Placental co-culture model reveals *Listeria monocytogenes* utilization of monocytes for vertical transmission

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PLACENTAL CO-CULTURE MODEL REVEALS *LISTERIA MONOCYTOGENES* UTILIZATION OF MONOCYTES FOR VERTICAL TRANSMISSION

BY

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PLACENTAL CO-CULTURE MODEL REVEALS *LISTERIA MONOCYTOGENES*

UTILIZATION OF MONOCYTES FOR VERTICAL TRANSMISSION

BY

BREANNA AMELUNKE

Submitted to the Faculty of the Graduate School of

Eastern Kentucky University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

2023
ABSTRACT

Listeria monocytogenes is an opportunistic pathogen that can cross the protective placental barrier and infect the fetus, leading to congenital disabilities or death. Extracellular L. monocytogenes infection of the placenta has been well studied; however, the bacteria have not been observed translocating this barrier intracellularly. As an intracellular pathogen, L. monocytogenes could infect phagocytic cells that can be transported across the placental barrier. A novel cell co-culture system comprised of BeWo and HPVEC cell lines was established to model the human placental barrier. Barrier integrity was confirmed using immunostaining of tight junctions and a FITC-dextran permeability assay. The model was then used to observe L. monocytogenes use of the “Trojan horse” method for vertical transmission. Human monocyte THP-1 cells were exposed to L. monocytogenes (MOI 100) for one hour to ensure infection. The co-culture model was exposed to varying treatments including the L. monocytogenes-infected THP-1s for two hours to allow translocation. Upon exposure to the infected cells, the placental model experienced a five-fold increase in barrier permeability. Hemocytometer counts revealed a significant increase in cell translocation with treatments that included THP-1s infected with L. monocytogenes. Model exposure to monocytes infected with L. monocytogenes resulted in an increase in sICAM-1 expression (~3-fold) and an increase in cell cytotoxicity by ~20%. Immunostaining of co-culture models exposed to L. monocytogenes-infected monocytes revealed severe disruption of occludin and claudin-1 tight junctions. This study demonstrates Listeria usage of the Trojan horse mechanism to reach and translocate the human placental barrier. Thus, Listeria infection can utilize both extracellular and intracellular forms of the pathogen to breach the placental barrier.
# Table of Contents

Introduction .......................................................................................................................... 1

*Listeria monocytogenes* .................................................................................................. 1

Pathogenic Disease ............................................................................................................ 2

Epidemiology ...................................................................................................................... 3

Infectious Dose .................................................................................................................. 3

Virulence Factors .............................................................................................................. 4

Species Specificity of Virulence Factors ......................................................................... 5

Infection Mechanisms ....................................................................................................... 7

The Human Placenta .......................................................................................................... 8

Placental Functions ............................................................................................................ 10

Anatomy ............................................................................................................................. 13

Placental Barrier ............................................................................................................... 14

*Listeria* Infection of the Human Placenta .................................................................... 16

Maternal Outcomes .......................................................................................................... 16

Fetal Outcomes ................................................................................................................ 18

Treatment and Prevention ............................................................................................... 20

Placental Tropism .............................................................................................................. 21

Known Mechanisms ......................................................................................................... 21

Unknown Mechanisms ..................................................................................................... 22
Bacterial Enumeration

Microscopy for Enumeration of Trojan Horses

Flow Cytometry

LDH Cytotoxicity Assay

ICAM-1 ELISA

Immunostaining of Cell-Cell Junctions

Quantification And Statistical Analysis

Results

Establishment and permeability characterization of a human placental co-culture model using BeWo and HPVEC cell lines

Lm-infected THP-1 cells increase translocation and promote infection of the placental barrier model

The presence of extracellular Lm with THP-1s increases translocation of monocytes

Lm-infected THP-1s disrupt tight junctions and increase permeability of placental barrier model

Flow cytometry quantitative measure of host cell Lm infection and translocation through co-culture model barrier

Discussion

The cell co-culture model is an effective in vitro model system for the human placental barrier

Lm-infected THP-1s translocate the placental barrier at higher levels than THP-1s
Placental barrier exposure to THP-1s correlates with increases in sICAM-1 expression
............................................................................................................................................ 56

*LM*-infected THP-1s disrupt tight junctions to promote infection of the placental barrier
................................................................................................................................................ 58

The presence of extracellular *LM* supports infection of the co-culture model ............. 60

Future Directions..................................................................................................................... 63

Appendices............................................................................................................................... 66

Appendix A: Supplementary Table....................................................................................... 67

Appendix B: Supplementary Figures .................................................................................. 70

Phase-Contrast Microscopy of Cell Lines ............................................................................. 71

FITC-Dextran Standard Curve ............................................................................................... 71

sICAM-1 ELISA Standard Curve............................................................................................ 72

Flow Cytometry Results ........................................................................................................ 72

Bacterial Enumeration Results for Extracellular *Listeria*.................................................. 73

References............................................................................................................................... 74
List of Tables

Table 1. Pathogens capable of utilizing host lymphocytes as Trojan horses during pathogenesis. ............................................................ 25

Table 2. Pathogens capable of breaching the placental barrier and causing congenital infection. ................................................................. 31

Table 3. Key resources used over the duration of this study. ............................................... 68
List of Figures

Figure 1. Species specificity of *L. monocytogenes* surface proteins InlA and InlB........... 6

Figure 2. *Listeria monocytogenes* intracellular life cycle schematic ................................. 7

Figure 3. Anatomy of the human placenta ................................................................. 14

Figure 4. Anatomy of the human placental barrier ...................................................... 16

Figure 5. The co-culture model of the human placental barrier .................................... 34

Figure 6. LDH cytotoxicity assay reaction ...................................................................... 39

Figure 7. Distribution of cell media and seeded cells after translocation assay .............. 42

Figure 8. Establishment of a cell co-culture model using BeWo and HPVEