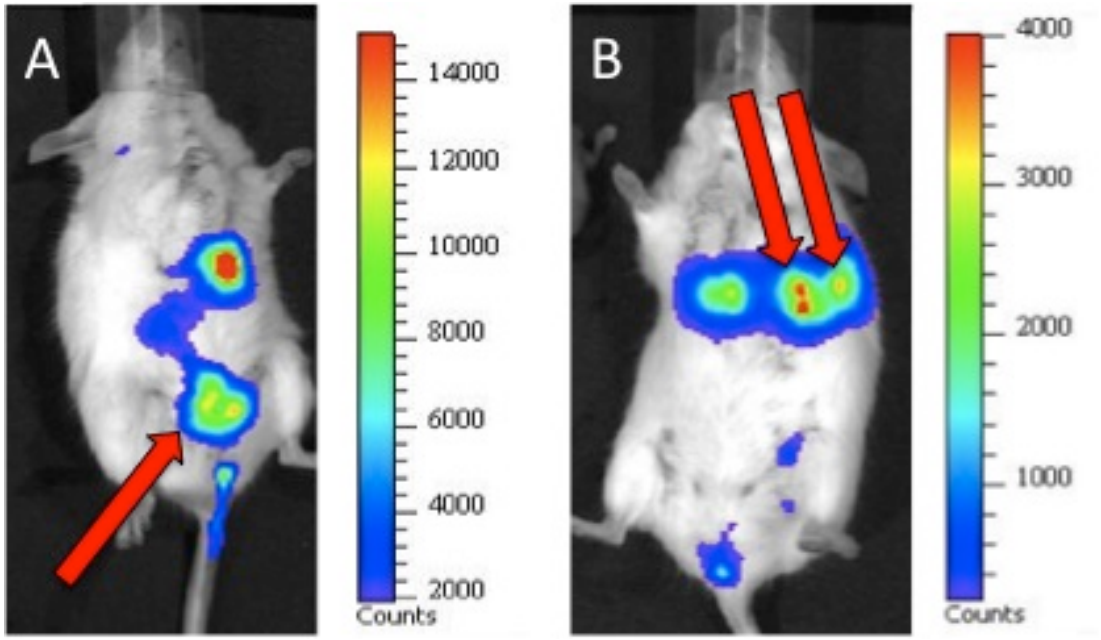
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Figure 1. Pre-partum *L. monocytogenes* infection causes fetal death and contaminated vaginal secretions. Mice were inoculated ig with 10^6 CFU of LM10403S. (A) Mouse was infected at 10-14 days of gestation and imaged 5 days later. Bioluminescence illustrates infection of fetal placental units (arrow) and discharge of contaminated secretions consistent with abortion. No live pups were born to mice infected at 10-14 days gestation. (B) Mouse was infected approximately 48 hours before its calculated whelping date and imaged 5 days later (3 days after delivering live pups – all of which died within 5 days of parturition). Bioluminescence illustrates contaminated vaginal secretions and infected axillary mammary glands (arrows). Images are representative from those obtained in 4 separate experiments with 4 mice per experiment.

Figure 2

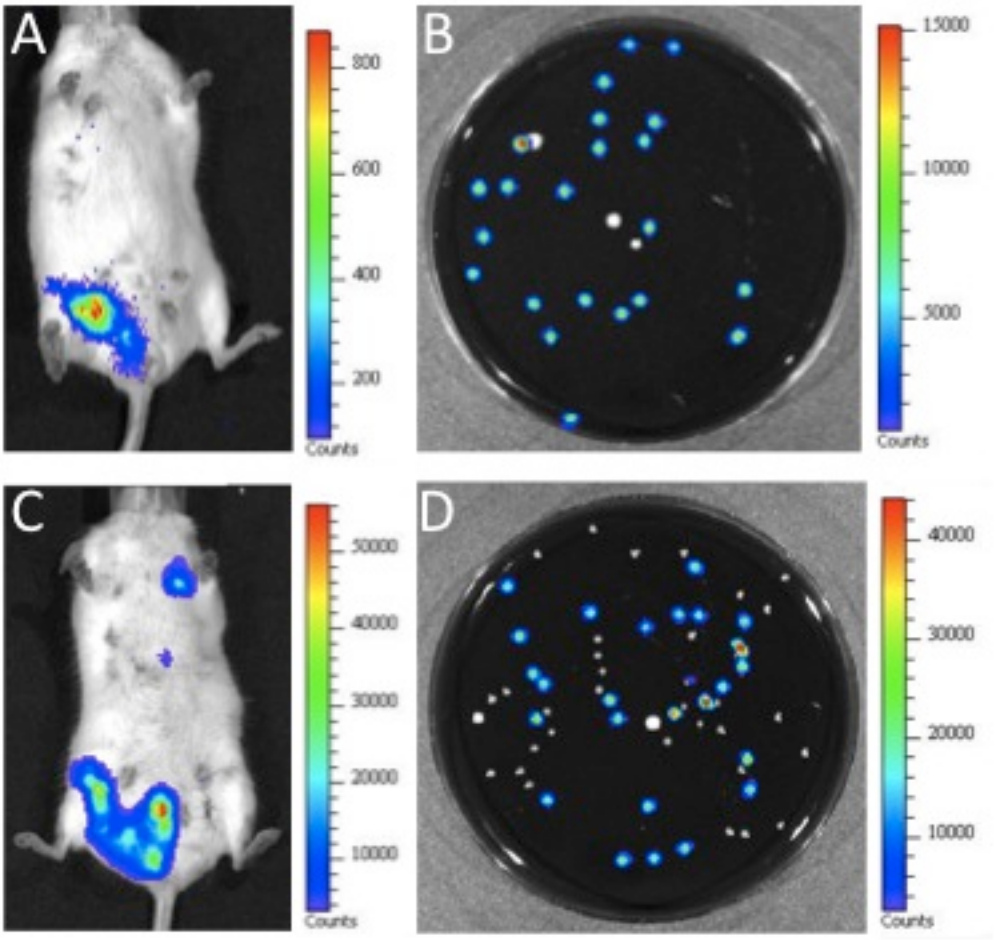
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Figure 2. Post-partum *L. monocytogenes* infection translocates to mammary glands and breast milk. Mice were inoculated ig with 10^6 CFU of LM10403S 3 days after parturition. The dams were removed from their pups for 2 hours to allow for mammary engorgement and injected intraperitoneally with 10 units of oxytocin to stimulate milk letdown to the gland cistern at (A) 72 hr and (C) 96 hr after infection with *L. monocytogenes*. Milk was expressed from luminescent glands onto sterile filter paper and placed in BHI media for a 24 hr enrichment culture prior to serial dilution in phosphate buffered saline and plating on blood agar. Plates incubated at 37°C for 24 hr and were imaged 24 hr later. Panel B illustrates post enrichment 10^{-8} dilution of milk expressed 72 hr after infection and Panel C a post enrichment 10^{-7} dilution of milk expressed 96 hr after inoculation. Plates exhibit luminescent *L. monocytogenes* colonies amongst non-luminescent colonies of bacteria. Pup mortality was not observed in dams infected 72 hr post-partum. Results are representative of 5 experiments with 2 mice per experiment.

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Chapter Four

Bacterial load and fetal inflammation is not dependent on IL-17a or IL-22 in pregnant mice infected with *Listeria monocytogenes*

Abstract

In this study, we first assessed the effect of intragastric infection of pregnant mice with *Listeria monocytogenes* on relative expression of select genes associated with T cell subsets. Multiplex quantitative PCR (qPCR) detected minimal changes in IFNγ, IL-17a, IL-22, CD3, and FoxP3 and no change in IL-4 in maternal livers from pregnant mice infected with *L. monocytogenes*. Relative gene expression was moderately increased in placental tissues for IFNγ, IL-4, IL-17a, IL-22, CD3, and FoxP3. To assess the roles of IL-17a and IL-22 in resistance to listeriosis during pregnancy, we compared the severity of maternal and fetal infection in IL-17a$^{(-/-)}$, IL-22$^{(-/-)}$, and IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice with that of wild type C57BL/6 mice. Intragastric infection with modest numbers of bacterial cells (10^5 CFU) caused reproducible maternal and fetal infection in all four mouse strains. We found greater microbial load in the bloodstream of pregnant IL-22$^{(-/-)}$ mice than pregnant wild type control mice. However, we found no significant difference in bacterial load in maternal or fetal tissues from pregnant IL-17a$^{(-/-)}$, IL-22$^{(-/-)}$, or IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice compared to pregnant wild type control mice. Nor did we observe histopathologic differences in severity of inflammation in maternal or fetal tissues from the various groups of mice. Although IL-17a and IL-22 are up-regulated in placental tissue, our study suggests that antibacterial resistance and the host inflammatory response are not dependent on IL-17a or IL-22 during infection of mice with *L. monocytogenes*.

Introduction

Listeria monocytogenes is commonly linked to foodborne disease outbreaks, especially in the industrialized nations of the world. Listeriosis results in an estimated 1600 cases per year in the U.S.A., which is relatively low compared to other bacterial and viral foodborne pathogens [1]. Despite its relatively low incidence, *L. monocytogenes* is associated with a high mortality, causing an estimated 250 deaths annually in the United States [1]. Immunocompromised or pregnant individuals are at higher risk for clinical disease due to listeriosis [2, 3]. Pregnant women represent 16% of *L. monocytogenes* clinical disease in the USA and have 20 times greater risk for developing moderate to severe clinical disease than the average adult [4, 5]. The 3rd trimester is the most common time of *L. monocytogenes* infection during pregnancy [6]. Although most infections are mild or asymptomatic, 20% of *L. monocytogenes* infections during pregnancy result in stillbirth and spontaneous abortion and 68% of live births to infected mothers result in neonatal infection [6]. It remains unclear why pregnant women cannot protect their fetuses against listeriosis.

Increased susceptibility to listeriosis during pregnancy has been hypothesized to be due to loss of homeostasis between pro- and anti-inflammatory T cell populations. In a non-pregnant animal, T helper (Th) 1 cells are significantly more abundant than Th2 cells, especially in response to infection with intracellular pathogens [7]. Pregnancy is associated with diminished Th1 cells, which are critical for host defense against listeriosis [3, 4, 8]. The balance among Th cell populations is important for defense

against infection with *L. monocytogenes,* because cytokines associated with the Th1 cell response (e.g. interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF_{α})), are critical for protective cellular immunity to *L monocytogenes* [9]. However, recent findings of additional distinct types of T cells, such as Th17 cells and T regulatory cells (reviewed in [10]), complicate assessment of the roles of T cells in protecting the fetus against *Listeria* infection during pregnancy.

Th17 cells are the predominant source of the cytokines IL-17a and IL-22 [11-13]. These cytokines have pro- and anti-inflammatory properties that are critical to inflammation and immune surveillance in mucosal and endothelial tissues. IL-17a is a pro-inflammatory cytokine, produced primarily by Th17 cells, which influences expression of multiple proinflammatory chemokines, cytokines, peptides, and other proteins involved in the acute phase response (reviewed in [14]). IL-17a also stimulates neutrophil differentiation, migration, and activation [15]. IL-17a has been detected in the human placenta during inflammation, and is associated with miscarriage and other pregnancy abnormalities [16]. IL-17a is found in the mouse placenta during normal pregnancy, but its presence in the placenta during *L. monocytogenes* infection has not been reported [17]. IL-22 is produced by several subsets of T cells, including Th17 cells, and has been implicated in host defense, inflammation, and tissue repair [18-20]. IL-22 regulates function of parenchymal cells, such as epithelial cells and hepatocytes [19, 21]. In some circumstances, IL-22 works synergistically with IL-17a and is reported to have multiple functions that are both pro- and anti-inflammatory (reviewed in [22]).

Here we test the hypothesis that *L. monocytogenes* infection during pregnancy causes alterations in the relative expression of inflammatory cytokines, including IL-17a and IL-22, associated with T cell populations. We further hypothesize that IL-17a and IL-22 have roles in resistance to listeriosis in pregnant mice. We test this latter hypothesis using gene knockout mouse strains.

Materials and Methods

Preparation of L. monocytogenes

L. monocytogenes strain 2203 (serotype 4b) was generously donated by Dr. Sophia Kathariou (Raleigh, NC). This is a clinical isolate from a food-borne outbreak that caused disease in 13 people, 11 of whom were pregnant. Five of the pregnant women experienced stillbirth, 3 were induced into premature labor, and 3 births resulted in neonatal infections [23]. *L. monocytogenes* cells were stored at -20°C on Cryobank™ Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of brain heart infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500 X g for 5 minutes), washed three times in phosphate buffered saline and kept on ice prior to inoculating mice. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial dilutions onto tryptic soy agar with 5% sheep blood (BD® Biosciences).

Strains of mice and inoculation

Female inbred C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) at 6 weeks of age and housed under microisolator caps at the School of Veterinary Medicine animal care facility. Breeding pairs of IL-17a$^{(-/-)}$ and IL-22$^{(-/-)}$ mice (C57BL/6 genetic background) were obtained from Dr. Iwakura (Univ. of Tokyo) and Genentech (San Francisco, CA), respectively. IL-17a$^{(-/-)}$ and IL-22$^{(-/-)}$ mice were housed and bred under microisolator caps under supervision of the Research and Animal Care Biotron facility. IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice were derived by breeding IL-17a$^{(-/-)}$ females with

IL-22$^{(-/-)}$ males. The IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice were bred to provide 6 week old female mice for experiments. All null mouse strains were genotyped (Table 1) to ensure their status using tail snip tissue submitted to an outside vendor (Transnetyx, Cordova, TN). Mice obtained from the breeding colony were acclimated for 1 week in the School of Veterinary Medicine animal care facility prior to being paired with a breeding male. Female mice were allowed to reach 7-10 days of gestation prior to use in an experiment. Mice received food and water ad libitum until 5 hours prior to an intragastric inoculation experiment, at which time food was removed from the cage. This was done to minimize the risk of delivery of the bacterial inoculum into stomachs that were engorged with mouse chow, which could lead to aspiration of the inoculum into the lungs. Mice were anesthetized by i.p. injection of sodium pentobarbital (40 mg/kg). When the mice were sedated, the listerial inoculum was introduced (as a total volume of 0.1 ml) via a 1.5 in.-long, 24 gauge, stainless steel oral esophageal tube attached to a 1-ml syringe.

Recovery of L. monocytogenes from tissues of infected mice

At the desired time points (10-14 days gestation), mice were humanely euthanized by asphyxiation with CO_2 followed by exsanguination and cervical dislocation. Blood was collected into a syringe containing sodium citrate as an anticoagulant. The blood was then serially diluted in sterile saline, plated (0.1 ml) on trypticase soy agar with 5% sheep's blood, and the plates incubated at 37°C. The abdominal cavity was then aseptically opened and portions of the spleen, liver, and fetoplacental units (FPU) (2-3 FPUs per pregnant mouse) were removed. These

tissues were weighed in sterile weigh boats and placed into separate sterile tissue tubes that contained 1 ml of cold, sterile saline. The tissues were homogenized with sterilized Zirconium Oxide beads using a Bullet Blender® (Next Advance, Averill Park NY). The homogenates were diluted in sterile saline, and plated on blood agar. The plates were allowed to dry and then incubated at 37°C for 48 hours. Colonies were counted and the data expressed as mean ± standard error of the mean (SEM) $\log_{10}$ CFU of *L. monocytogenes* per gram of tissue (wet weight).

Histopathology

At the time of necropsy, portions of the spleen, liver, and fetoplacental units were removed, placed in plastic cassettes, and fixed in 10% buffered formalin. Following fixation and embedding into paraffin, the tissues were serial sectioned, mounted on glass slides, and stained with hemotoxylin and eosin or a tissue gram stain. The sections were coded and evaluated by a veterinary pathologist who is board certified by the American College of Veterinary Pathologists (H.S.). Liver sections were scored based on number of focal inflammatory lesions per five -100x fields. Pathological changes in spleen and fetoplacental units were scored on a 0-3 scale with 0 defined as no lesions present, 1 mild to moderate inflammation, 2 moderate to severe inflammation, and 3 severe inflammation and necrosis.

Cytokine response to L. monocytogenes infection

At the time of necropsy, 80-100 mg pieces of maternal liver and entire placentas were removed, placed in cryogenic vials (Corning, Corning NY), snap-frozen in liquid

nitrogen, and stored at -80°C. RNA was extracted from tissue samples using the Applied Biosystems RNAqeuous kit (Life Technologies, Carlsbad CA) following the manufacturer's protocol. RNA concentration was determined using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE). RNA purity was assessed by $OD_{260/280}$, and all samples greater than 2.0 or less than 1.8 were discarded. RNA integrity was tested by running the sample on an agarose gel with ethidium bromide fluorescent staining [24]. One microgram of RNA was heated to 70°C for 10 minutes prior to transcription to cDNA, using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies) with an Eppendorf Mastercycler EP Gradient S thermocycler (Eppendorf, Hamburg). Relative gene expression of IFNγ, IL-4, IL-17a, IL-22, CD3, and FoxP3 between experimental and control groups of tissues, using beta actin (Actβ), 60S ribosomal protein L8 (RPL8), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes for normalization, were measured by real-time PCR using the Applied Biosystems StepOne Plus RT PCR system and analyzed with StepOne Software v2.1 (Life Technologies). Samples were loaded in triplicate into a 96-well optical reaction plate (Applied Biosystems), and sealed with optical sealing tape (MicroAmp Optical Adhesive Film, Applied Biosystems). The Power SYBR Green master mix and AmpErase Uracil N-glycosylase were used (Applied Biosystems) with a 1:50 dilution of cDNA from the qPCR reaction. Each reaction was optimized according to the manufacturer's protocol. Primer concentrations can be found in Table 2. The cytokine primer sequences were constructed using the National Center for Biotechnology Information (NCBI) Gene online software and manufactured by the University of Wisconsin Biotechnology Center

(Madison, WI). All primer sets were tested on cDNA isolated from control murine liver and placental tissues using regular PCR and products were sequenced by the University of Wisconsin Biotechnology Center. Sequences were then analyzed using NCBI Gene online software to ensure accurate amplification of the desired cytokines. The parameters for development of the primer sequences consisted of a primer length of 15-25 bp, an amplicon of 50-150 bp, a 30-80% GC content, and a melting point of about 60°C [24]. Primer sequences and amplicon size can be found in Table 2. Specific PCR amplification was confirmed by a dissociation curve, PCR products with a melting temperature lower than 81°C were not included in the relative quantification analysis [24]. Relative gene expression was then calculated using the $\Delta\Delta C_t$ method [24].

Statistical analysis

For comparison of bacterial burden and inflammation between groups of mice, non-parametric statistical analysis was done using a Kruskal-Wallis test of analysis of variance (ANOVA) followed with a Dunn's Multiple Comparison test of groups (Instat, GraphPad). Statistical significance for all comparisons was set at $P<0.05$.

Results

Expression of inflammatory cytokine mRNAs in maternal and fetal tissues from L. monocytogenes infected pregnant mice.

Quantitative PCR analysis showed minimal changes in expression of T cell associated genes in maternal liver from *L. monocytogenes* infected pregnant C57BL/6 mice, compared to uninfected pregnant mice. IL-4 and IL-17a expression did not differ and were slightly decreased, respectively, in infected pregnant mice compared to uninfected mice (Fig 1A). The $\Delta\Delta C_t$ method used to present the data in Fig. 1 can detect significant differences of 10 fold or greater [25]. The increases seen for IFNγ, IL-22, CD3, and FoxP3 were near the level of detection for the $\Delta\Delta C_t$ method.

Placental tissues from *L. monocytogenes* infected mice had increased expression of inflammatory cytokine mRNA as compared to uninfected pregnant mice (Figure 1B). As with maternal liver tissue, the relative expression difference ($\Delta\Delta C_t$) of several cytokines of interest (including IFNγ, IL-17a, and IL-22) were slightly increased. IL-4, CD3, and FoxP3 expression were moderately (>10-fold) increased in *L. monocytogenes* infected placental tissue compared to uninfected fetal tissue. ΔC_t values for infected vs. uninfected placental tissues suggested a trend towards increased relative gene expression, but did not achieve statistical significance (data not shown).

IL-17a and IL-22 are not required for resistance to L. monocytogenes in pregnant mice.

The modest increase in relative expression of IL17a and IL-22 in placental tissues support our hypothesis that IL-17a and IL-22 are involved in the protective

inflammatory response to *L. monocytogenes* infection during pregnancy. We utilized mouse strains with gene deletions for IL-17a$^{(-/-)}$, IL-22$^{(-/-)}$, and IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ to test the role of these cytokines in the maternal and fetal response to intragastric infection with *L. monocytogenes*. Overall, we observed similar levels of *L. monocytogenes* infection in pregnant IL-17a$^{(-/-)}$, IL-22$^{(-/-)}$, IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$, and wild type C57BL/6 mice. In each set of experiments, both control C57BL/6 and gene knockout groups of mice displayed mild to moderate signs of systemic illness (lack of grooming, movement, hunched posture) by the end of the 72 hour infection period. We observed no significant increase in microbial load in maternal and fetal tissues from pregnant IL-17a$^{(-/-)}$ (Figure 2A) or IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice (Figure 2C) compared to wild type C57BL/6 mice. We did, however, observe a significantly greater bacteremia in IL-22$^{(-/-)}$ than wild type pregnant mice (Figure 2B).

Portions of the tissues removed at necropsy were submitted for histopathological analysis. Focal inflammatory lesions in the liver were counted and the results compared between control C57BL/6 and gene knockout groups of pregnant mice (Figure 3A). No significant differences were seen in the number of liver lesions in control vs. experimental groups of mice ($p \geq 0.7$ among all experiments). We also quantified the severity of splenic inflammation using a scoring system detailed in the Materials and Methods, (Figure 3B). We observed no significant difference in splenic inflammation among any of the groups of mice ($p \geq 0.8$ in all experiments). Nor did we discern a significant difference in inflammation and necrosis in the FPUs of *L. monocytogenes*

infected control C57BL/6 mice compared to gene knockout mice ($p \geq 0.1$ – data not shown).

Discussion

Using qPCR, we found little change in the relative expressions of T cell associated genes in maternal liver of *L. monocytogenes* infected pregnant mice. However, the results of this study show that IFNγ, IL-4, IL-17, IL-22, and FoxP3 are moderately up-regulated in placental tissue following intragastric infection of mice with *L. monocytogenes*. Cluster of differentiation (CD3) is a four-chain protein complex that is part of the T cell receptor, and a marker for activated T cells. CD3 has been reported to be up-regulated in *L. monocytogenes* infection [26]. Here we show that CD3 expression is up-regulated in placental tissue, which is consistent with our findings of increased relative expression of T cell associated genes. IL-4 expression was similar to IFNγ, which is consistent with the expected Th2:Th1 ratio during pregnancy [3, 4, 7, 8]. One theory for the change from a Th1 to Th2 predominant response is due to elevated levels of progesterone in placental tissue [27]. Progesterone stimulates peripheral blood T cells to differentiate into Th2 lineage cells via the progesterone-induced blocking factor (PIBF) [28, 29]. PIBF is thought to act via a specialized IL-4 receptor to active a signaling pathway that induces Th2 cytokines (IL-3, IL-4, and IL-10) [30, 31].

IL-17a is part of the IL-17 family, which influences the production of chemokines (CXCL1, CXCL2, CXCL5, CXCL8) and cytokines (IL-6, GM-CSF, and G-CSF) that are responsible for recruitment, differentiation, and activation of neutrophils [11, 32]. Listeriosis is typified by mixed infiltration of macrophages and neutrophils into tissues. One exception is the placenta, which experiences primarily neutrophilic inflammation [33, 34]. Our results show that IL-17a expression is up-regulated in murine placental

tissue following intragastric infection with *L. monocytogenes* compared to uninfected control placental tissue. This finding is consistent with our hypothesis that IL-17a is involved in placental inflammation during early *L. monocytogenes* infection.

IL-22, like IL-17a, is produced primarily by Th17 cells, which in turn are regulated by IL-23 during *L. monocytogenes* infection [35]. Because IL-22 has both pro and anti-inflammatory functions in epithelial tissues, and in certain circumstances works synergistically with IL-17a, we hypothesized that it would play a significant role in resistance to intragastric *L. monocytogenes* infection [19, 21, 22]. Previous publications reported that IL-22 deficient mice do not differ in bacterial clearance of *L. monocytogenes*, or tissue protection [36, 37]. However, both of these studies used mice infected intravenously rather than via the gastrointestinal tract, which is the natural route of infection. We chose to examine the role of IL-22 in an intragastric infection model, which more closely follows the physiologic route of infection. We found relative expression of IL-22 is moderately increased in placental tissue following *L. monocytogenes* infection compared to placental tissue from uninfected control C57BL/6 mice. This finding supported our hypothesis that IL-22 is involved in the early pathogenesis of listeriosis in the pregnant mouse.

Forkhead box p3 (FoxP3) is part of the forkhead box (FOX) gene family that regulates development of cells. FoxP3 regulates development and function of CD4+CD25+ Treg cells, which prevent autoimmunity and regulate maternal tolerance to the fetus [38, 39]. Previous studies have shown that FoxP3+ regulatory T cells are

required to maintain pregnancy, but compromise host defense against prenatal bacterial pathogens [40]. However, regulatory T cell function is not always able to suppress the host immune response. One example of this was provided by a study that showed that the suppressive function of T reg cells can be over-ridden by *L. monocytogenes* infection in murine cardiac allograft models [41]. Our results show that relative FoxP3 expression in murine placental tissue is moderately elevated, as is fetal inflammation. This is consistent with previous murine allograft models and suggests that inflammation as a result of intragastric *L. monocytogenes* infection over-rides the suppressive regulatory T cell function.

A limitation of this study is that multiplex qPCR was performed at a single time point (72 hours) after intragastric infection. We chose this time point because we observe consistent microbial load and histopathology at 72 hours post infection in 10-14 day pregnant C56BL/6 mice [42]. It is possible that the inflammatory cascade results in more pronounced relative gene expression changes earlier in the course of infection than what we report here, but we did not perform qPCR analysis on mice euthanized at earlier time points.

We attempted to quantify antigen-specific T cells expressing the cytokines of interest. However, we did not recover sufficient numbers of antigen-specific T cells from murine placental tissue to allow us to distinguish various T cell subsets. We also attempted to quantify cytokine protein in tissues using a multiplex fluorescent bead

array. However, we obtained highly variable results, probably due to various confounding factors in placental tissue.

Despite evidence for increased relative expression of IL-17a and IL-22 in placental tissue of *L. monocytogenes* infected pregnant mice, the severity of early infection and inflammation of maternal and fetal tissues were not dependent on either IL-17a or IL-22. One exception was the microbial load in the bloodstream of IL-22 (-/-) pregnant mice, which was significantly greater than *L. monocytogenes* infected pregnant wild type mice. Perhaps this observation indicates that the presence of IL-22 on epithelial surfaces, especially gastrointestinal epithelia, could affect the ability of *L. monocytogenes* to translocate across the gut mucosa and cause bacteremia [19-21]. However, the overall bacterial burden was particularly high in the IL-22 (-/-) mice experiments, which could account in part for the increased bacteremia. We did not observe this effect in the IL-17a (-/-)/IL-22 (-/-) mice. It is possible that IL-17a and IL-22 have effects earlier or later in the host response, of which we did not examine.

We hypothesized that inflammation would be decreased in the placental tissue of IL-17a$^{(-/-)}$ mice, due to the role of IL-17a in neutrophil recruitment and activation, and the importance of neutrophilic inflammation in placental tissue during *L. monocytogenes* infection [33, 34]. As expected, histopathology showed that neutrophilic inflammation was a significant finding in maternal and fetal tissues. However, inflammation in maternal and fetal tissues and bacterial burden were similar among IL-17a$^{(-/-)}$, IL-22$^{(-/-)}$, and wild type mice. Nor did the IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ mice exhibit any differences in

inflammation of maternal or fetal tissues. However, due to the constraints of our experimental system, we cannot completely disprove our hypothesis as we only looked at one time after infection for reasons stated above. Further experiments would be required to test roles of IL-17 and IL-22 at early and later times after *L. monocytogenes* infection.

In conclusion, the results from this study indicate that intragastric infection of pregnant (10-14 days of gestation) C57BL/6 mice with *L. monocytogenes* causes increased relative expression of IL-17a and IL-22 in placental tissues. However, neither bacterial burden nor inflammation in fetal tissues are dependent on IL-17a, or IL-22 in this experimental system.

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Figure 1

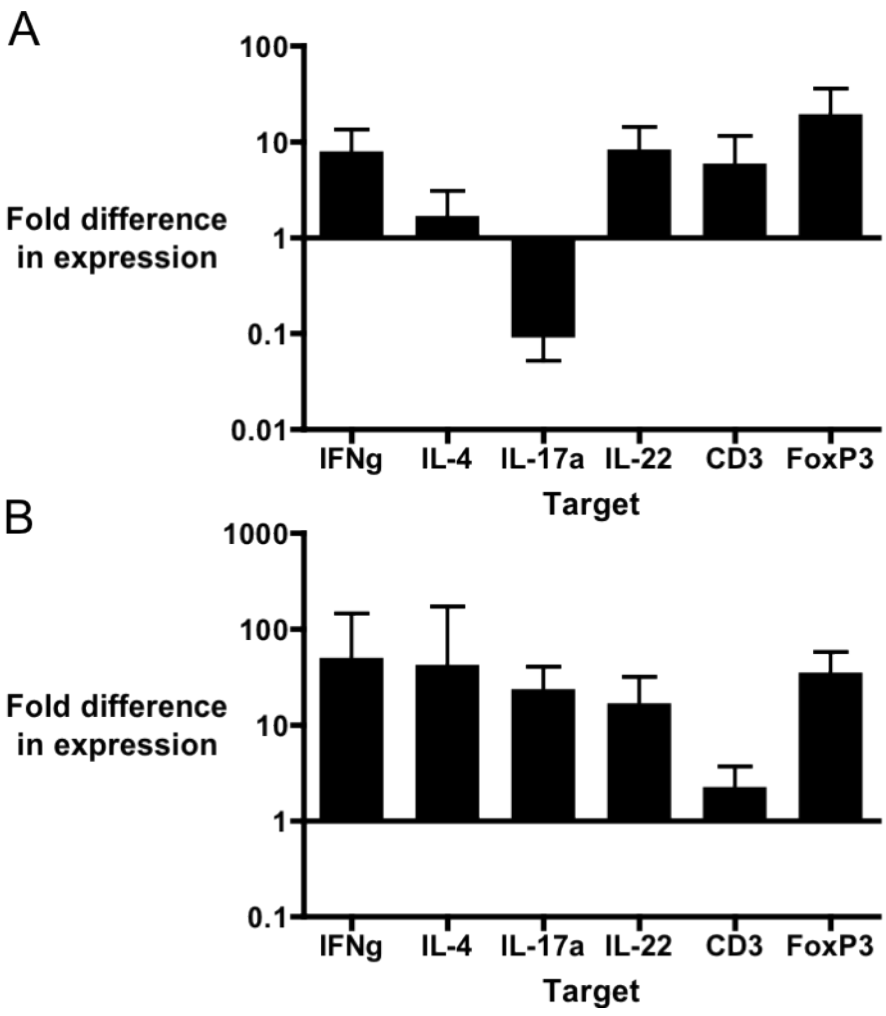
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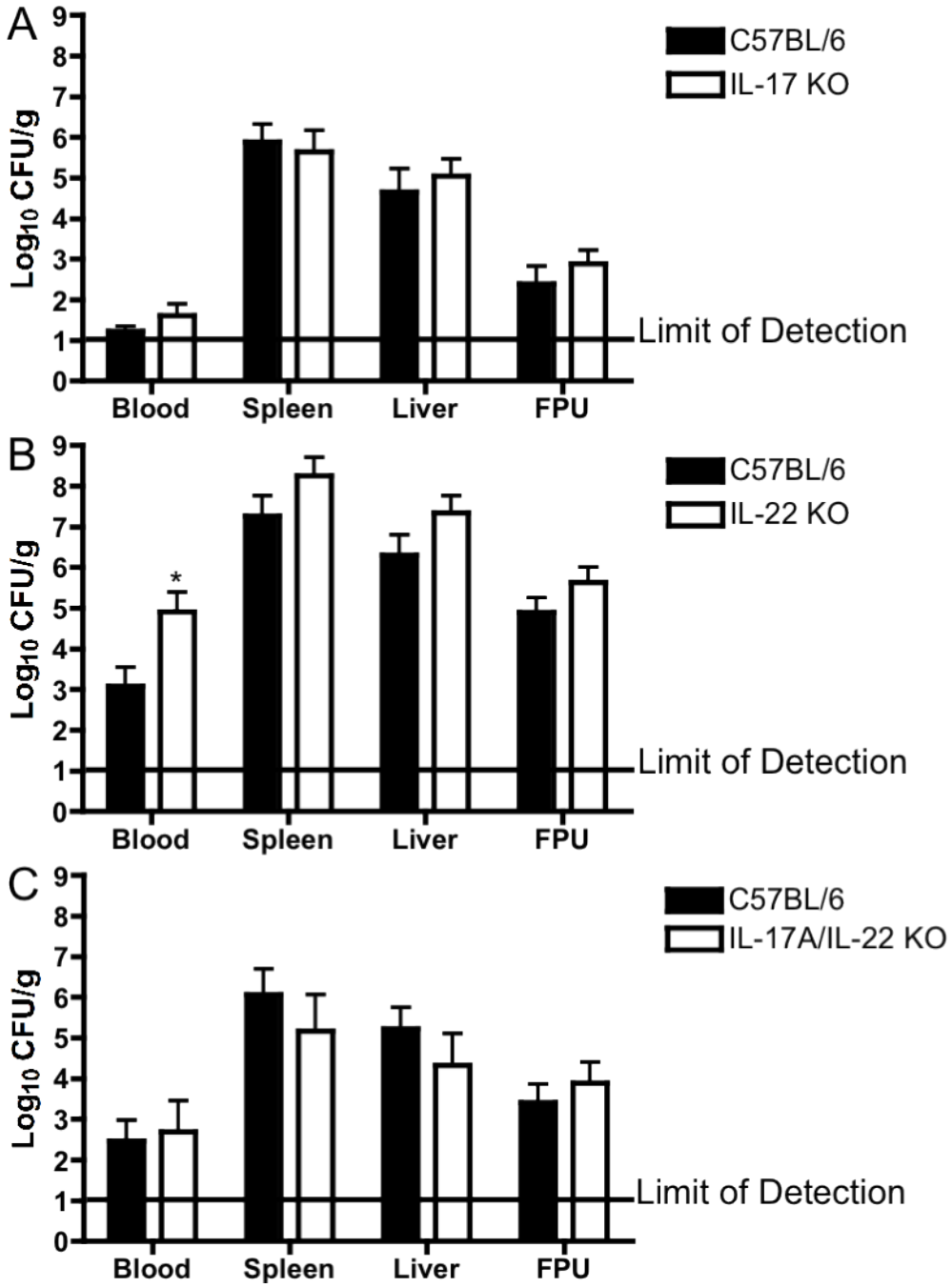
Figure 1. Changes in expression of T cell associated mRNAs in maternal liver and placental tissue following oral infection with *L. monocytogenes*. Pregnant C57BL/6 mice (10-14 days gestation) were infected by intragastric lavage with 10^5 CFU LM2203 in 100μL of 1% saline. Tissues were harvested 72 hours post infection for RNA isolation and qPCR analysis. The ΔΔCt method was used to calculate relative expression, using 3 reference genes (ActB, GAPDH, and RPL8) between *L. monocytogenes* infected and uninfected mice. (A) mRNA expression in liver tissue from *L. monocytogenes* infected pregnant mice (n=8 from 3 separate experiments) is compared to uninfected control pregnant mice (n=10 from 3 separate experiments.) (B) mRNA expression in placental tissue from *L. monocytogenes* infected pregnant mice (n=15 from 3 separate experiments) is compared to uninfected pregnant control mice (n=9 from 3 separate experiments.) Data are expressed as the mean ± SD.

Figure 2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B
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Log10 CFU/g
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IL-22 KO
IL-17A/IL-22 KO
Limit of Detection
Blood
Spleen
Liver
FPU
*

Figure 2. Bacterial burden in maternal and fetal tissues is not dependent on IL-17a, or IL-22. Mice were infected with *L. monocytogenes* as described in the Materials and Methods. Mice were euthanized 72 hours later and blood, spleen, liver and fetoplacental units (FPUs) were removed (2-3 per mouse) and processed for bacterial culture. Data are presented as the mean ± SEM $\log_{10}$ CFU/g of tissue or $\log_{10}$CFU/ml of blood. (A) Data from 3 separate experiments are combined for a total of 9 pregnant C57BL/6 (25 FPU) and 15 pregnant IL-17a$^{(-/-)}$ (33 FPU) mice. (B) Data from 2 separate experiments are combined for a total of 12 pregnant C57BL/6 (23 FPU) and 9 pregnant IL-22$^{(-/-)}$ (23 FPU) mice. (C) Data from 2 separate experiments are combined for a total of 10 pregnant C57BL/6 (22 FPU) and 9 pregnant IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ (19 FPU) mice. The asterisk indicates $p<0.05$ compared to wild type mice.

Figure 3

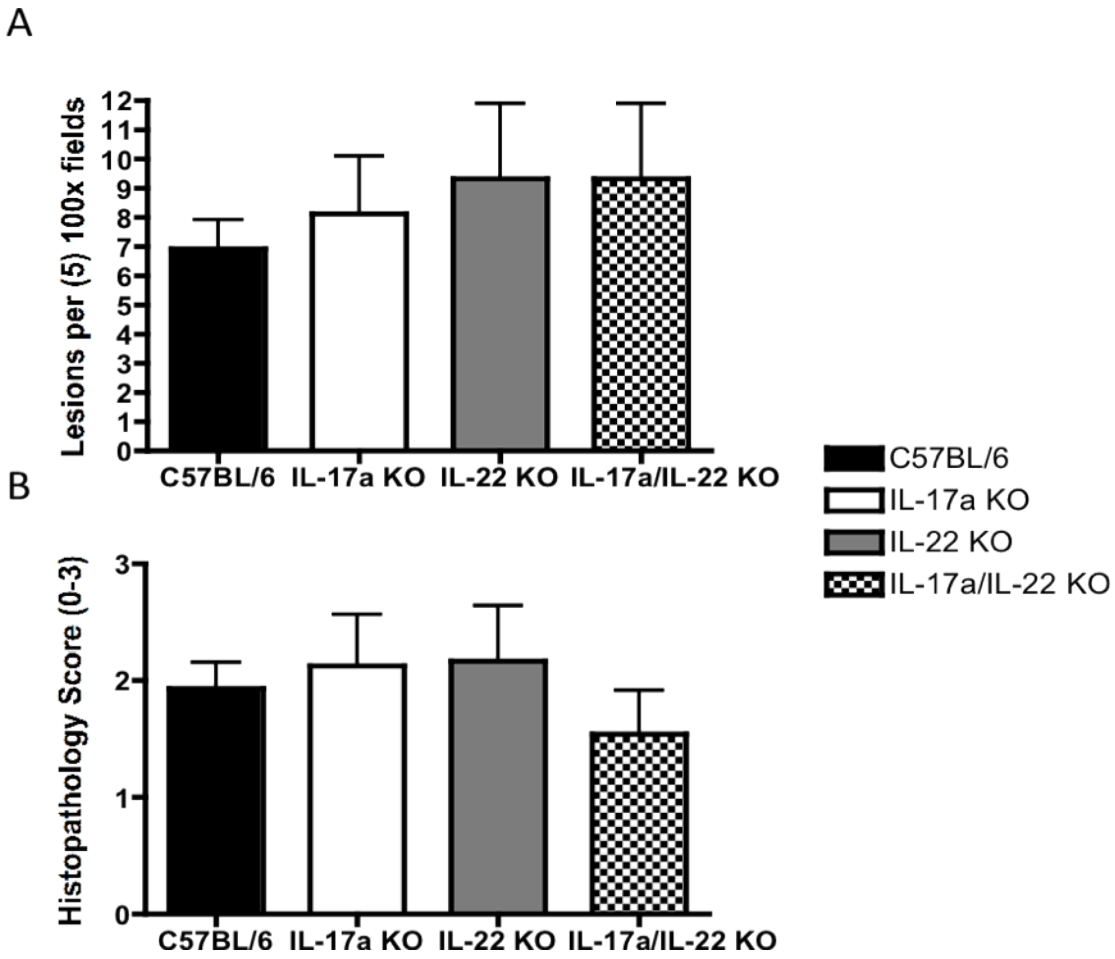
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Figure 3. Severity of inflammation in maternal liver and spleen is not dependent on IL-17a or IL-22 during *L. monocytogenes* infection. Mice were infected as described in the Materials and Methods. Portions of spleen and liver tissues were fixed in 10% buffered formalin and prepared for histopathological analysis. Liver and spleen sections were sectioned, stained and scored as described in the Materials and Methods. (A) Inflammatory lesions per five 100x fields of maternal liver. Tissues were from the same mice illustrated in Fig. 2. We observed no significant difference between *L. monocytogenes* infected pregnant C57BL/6 mice (n=24) and pregnant IL-17a$^{(-/-)}$ (n=8), IL-22$^{(-/-)}$ (n=6), and IL-17a$^{(-/-)}$/IL-22$^{(-/-)}$ (n=12) mice. (B) Spleen tissue from the same mice were scored for severity of inflammation (0-3) as described in the Methods. We observed no significant difference in splenic inflammation between control C57BL/6 wild type mice and IL-17a or IL-22 gene knockout mice. The asterisk indicates $p<0.05$.

Table 1

Primer specificity	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product Size
IL-17a WT	ACTCTTCATCCACCTCACACGA	GCCATGATATAGACGTTGTGGC	1300 bp
IL-17a$^{(-/-)}$	ACTCTTCATCCACCTCACACGA	CAGCATCAGAGACTAGAAGGGA	500 bp
IL-22 WT	CAGCTGGCGGCCAAAGTCCC	GATACAGGTGCAGCTAAGCGAG	196 bp
IL-22$^{(-/-)}$	CTCAGACCTCTACAGACAATCATC	GATACAGGTGCAGCTAAGCGAG	374 bp

Table 1 Primers used to genotype IL-17a and IL-22 null mice.

Table 2

Primer specificity	Forward primer (5'→3')	Reverse primer (5'→3')	cDNA amplicon size	Concentration used (Liver/Placenta uM)
Actβ*	TGTGATGGTGGGAATGGGTCAGAA	TGTGGTGCCAGATCTTCTCCATGT	140 bp	200/400
GAPDH*	CTTTGTCAAGCTCATTTCCTGG	TCTTGCTCAGTGTCCTTGC	133 bp	400/400
RPL8*	CTACGTGCTGTGGACTTCG	CGGCCAGGGTCATGAATG	77 bp	600/200
IFNγ	GGCTGTTACTGCCACGGCACA	CACCATCCTTTTGCCAGTTCCTCCA	130 bp	600/600
IL-4	CGAATGTACCAGGAGCCATATC	TCTCTGTGGTGTTCTTCGTTG	149 bp	600/600
IL-17a	TCCAGAATGTGAAGGTCAACC	TATCAGGGTCTTCATTGCGG	129 bp	600/600
IL-22	AGCTTGAGGTGTCCAACTTC	GGTAGCACTCATCCTTAGCACTG	150 bp	600/600
CD3	TGGAGCAAGAATAGGAAGGC	CATAGTCTGGGTTGGAACAG	115 bp	600/600
FoxP3	AAGTACCACAATATATGCGACCC	TCTGAAGTAGGCGAACATGC	132 bp	600/400

*Denotes reference gene

Table 2: Primers Used for real time PCR.

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Chapter Five

Listeria monocytogenes infection of human choriocarcinoma JEG-3 cells reduces migration and invasion

Abstract

Listeria monocytogenes is a facultative intracellular bacterium associated with foodborne disease outbreaks. Pregnant women, and more specifically their fetuses, are at particular risk. The mechanisms by which *L. monocytogenes* infection disrupt homeostasis in the placenta are incompletely understood. JEG-3 cells are a human choriocarcinoma cell line that is used as a model for extravillous trophoblast cells. The latter invade maternal decidua to attach the fetal placenta and direct maternal blood flow to the fetus. In this study, we tested the ability of *L. monocytogenes* to invade, multiply within, and alter the function of JEG-3 cell monolayers. *L. monocytogenes* readily infected JEG-3 cells and multiplied to a peak number of intracellular organisms at 10 hours post-infection. *L. monocytogenes* infection decreased transepithelial electrical resistance (TEER) of the JEG-3 monolayer as compared to uninfected JEG-3 cell monolayers. *L. monocytogenes* infected JEG-3 cells also displayed decreased migration and invasion through a fibronectin layer. Finally, *L. monocytogenes* infection decreased JEG-3 cell metabolic activity, as measured by Alamar Blue reduction, and caused cell death as measured by lactate dehydrogenase (LDH) release. These findings suggest that *L. monocytogenes* infection of extravillous trophoblast cells may compromise functions required by the fetoplacental unit for maternal vascular remodeling and pregnancy success.

Introduction

Listeria monocytogenes is a significant foodborne pathogen that has up to a 30% mortality rate [1]. Listeriosis can present with a variety of clinical syndromes including gastroenteritis, meningioencephalitis, and maternal-fetal infections [2-4]. Pregnant women have a 20 fold greater risk than the average adult to acquire invasive listeriosis, and represent 16% of reported listeriosis cases in the USA [5, 6]. This increased risk is hypothesized to be caused, in part, by alterations in the immune system to facilitate immune tolerance and prevent rejection of the fetal allograft. However, other mechanisms may be important [7-10].

L. monocytogenes infection during pregnancy targets the maternal-fetal interface, particularly placental tissues. Despite the extensive body of knowledge surrounding *L. monocytogenes* and its pathogenesis, little is known about the underlying mechanisms of fetal and placental infection. This is true in both humans and ruminant species, which are also susceptible to fetal infection and abortion. The most common routes of infection for the fetus and neonate of all species are thought to be: 1.) hematogenous, i.e., if the mother experiences a bacteremia; or 2.) ascending infection from the birth canal to the amniotic fluid and placenta [1, 11]. Late term infections tend to result in stillbirth or neonatal listeriosis. In general, bacterial infection early in gestation is thought to result in spontaneous abortion [12]. Although early aborted tissues are rarely submitted for bacterial isolation *L. monocytogenes* is implicated in approximately 3% of spontaneous abortions in both humans and cattle [13-15].

There are several possible explanations for how *L. monocytogenes* crosses the maternal-fetal barrier. Specific cell types that have been shown to be targets for listerial invasion into the fetal placenta include syncytiotrophoblast and extravillous trophoblast cells [16-18]. Studies suggest the mechanism for listerial cell entry into these host cells is mediated by interactions between the bacterial cell wall proteins internalin A and internalin B, and E-cadherin and c-Met-tyrosine kinase, respectively, on the mammalian cells [19, 20].

Listerial invasion of syncytiotrophoblast and extravillous trophoblast cells could have serious consequences for fetal health and pregnancy success (Figure 1). Humans have a hemonochorial villous placenta, which presents a minimal barrier between maternal and fetal components [21]. Syncytiotrophoblasts line the villi, which are bathed in maternal blood. Extravillous trophoblast cells anchor the fetal placenta to the decidual tissue, and are essential in remodeling of the uterine vasculature to increase blood flow to the placental surface (reviewed in [22] and [23]). It has been shown by others that *L. monocytogenes* can infect primary cultures of extravillous trophoblast cells [17, 18]. Although these previous studies identified trophoblast cells as potential sites of bacterial entry to the fetus during *L. monocytogenes* infection, they did not examine how *L. monocytogenes* infection alters the function of these cells, and how this may relate to fetal health.

In this study, we use JEG-3 cells as a model for human extravillous trophoblast cells. Other investigators have shown that although derived from a choriocarcinoma,

JEG-3 cells have similar gene expression and present many of the same biological and biochemical characteristics as normal extravillous trophoblast cells [24, 25]. In this study we demonstrate that JEG-3 cells are readily infected with *L. monocytogenes* in vitro. *L. monocytogenes*-infected monolayers exhibit decreased monolayer integrity, as measured by transepithelial electrical resistance (TEER). *L. monocytogenes* infection also decreased cell migration and invasion through fibronectin, and ultimately impaired JEG-3 cell metabolic activity, leading to cell death. Overall these actions would be deleterious to fetal development.

Materials and Methods

Preparation of L. monocytogenes

L. monocytogenes strain 2203 (serotype 4b) was generously donated by Dr. Sophia Kathariou (Raleigh, NC). This is a clinical isolate from a food-borne disease outbreak that caused disease in 13 people, 11 of whom were pregnant, and all experienced adverse pregnancy outcomes [26].

L. monocytogenes cells were stored at -20°C on Cryobank™ Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of brain heart infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500 X g for 5 minutes), washed three times in phosphate buffered saline and kept on ice prior to inoculating mice. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial dilutions onto tryptic soy agar with 5% sheep blood (BD® Biosciences).

Chemicals and media

Dulbecco's Modified Eagle Medium (DMEM), Hank's balanced salt solution (HBSS), and trypsin were purchased from Cellgro (Kansas City, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lauranceville, GA). NP-40 detergent was purchased from USB Corporation (Cleveland, OH). Brain heart infusion broth was purchased from Difco (BD Biosciences, Franklin Lakes, NJ). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

JEG-3 extravillous trophoblast cells

The human choriocarcinoma-derived JEG-3 cells were used as a model of human extravillous trophoblast cells and were obtained from the American Type Culture Collection (ATCC) (Rockville, MO) [27, 28]. The cells were cultured in DMEM supplemented with 10% FBS and were passaged by brief enzymatic digestion using 0.1% trypsin-EDTA.

Invasion and growth of L. monocytogenes in JEG-3 cells

For invasion and growth experiments, JEG-3 cells were cultured in 24-well plates for 48 hours. The monolayers were incubated with *L. monocytogenes* strain 2203 at a multiplicity of infection (MOI) of 5 for 60 minutes. The 24-well plates were then washed extensively with warm HBSS. Some wells were then lysed with TritonX-100 for 5 minutes and the lysates were diluted in phosphate buffered saline and plated on tryptic soy agar supplemented with sheep blood to determine the initial invasion by *L. monocytogenes*. The remaining wells received DMEM media supplemented with 10% FBS and 200μg/ml gentamicin. At selected time points, medium, was removed from wells and the monolayers lysed to estimate numbers of intracellular *L. monocytogenes* as indicated above. The medium was changed daily throughout the experiment.

TEER measurements

JEG-3 cells were seeded onto translucent polyethylene terephthalate (PET) cell culture inserts (3.0μm average pore size; BD Falcon, San Jose, CA) and incubated at 37°C with daily medium changes (1 ml of medium on the bottom side of the insert and

700µl on the top side of the insert) for 3 days in DMEM with 10% FBS. TEER was measured using a voltmeter and End-ohm six-chamber cup electrodes (World Precision Instruments, Sarasota, FL). Initial TEER values for each monolayer were determined prior to the beginning of each experiment. Subsequent recordings obtained during the course of the experiment were normalized to this initial reading. Only monolayers with initial readings ≥80 Ω were used in experiments. JEG-3 cells were treated with *L. monocytogenes* (MOI of 5) for 1 hour, washed extensively with HBSS, and then incubated with DMEM supplemented with 10% FBS and 200µm/ml gentamicin. Resistance measurements were taken at 4, 8, 12, 24, 48, and 72 hours post infection and recorded manually.

Extravillous trophoblast invasion and migration assays

Cell migration and invasion was performed using translucent PET cell culture inserts (8.0µm average pore size; BD Falcon, San Jose, CA) (Figure 4). For the invasion assay, the filters were pre-coated with fibronectin (BD Biosciences). This was done by adding 200 µl of 20 µg/ml fibronectin solution to the top of the filter, followed by incubation for 1 hour at 37°C. After incubation, all excess liquid was removed. For both invasion and migration assays, 400 µl of cell suspension (1 x 10^5 cells/ml) was placed on top of the filters and the later placed into a 24 well plate, whose wells contained 600 µl of medium. Cells were incubated for 4 hours at 37°C and then incubated for 1 hour with *L. monocytogenes* (MOI of 5) added to the top of the filter cup. Monolayers were washed extensively with HBSS, and fresh DMEM supplemented with 10% FBS and 200µm/ml gentamicin was added. Control cell cultures were not exposed to *L.*

monocytogenes. Cell cultures were incubated at 37°C for 24 hours to allow migration though the filter pores and invasion through the fibronectin layer and to the bottom side of the filter. After the 24 hour period, remaining cells on the top of the filter were removed with a sterile cotton swab. Alamar Blue dye was added to the medium (1:10) and the plate was incubated at 37°C for 24 hours [29]. Alamar Blue is a water-soluble dye, which is non-toxic to cells and is used to monitor cell growth over time [30, 31]. Alamar Blue is added to the media in the oxidized state, which has peak absorbance at 600 nm. Normal cellular metabolism reduces the dye, which has a peak absorbance of 570 nm. Viable cells in experimental and control cultures are then measured by percentage of Alamar Blue reduction. After the 24 hour incubation (48 hours post infection), 200 µl of conditioned culture media was transferred to a new 96 well plate and absorbance was read at 570 and 600 nm using a plate reader (Synergy HT, Biotek, Winooski, VT). The number of cells that migrate or invade across the filter are expressed as a percentage of Alamar Blue reduction, and compared to a standard curve of cells grown in a 24 well plate alone to extrapolate percent migration and invasion [29, 31].

Cell metabolic activity and viability

Cell metabolic activity was measured with Alamar Blue dye as previously described [29]. Briefly, JEG-3 cells (50,000) were seeded into 24 well plates. After incubation for 4 hours at 37°C cells were incubated with *L. monocytogenes* (MOI of 5) for one hour, washed extensively with HBSS, and medium was replaced with DMEM supplemented with 10% FBS and 200µm/ml gentamicin. After a 24 hour incubation,

Alamar Blue dye was added (1:10) to the cell culture medium [29]. After a 24 hr incubation at 37°C to allow viable cells to reduce the Alamar Blue dye, 200 µl of conditioned culture media was transferred to a new 96 well plate. The absorbance of the control and experimental wells was read at 570 and 600 nm using a plate reader.

Cell death was assessed by measuring LDH release. JEG-3 cells were seeded onto translucent (PET) cell culture inserts (3.0µm average pore size) and cultured with daily media changes (1 ml of media on the bottom side of the insert and 700µl on the top side of the insert) for 3 days in phenol red-free DMEM with 10% FBS. JEG-3 cells were treated with *L. monocytogenes* (MOI of 5) for 1 hour, washed extensively with HBSS, and fresh phenol red-free DMEM supplmented with 10% FBS and 200µm/ml gentamicin was added. Media was removed at selected time points to measure LDH release using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI) according to manufacturer's protocol. Maximum cell death was achieved by adding 1% NP-40 detergent to appropriate wells for 45 minutes prior to harvest of the media.

Statistical analysis

For invasion and growth experiments, data from at least three experiments were analyzed by ANOVA followed by the Tukey post test for all groups. The Mann-Whitney test was used for TEER, migration and invasion, cell metabolism, and cell viability experiments (Instat, GraphPad). Statistical significance for all comparisons was set at $P < 0.05$.

Results

Listeria monocytogenes invades and grows within JEG-3 extravillous trophoblast cells

As illustrated in Figure 1, the placental maternal-fetal interface consists of an invagination of fetal extravillous trophoblasts and syncytiotrophoblasts, that are in direct contact with maternal tissue and blood, respectively [32]. Previous studies suggested that both types of trophoblast cells are targets for *L. monocytogenes* infection [16-18].

Figure 2 demonstrates that JEG-3 cells, which model extravillous trophoblast cells, can be infected with *L. monocytogenes* and support intracellular listerial multiplication in vitro. *Listeria monocytogenes* was able to establish infection after only a one hour incubation with JEG-3 cells. The bacterial burden reached a peak at 10 hours of infection and then gradually declined at 24 hours post infection. Visual microscopic inspection of the monolayers revealed cytopathic changes such as cellular debris and cells lifting off the monolayer in *L. monocytogenes*-infected monolayers 24 hours after inoculation (data not shown).

Listeria monocytogenes infection of extravillous trophoblast cells decreases monolayer integrity.

The visible cytopathic changes in infected monolayers led us to determine if functional alterations in the monolayers also occurred during infection. We assessed the effects of *L. monocytogenes* infection on JEG-3 cell monolayers by measuring TEER across the surface area of the cell monolayer. Total resistance is made up of both transcellular and paracellular electrical resistance. As the integrity of the monolayer diminishes, electrical conductivity across it increases and TEER declines. *L.*

monocytogenes infection of JEG-3 monolayers decreased electrical resistance (TEER) over a 72 hour time period. As shown in Figure 3, TEER values for *L. monocytogenes* infected cells began to decline between 12 and 24 hours of infection. These findings contrast with uninfected monolayers, which exhibited steadily increasing TEER. This response reflects the continued proliferation of uninfected JEG-3 cells.

Listeria monocytogenes infection of extravillous trophoblast cells diminishes cell motility.

JEG-3 cells are used as a model for extravillous trophoblast cells, which attach fetal placental tissue to maternal decidual tissue, and migrate and invade into decidual tissue to remodel the maternal spiral arteries [32, 33]. We hypothesized that *L. monocytogenes* infection of JEG-3 cells impairs their ability to migrate and invade. We tested migration of JEG-3 cells through 8μm pore PET cell culture inserts, and through the same cell culture inserts that were coated with fibronectin. Alamar Blue reduction was used to quantify the cells that had migrated or invaded across the cell culture insert (Figure 4). Viable cells reduce Alamar Blue, which can be measured with increased absorption (570nm and 600nm), as part of their normal metabolic activity. As illustrated in Figure 5, JEG-3 cells incubated with *L. monocytogenes* displayed decreased migration across the cell culture insert compared to uninfected JEG-3 cells. We also observed decreased invasion of *L. monocytogenes* infected JEG-3 cells through a fibronectin layer compared to control (Figure 5).

Listeria monocytogenes infection of extravillous trophoblast cells decreases cell metabolism and increases cell death.

The above experiments showed reduced function of JEG-3 cells (decreased TEER, migration, and invasion). These results suggested that infected JEG-3 cells were not proliferating and experienced cell damage following *L. monocytogenes* infection. We also determined if *L. monocytogenes* infection causes diminished JEG-3 cell metabolic activity or cell death. Using Alamar Blue reduction to quantify JEG-3 cell metabolic activity we show that *L. monocytogenes* infected JEG-3 cells exhibit decreased cell activity within 48 hours of infection, compared to uninfected control cell cultures (Figure 4b (illustration of color change) and 6a). Furthermore, *L. monocytogenes* infection caused significant cell death (as measured by LDH release) compared to uninfected control in JEG-3 cells (Figure 6b).

Discussion

This study demonstrates that *L. monocytogenes* infection of the human JEG-3 choriocarcinoma cell line reduces monolayer TEER, decreases migration and invasion through a filter or fibronectin, decreases cell metabolic activity, and causes cell death. Our finding that *L. monocytogenes* readily infects JEG-3 cells, with peak numbers of intracellular bacteria at 10 hours after infection, is similar to a previous report that used BeWo cells (human choriocarcinoma derived cell line) and primary human extravillous trophoblast cells [17]. In that study primary human extravillous trophoblast cell culture restricted intracellular *L. monocytogenes* growth. Zelovich et al. suggested that extravillous trophoblast cells inhibit the growth of *L. monocytogenes* better than choriocarcinoma-derived cell lines because the former produces high levels of progesterone [17]. We do not believe this explains the intracellular *L. monocytogenes* growth in JEG-3 or BeWo cells because both are robust producers of progesterone [25, 34].

Listeria monocytogenes is not the only intracellular pathogen that invades fetal trophoblast cells. Human cytomegalovirus is the most common viral pathogen in the pregnant uterus and readily invades all types of trophoblast cells [35, 36]. *Chlamydia* sp., (e.g. *C. abortus*) are Gram-negative bacteria that are endemic in ruminant and rodent populations. *C. abortus* spreads through oral and sexual contact, and causes abortion in women exposed to infected ruminant and rodent fecal material and abortive tissues [37]. It was reported that intracellular growth of *C. abortus* in extravillous trophoblast cells is related to expression of an innate immunity molecule, SLPI, which is

thought to control tissue inflammation and pathology [38]. *Campylobacter rectus* has also been shown to invade extravillous trophoblast cells [39]. *C. rectus* is an oral Gram-negative, anaerobic, motile bacterium that can disseminate to the fetus and cause abortion or pre-term delivery [40]. The pathophysiology of *C. rectus*'s ability to cause abortion has been suggested to include up-regulation of IL-6 and TNF in extravillous trophoblast cell layers [39].

There are several possible explanations for why JEG-3 cells do not sustain intracellular growth of *L. monocytogenes.* Disruption of the monolayer, as demonstrated in our TEER experiments, would diminish direct contact among JEG-3 cells and thus reduce the opportunities for listerial cells to move from one JEG-3 cell to the next. We also cannot exclude the possibility that the diminished metabolic activity of infected JEG-3 cells creates a cytoplasmic environment that is not suitable for sustained proliferation of *L. monocytogenes*. JEG-3 cells are known to produce multiple immunomodulating molecules including, but not limited to, B7 proteins, maltooligosaccharides, and other unknown factors [41-43]. However, no specific antimicrobial properties or compounds have been reported to be associated with, or produced by, JEG-3 cells.

The decreased TEER of *L. monocytogenes* infected JEG-3 cell monolayers could reflect extracellular (e.g. damage to tight and gap junctions), or transcellular events (damaged or dead cells). These changes would have negative consequences for the fetus, because trophoblast cells are an important component of the innate immune

barrier during pregnancy [44]. To the author's knowledge, the effects of bacterial infection of trophoblast cells on TEER have not been previously reported. However, TEER has been used to show that different types of growth medium change the electrical resistance across a JEG-3 monolayer [45]. Ikeda et al. also showed that different growth media change the expression of gap junctional and adherence proteins, including E cadherins on the surface of JEG-3 cells. InlA expressed by *L. monocytogenes* binds to E cadherin on host cells [20]. Ikeda et al. concluded that altering the JEG-3 cell environment caused change to the integrity of the JEG-3 monolayer, facilitating drug delivery across the monolayer to the basement membrane. In our study, paracellular damage from *L. monocytogenes* infection may be due to alterations in gap junctional and adhesion proteins, which are present in human trophoblasts [46, 47]. Most likely the damage is both paracellular and transcellular, with the end result diminishing the ability of fetal trophoblast cells to serve as a barrier to bacterial cells. As a result, immune cells in the maternal bloodstream could enter the fetal placental villi and the fetal blood circulation to infect and initiate inflammation in fetal parenchymal tissues.

The results of the migration and invasion experiments, together with the cell metabolic activity and cell death experiments, show that *L. monocytogenes* infection diminishes the functional capacity of JEG-3 cells. We measured JEG-3 migration and invasion 24 hours after infection (Figure 5). Reduction of cell migration and invasion in our experiments are not a result of cell death because we did not observe significant cell death in infected cells compared to uninfected control until 48 to 72 hours after *L.*

monocytogenes infection (Figure 6B). Similar results have been shown previously with human cytomegalovirus infection. Tao et al. showed that primary human extravillous trophoblast cells, when infected with human cytomegalovirus, displayed diminished invasion function [48]. If we extrapolate our results to an *in vivo* scenario, they suggest that *L. monocytogenes* infection decreases the ability of extravillous trophoblast cells to attach to and invade the maternal decidual tissue. This would have implications for the integrity of the implantation site and ability of the fetus to orchestrate spiral artery formation to increase the oxygen and nutrient availability from the mother that are needed to maintain normal pregnancy [23, 46].

It has been reported that extravillous trophoblast cells have bacteriocidal capacity against *L. monocytogenes*, that may prevent the pathogen from crossing the basement membrane in the fetal placental villi and infecting the fetus [17, 18]. The results of the present study suggest that attention should also be paid to the functionality of these cells following *L. monocytogenes* infection, rather than simply focusing on the trophoblast cells as a point of entry to infect the fetus. Further work is needed to define how *L. monocytogenes* infection affects attachment and invasion of fetal extravillous trophoblasts to the decidual tissue. Furthermore extravillous trophoblast cells have roles in altering the maternal adaptive immune system to maintain pregnancy, specifically by down regulating production of CD8+ cytotoxic T cells, which may also be relevant for the issue of pregnancy loss (reviewed in [49] and [50]). Future studies are warranted to examine specific cell functions of extravillous trophoblast in terms of adhesion, invasion,

and modulation of innate and adaptive maternal immunity in response to infection with *L. monocytogenes*.

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Figure 1

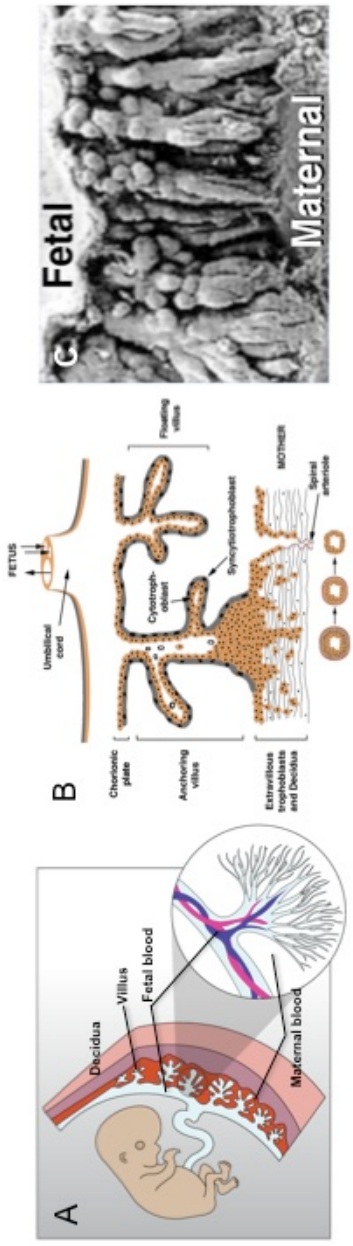
C
Fetal
Maternal
B
FETUS
Umbilical cord
Chorionic plate
Cytotroph-oblast
Anchoring villus
Floating villus
Syncytiotrophoblast
MOTHER
Spiral arteriole
Extravillous trophoblasts and Decidua
A
Decidua
Villus
Fetal blood
Maternal blood

Figure 1. Human placenta structure. (A) Developing fetus with placental villi bathed in maternal blood in the intervillous space. These tissues are attached to the decidual tissue in the uterus. The inset illustrates an enlargement of a fetal villus, which facilitates oxygen exchange and nutrient transport to the fetal circulation via the umbilical arteries (blue) and vein (red). (B) Enlargement of panel A shows different trophoblast cell types. Extravillous trophoblast cells anchor the fetal placenta to the decidual tissue, influence the maternal immune system to allow fetal tolerance, and alter maternal blood flow in the spiral arteries to increase nutrient availability to the fetus. Extravillous trophoblasts arise from the trophoblast cell columns, while villous cytotrophoblast cells differentiate from syncytiotrophoblast layer overlying the villus. The syncytiotrophoblast cell layer is responsible for gas and nutrient exchange from the maternal blood in the intervillous space to the fetal blood in capillaries located in the villous stroma. (C) Scanning electron micrograph of the maternal-fetal interface of a rhesus monkey showing anchoring villi of fetal placenta attached to maternal decidual tissue (reproduced from [51]).

Figure 2

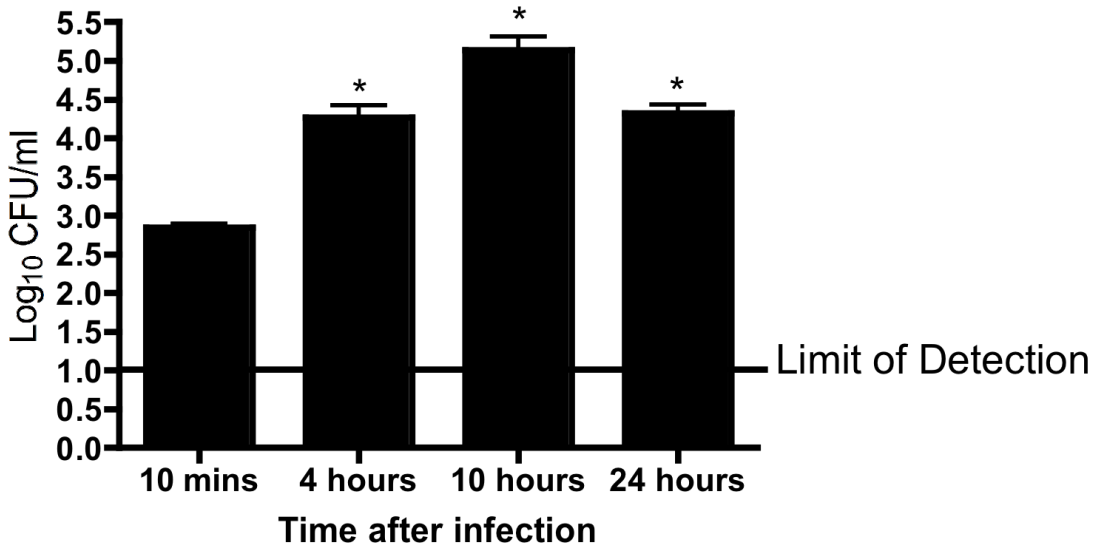
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Log10 CFU/ml
*
*
*
Limit of Detection
10 mins
4 hours
10 hours
24 hours
Time after infection

Figure 2. *Listeria monocytogenes* invades JEG-3 cells. JEG-3 cells were incubated with 10^5 CFU of LM2203 for 1 hour, then extensively washed with HBSS. Gentamicin (200μg/ml) was then added to fresh medium to prevent extracellular bacterial growth. At selected time points, cells were lysed with Triton-X detergent and intracellular microbial load quantified by diluting and plating lysates on blood agar. Data are expressed as mean ± SEM of 6 replicates at each time point from one representative experiment of 3 separate experiments that were performed. Asterisks indicate $p<0.01$ for intracellular growth time points compared to the initial 10-minute invasion time point.

Figure 3

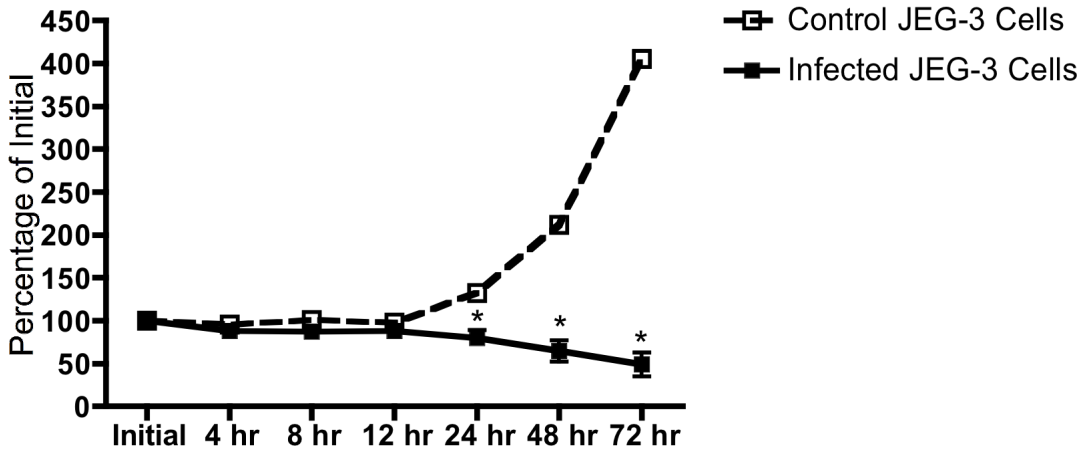
Control JEG-3 Cells
Infected JEG-3 Cells
Percentage of Initial
450
400
350
300
250
200
150
100
50
0
Initial
4 hr
8 hr
12 hr
24 hr
48 hr
72 hr
*
*
*

Figure 3. *Listeria monocytogenes* infection decreases JEG-3 cell monolayer TEER. JEG-3 cells were cultured in a trans-well system on top of a 3μm filter. The JEG-3 monolayers were infected with 10^5 CFU of LM2203 for 1 hour, then extensively washed with HBSS. Gentamicin (200μg/ml) was then added to fresh medium to prevent extracellular bacterial growth. At selected time points, TEER was measured and calculated as the percentage change from the initial measurement. Data are expressed as mean ± SEM of 3 replicates for control and 9 replicates for infected wells at each time point and are from one representative experiment of 3 separate experiments that were performed. Asterisks indicate $p<0.01$ for infected JEG-3 cells.

Figure 4

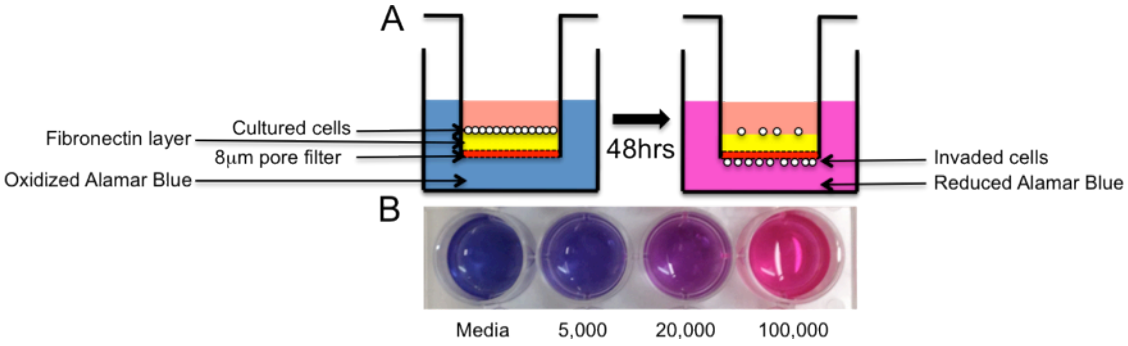
A
Fibronectin layer
Cultured cells
8μm pore filter
Oxidized Alamar Blue
48hrs
Invaded cells
Reduced Alamar Blue
B
Media
5,000
20,000
100,000

Figure 4. Illustration of the cell migration and invasion assay. (A) JEG-3 cells were added to the top of 8 μm PET cell culture inserts (migration), or inserts with a fibronectin layer (invasion), and placed into wells of a 24 well plate containing cell culture media with 10% FBS. After 24 hr incubation at 37°C, cells on the top of the filter were removed with a sterile swab, Alamar Blue was added to the medium in the bottom well, and wells were incubated for a further 24 hrs. (B) Examples of color change after 24 hr incubation with Alamar blue in wells with increasing numbers of JEG-3 cells.

Figure 5

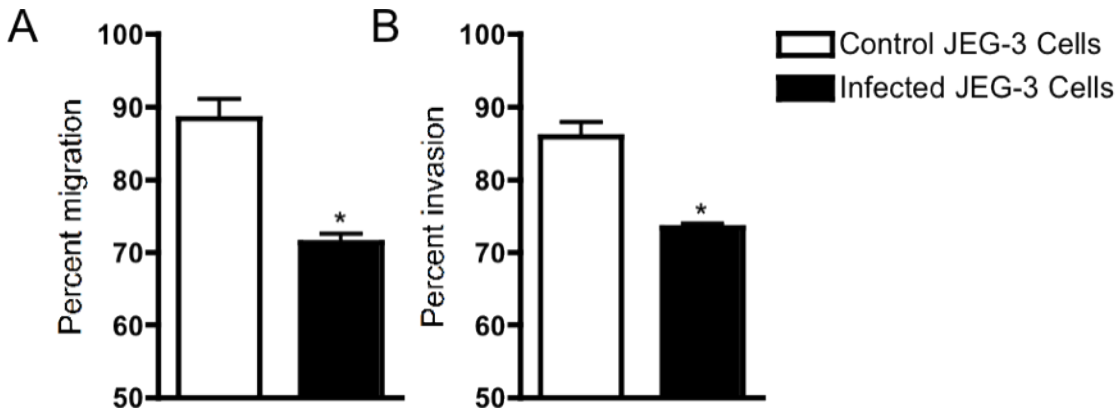
A
B
100
90
80
70
60
50
Percent migration
Percent invasion
*
*
Control JEG-3 Cells
Infected JEG-3 Cells

Figure 5. *Listeria monocytogenes* infection diminishes JEG-3 cell migration and invasion. JEG-3 cells were cultured in a trans-well system (50,000 cells). After 4 hours incubation, cells were infected with 10^5 CFU LM2203 for 1 hour, then extensively washed with HBSS. Fresh medium with gentamicin (200μg/ml) was then added to prevent extracellular bacterial growth and the culture was incubated for 24 hours. The tops of the filters in the transwell system were wiped with a sterile cotton swab to remove cells that had not migrated through the 8μm pore filter. Percent Alamar Blue reduction was compared between cells in the transwell (Figure 4a) and cells grown in a well without transwell system (Figure 4b) to establish percent migration and percent invasion. Percent of migration (A) and invasion (B) is shown in control, non-infected transwells and experimental, *L. monocytogenes* infected (48 hours duration of infection) transwells. Data are expressed as mean ± SEM of 4 replicates at each time point from 3 separate experiments. Asterisk indicates $p<0.05$ compared to uninfected JEG-3 cell migration (A) and invasion (B).

Figure 6

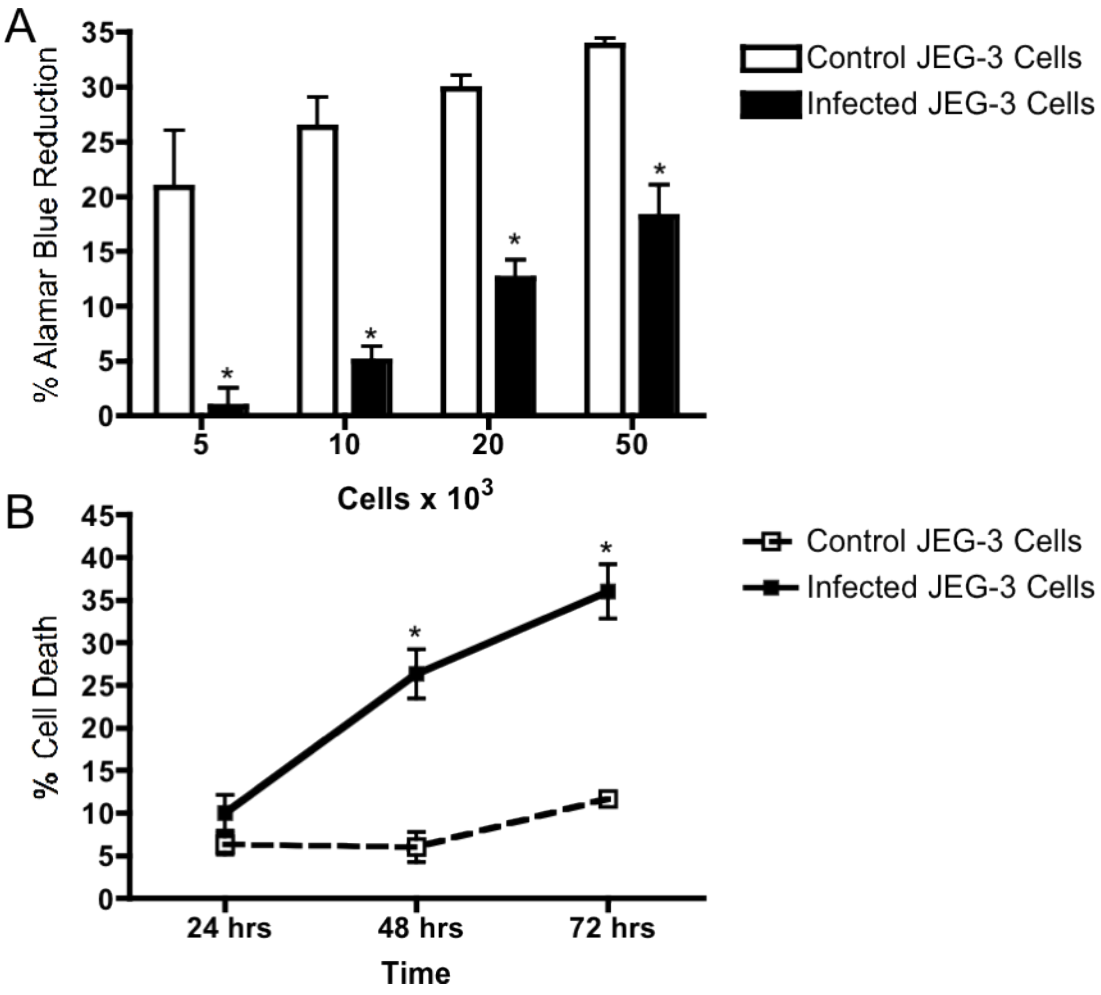
A
% Alamar Blue Reduction
35
30
25
20
15
10
5
0
5
10
20
50
Cells x 10^3
Control JEG-3 Cells
Infected JEG-3 Cells
*
B
% Cell Death
45
40
35
30
25
20
15
10
5
0
24 hrs
48 hrs
72 hrs
Time
Control JEG-3 Cells
Infected JEG-3 Cells

Figure 6. *Listeria monocytogenes* infection decreases cell metabolism and increases cell death in JEG-3 cells. Monolayers of JEG-3 cells were infected with 10^5 CFU of LM2203 for 1 hour, and then extensively washed with HBSS. Fresh medium with gentamicin (200μg/ml) was then added to the medium to prevent extracellular bacterial growth. (A) Metabolic activity is detected with Alamar Blue assay. Data represent percent Alamar Blue reduction of infected cells (48 hours of infection) compared to uninfected control cells. (B) LDH release from *L. monocytogenes* infected cells, compared to uninfected control cells. Data are expressed as percentage maximum LDH activity in wells treated with 2% NP-40 detergent. Data are expressed as mean ± SEM of 6 replicates at each time point for one representative experiment from 3 separate experiments. Asterisks indicate $p<0.01$ compared to uninfected JEG-3 cell Alamar Blue reduction and LDH release.

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Chapter Six

Summary and Conclusion

The overall objective of this thesis was to examine mechanisms of the innate and adaptive immune responses to *L. monocytogenes* infection during pregnancy and the periparturient period. Our central hypothesis was that *L. monocytogenes* infection initiates innate and adaptive immune responses in the placenta that promote severe inflammation, which is deleterious to fetal health. The first Aim was to test the hypothesis that intragastric infection of the pregnant mouse with *L. monocytogenes* would produce reproducible fetal infection and abortion. This work also showed that pregnancy reduces genetically mediated resistance to *L. monocytogenes* during pregnancy. We also found, using bioluminescence imaging of mice infected post-partum, that *L. monocytogenes* is shed in the breast milk for multiple days following infection. Using the same mouse model, our second Aim was to test the hypothesis that *L. monocytogenes* infection during pregnancy is associated with elevated levels of IL-17a and IL-22, which promote maternal and fetal inflammation. We found that IL-17a and IL-22 relative gene expression is moderately increased in placental tissue of mice infected with *L. monocytogenes* compared to uninfected control mice. However, using gene knockout mice for IL-17a and IL-22, we found that antimicrobial resistance and maternal and fetal inflammation are not dependent on IL-17a nor IL-22. In our mouse studies, the vast majority of microbial load and inflammation due to *L. monocytogenes* infection in the fetoplacental unit is in the placenta. This prompted us in Aim 3 to

examine the effect of *L. monocytogenes* infection on placental epithelial cells in vitro. Specifically, we tested the hypothesis that *L. monocytogenes* infection of JEG-3 human choriocarcinoma cells reduces cell functionality and causes cell death. We found that *L. monocytogenes* infected JEG-3 cells, which are a model for extravillous trophoblast cells; the infected cells have decreased migration and invasion function, decreased cell metabolic activity, and eventually suffer from increased cell death.

The *L. monocytogenes* strain used in all experiments, with the exception of bioluminescence imaging, was quite virulent in our in vivo and in vitro experiments. This strain was donated by Dr. Sophia Kathariou (North Carolina State University) who isolated it in 2001 in from a listeriosis outbreak that caused disease in 13 people, 11 of whom were pregnant [1]. This strain (LM2203 or WS1) is interesting because it is a serotype 4b strain but is distinct from epidemic clone groups 1 and 2, as identified by pulse-field electrophoresis and DNA probe hybridization [2]. Epidemic clones of *L. monocytogenes* are typed with 7 DNA probes and LM2203 shares only 2 DNA hybridization sites with epidemic clone groups 1 and 2 [2]. There are 2 other known *L. monocytogenes* strains with identical genotyping, isolated in 2003 and 2004. However, both of these strains were from sporadic listeriosis cases whereas LM2203 was associated with an epidemic [2]. Further genomic characterization of this strain could be helpful for future studies of *L. monocytogenes* virulence and characterization of strains involved in future epidemic outbreaks.

The use of the pregnant mouse as a model for *L. monocytogenes* infection during human pregnancy is subject to continued debate. Murine E-cadherin has a

different amino acid sequence from human E-cadherin and does not interact strongly with InlA [3]. It is assumed by many that without E-cadherin binding to InlA, the mouse does not accurately reflect *L. monocytogenes* invasion of human epithelial surfaces in the gastrointestinal tract and the placenta. However, we have shown that the pregnant mouse can be reliably infected intragastrically with relatively low numbers of *L. monocytogenes* cells to cause infection and inflammation in the fetoplacental unit [4]. Therefore mice provide a useful model to ask mechanistic questions, other than listerial cell entry into host epithelial cells. The mouse will likely remain a valuable animal model for listeriosis during pregnancy until genetic, immunological and molecular biology tools are developed for other potential model species, such as the guinea pig or the gerbil.

We found that pregnancy negated the genetically mediated resistance of C57BL/6 mice to *L. monocytogenes* infection. Resistance of non-pregnant C57BL/6 mice to intragastric *L. monocytogenes* infection was previously reported by our laboratory [5]. Genetic resistance is regulated principally by the *Hc* locus on chromosome 2 and mice with the resistant allele are less susceptible to various routes of *L. monocytogenes* challenge [6, 7]. Czuprynski et al. proposed that the effect of the resistant *Hc* allele for C57BL/6 mice infected with *L. monocytogenes* was greater accumulation of inflammatory neutrophils and macrophages, with increased antibacterial activity compared to *L. monocytogenes* infected A/J mice [7]. Gervais et al. suggested that the *Hc* susceptible allele causes a deficiency in the C5 component of complement, based on experiments that transferred C5-rich serum to enhance resistance of A/J mice to *L. monocytogenes* infection [6]. It is important to note that

although we did not observe a difference in microbial resistance, pregnant A/J mice had more severe inflammation in maternal tissues than pregnant C57BL/6 mice when infected with *L. monocytogenes*. In contrast; inflammation of fetal tissues was not significantly different between *L. monocytogenes* infected A/J and C57BL/6 pregnant mice.

There are three possible explanations for why we observed a significant decrease in anti-listerial resistance in C57BL/6 and A/J pregnant mice. The first possibility is that the *L. monocytogenes* strain that was used for infection possesses an unknown virulence mechanism, which allows it to be resistant to C5 mediated opsonization and white blood cell recruitment in C57BL/6 mice. The second possibility is that altered immune responses of pregnancy diminish the magnitude and effectiveness of the neutrophil and macrophage response early after infection, which results in an overwhelming bacterial infection. The third possibility is that C57BL/6 and A/J are susceptible to fetal infection and inflammation from *L. monocytogenes* infection during pregnancy by separate undetermined mechanisms. Perhaps A/J mice have a greater microbial burden because of diminished complement function and professional phagocyte recruitment and activation, while other mechanisms explain the increased microbial burden, but lack of inflammatory lesions in maternal tissues, of pregnant C57BL/6 mice [6, 7].

The use of bioluminescence imaging to visualize intragastric *L. monocytogenes* infection in maternal and fetal tissues was a novel approach and has potential uses for

future studies of pathogenesis. One could also use this approach to investigate potential therapeutic interventions that could be translated to treatment of pregnant women with listeriosis. We found variable bioluminescence in the fetuses of *L. monocytogenes* infected mice, although the microbial load in fetoplacental units was not significantly different. A possible explanation for this is the luminescent strain of *L. monocytogenes* that we used only emits bacterial luciferase during log phase bacterial growth [8]. Together with histopathological evidence that inflammation is variable among infected fetuses in the same pregnant mouse, we infer that each fetoplacental unit in the multipregnant uterus experiences a distinct infection and immune response to *L. monocytogenes* infection. This is similar to what has been observed in a limited number of reports of *L. monocytogenes* infection of multi-pregnant women, but has not been reported in other animal infection models [9-11].

Bioluminescence imaging experiments showed another novel finding of *L. monocytogenes* infection in the mouse, shedding of bacterial cells in breast milk. Historically, breast milk is not considered a risk factor for transmission of *L. monocytogenes* to a nursing infant. We find this attitude surprising considering the known high risk for transmission of viral pathogens and increasing awareness for methicillin resistant *Staphylococcus aureus* (MRSA) and Group B *streptococci* transmission to infants via breast milk [12-19]. Our findings warrant further efforts to define the risk of *L. monocytogenes* contamination of breast milk from mothers with listeriosis, or mother with infants suffering from neonatal listeriosis. The current standard of care is to feed fresh or fresh-frozen breast milk without testing or processing in

neonatal intensive care units. Further, contamination of the hospital environment and equipment (e.g. shared breast pumps) should also be considered. Future studies should focus on improving the mouse infection model or switching to an animal that produces higher volumes of milk to more readily study shedding and survival of *L. monocytogenes* in breast milk. To improve the mouse infection model, more detailed characterization of infected mammary glands, and demonstrating transmission of *L. monocytogenes* to pups are important. Constructing a better luciferase emitting *L. monocytogenes* strain would also be useful for these studies. Ideally, a virulent serotype 4b strain of *L. monocytogenes* should be used, with a plasmid containing firefly luciferase inserted into the bacterial chromosome, so that it is constitutively expressed. Firefly luciferase has significantly higher tissue penetration. If it were constitutively expressed, the sensitivity of the bioluminescence assay would improve greatly [20]. We attempted to construct a firefly luciferase emitting strain of LM2203 with a collaborator but abandoned the efforts after several attempts were unable to insert the firefly luciferase containing plasmid.

The second aim of the project was to test the hypothesis that *L. monocytogenes* infection during pregnancy is associated with elevated levels of IL-17a and IL-22, which promote inflammation in maternal and fetal tissues leading to fetal death and abortion. Our interest in IL-17a was due to its ability to stimulate neutrophil differentiation, migration, and activation [21]. Considering that the inflammatory cell infiltrate during *L. monocytogenes* infection of placental tissue is primarily neutrophilic, IL-17a was predicted to play a role in placental inflammation during *L. monocytogenes* infection [22,

23]. We also examined IL-22, which like IL-17a, is produced by Th17 cells, and whose expression is up-regulated in intestinal epithelial cells during *L. monocytogenes* infection [24]. IL-22 was previously reported to have no effect on bacterial clearance in non-pregnant mice infected with *L. monocytogenes*, but these experiments were done with intravenous infection methods. Our experiments used intragastric *L. monocytogenes* infection, which more closely resembles the natural route of infection. We found moderate increases in relative expression of genes associated with activated T cells (CD3+), Th1 (IFNγ), Th2 (IL-4), Treg (FoxP3), and Th17 (IL-17a and IL-22) in placental tissue of mice infected with *L. monocytogenes* compared to uninfected control. We next used *L. monocytogenes* infection of gene knockout mice to determine if IL-17a and IL-22 had protective or detrimental roles for the fetus. Despite evidence for increased relative expression of IL-17a and IL-22 in placental tissue of *L. monocytogenes* infected pregnant mice, the severity of infection and inflammation of maternal and fetal tissues were not dependent on either IL-17a or IL-22. Our data suggests that redundancy in the complex inflammatory response outweighs the roles of IL-17a and IL-22 in the placenta of mice infected with *L. monocytogenes*. We cannot rule out possible roles of IL-17a and IL-22 earlier in infection because all data were collected at 72 hours after infection. Our findings of no increase in severity of infection in either IL-17a$^{(-/-)}$ or IL-22$^{(-/-)}$ mice were disappointing and led us to abandon continued efforts to determine roles for IL-17a and IL-22 during *L. monocytogenes* infection. We cannot rule out possible roles of IL-17a and IL-22 earlier in gestation. Further studies are needed to determine whether Th17 cell cytokines have significant roles in early embryonic death due to *L. monocytogenes* infection.

An alternative approach to examining the Th17 cell response to *L. monocytogenes* infection during pregnancy is to increase numbers of Th17 cells by exposing naïve mice to a low dose of *L. monocytogenes*, essentially vaccinating them, and re-infecting them during pregnancy. We hypothesize that this will increase Th17 cell differentiation and IL-17a and IL-22 expression in the event of a second *L. monocytogenes* infection. Part of the rationale for this is that Th17 cells arise from memory CD4+ cells and IL-17a and IL-22 are thought to be critical for vaccine-induced memory immune responses and to protect against mucosal pathogens [25, 26]. In preliminary experiments, we examined how a secondary immune response to *L. monocytogenes* affects pregnancy. To do so, non-pregnant C57BL/6 mice were infected intragastrically with a low dose (10^3 CFU) of LM2203. Four weeks later, mice were bred and allowed to reach 10-14 days of gestation. At that time they were re-infected with 10^5 CFU of LM2203. When euthanized 72 hours later, the mice showed no signs of systemic disease and the microbial load in maternal livers and spleens were significantly lower than pregnant mice experiencing their first *L. monocytogenes* infection (Appendix, Figure 1). Although fetal tissues from these re-infected mice did not yield detectable CFU, their fetoplacental units exhibited significant inflammation and placental necrosis (Appendix, Figure 2). One explanation for these findings is that a primary *L. monocytogenes* infection primes a memory $CD4^+$ T cell response (including Th17 cells) that leads to a strong Th17 response during secondary infection. The latter results in severe placental inflammation and necrosis. We did not measure antigen specific T cells, nor relative gene expression in these pilot experiments. Future studies

are needed to determine if Th17 cells have a more significant role in a memory immune response to *L. monocytogenes* infection during pregnancy.

The third and final Aim of this project built on the findings of Specific Aims 1 and 2, which showed that the majority of microbial load and inflammation in the fetoplacental units was in the placenta. We surmised that infection of placental tissue also has detrimental effects on fetal health and designed experiments to test the effects of *L. monocytogenes* infection on JEG-3 cells, which are a model for extravillous trophoblast cells. We tested the hypothesis that *L. monocytogenes* infection of JEG-3 human choriocarcinoma cells would reduce cell functionality and cause cell death. We showed that *L. monocytogenes* readily infected JEG-3 cells, which supported listerial cell multiplication for approximately 10 hours of incubation, followed by a decline in listerial numbers. One possible explanation for the decline is that the infected JEG-3 cell monolayer becomes degraded. As the cells die and lift away, gaps form in the monolayer that impair cell to cell spread of *L. monocytogenes*. Another possibility is that JEG-3 cells have bacteriostatic capabilities, similar to primary cultures of extravillous trophoblasts [27]. These capabilities could be a function of JEG-3 cellular physiology, or under autocrine or paracrine signaling control. This would be similar to a report that TNFα and IFNγ exposure inhibit intracellular growth of *L. monocytogenes* in a murine hepatocyte cell line [28]. Before gaps became visible via light microscopy, we showed that the TEER of the monolayer decreases after *L. monocytogenes* infection, which suggests that infection alters monolayer integrity. We also showed that the migration and invasion capability of JEG-3 cells was compromised due to *L. monocytogenes*

infection within 24 hours of incubation. Degradation of the monolayer and decreased migration and invasion is accompanied by decreased JEG-3 cell metabolic activity by 24 hours after *L. monocytogenes* infection, and increased cell death starting at 24 hours after infection. These results suggest that *L. monocytogenes* infection of the extravillous trophoblast has at least two consequences: 1) altering the innate barrier to pathogens from the maternal blood stream to the placental circulation; and 2) diminished migration and invasion of trophoblasts into the decidual tissue. Possible sequelae include: 1) infection of fetal tissues; 2) the loss of anchoring to the decidua; and 3) the loss of the fetus's ability to influence decidual spiral artery remodeling and repress maternal cell mediated immunity [27, 29-31]. Future work to extend these findings will include photomicrographs of monolayer degradation, measurement of inflammatory cytokines with a Luminex based array (Millipore, Billerica MA), and measuring human chorionic gonadotropin in JEG-3 cells infected with *L. monocytogenes*. Future studies of migration and invasion using primary culture human extravillous trophoblast would also be important [27].

The in vivo experiments in this project focused on one time point after *L. monocytogenes* infection, with the exception of the secondary infection in two infection experiments. We chose this time point from previous experiments, which showed a peak in microbial load and tissue inflammation 72 hours after intragastric *L. monocytogenes* infection. Although the majority of our data surrounding the adaptive immune response was negative, I would not rule out a role for Th17 cells, or IL-17a and IL-22, in listeriosis. Because humans and domestic ruminants are consistently exposed

to *L. monocytogenes*, whether in food or in the environment, future studies should use animals exposed multiple times to low doses of *L. monocytogenes* prior to a large infective dose during pregnancy. These studies could help determine whether transient, repeated exposure to *L. monocytogenes* changes the innate and adaptive immune response to a large dose or virulent strain of *L. monocytogenes* in the event of an outbreak. I also think that repeated exposure experiments should include *L. monocytogenes* infections earlier in gestation. Based on what we found in the JEG-3 experiments, *L. monocytogenes* infection early in gestation could impair the extravillous trophoblast cells ability to invade and influence events in decidual tissue leading to poor implantation and early embryonic loss. Early embryonic loss is a problem in human and veterinary reproductive medicine, the mechanisms of which are largely unknown and undiagnosed. Because *L. monocytogenes* is a ubiquitous facultative pathogen frequently found in food, I would hypothesize that *L. monocytogenes* could be an undiagnosed cause of early embryonic loss.

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Appendix

Figure 1

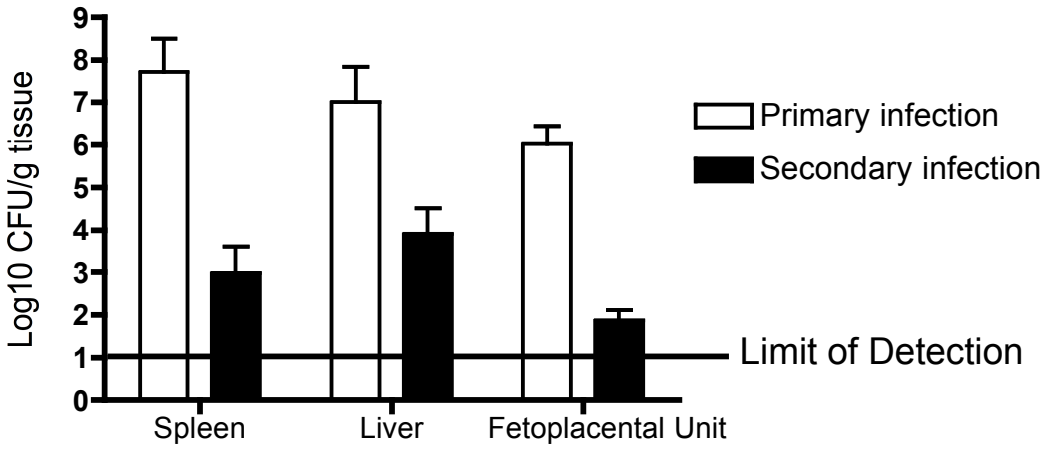

Figure 1: Intragastric infection with 10^5 CFU of LM2203 causes repeatable maternal and fetal infection and immunized mice exhibit lower microbial load in all tissues when re-infected. Primary infection of pregnant mice (10-14 days gestation) by intragastric infection of 10^5 CFU LM2203. Organs were harvested 72 hours post infection for bacterial culture. Mice were infected twice (secondary infection); they were infected orally with 10^3 CFU of LM2203, allowed to rest for 4 weeks, bred, and infected again with 10^5 CFU of LM2203. Three fetoplacental units (FPU) per mouse were cultured. Data points are mean ± SEM of 10 mice.

Figure 2

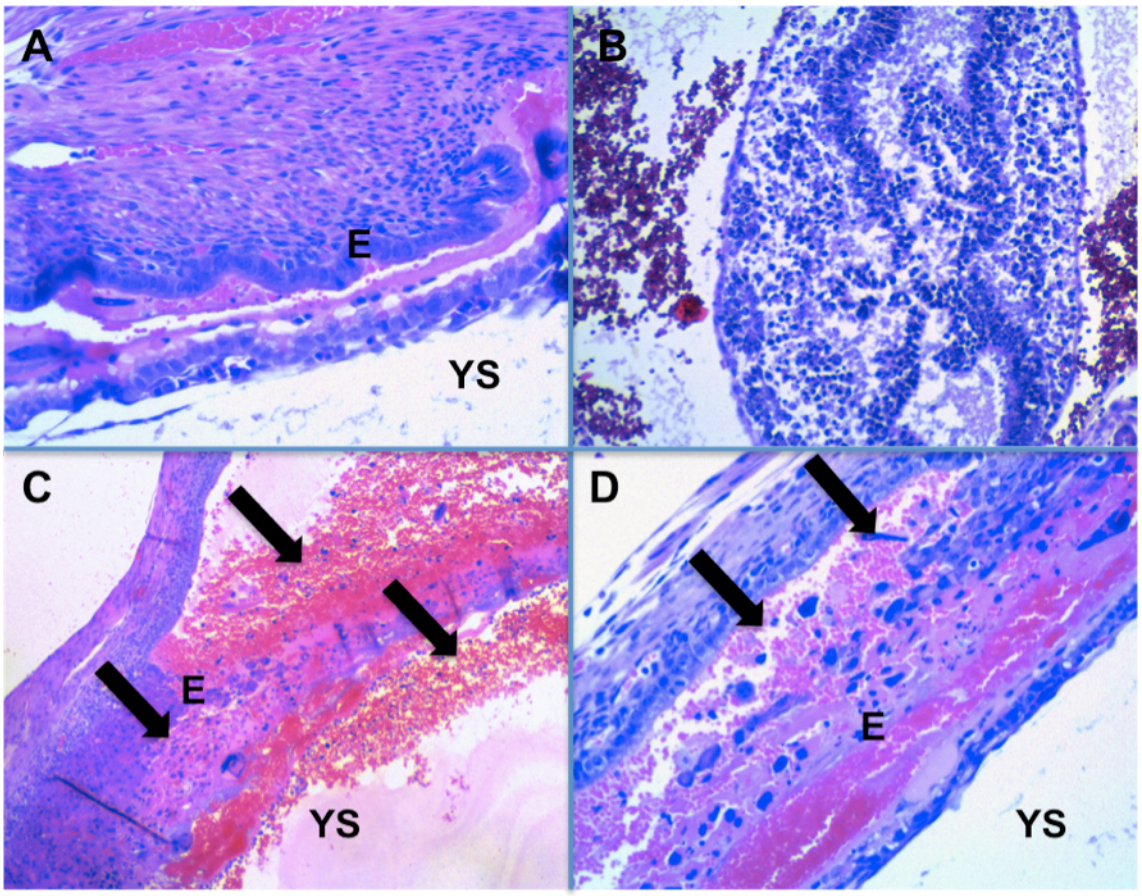

Figure 2: Primary infection of pregnant mice is associated with severe generalized hemorrhage and placental necrosis. Immunized mice exhibit severe multifocal placental necrosis when re-infected. (A) H&E staining – 100X of yolk sac (YS) - endometrium (E) interface from a non-infected (control – 10 days gestation) C57BL/6 mouse. (B) H&E stain – 100X of fetoplacental unit with generalized necrosis of fetal tissues. (C) H&E stain – 200X of yolk sac-endometrium interface with severe generalized placental necrosis and hemorrhage (arrows) from a mouse infected with 10^5 CFU of LM2203. (D) H&E stain – 100X of fetal tissue with severe multifocal placental necrosis (arrows) from a mouse infected twice with LM2203.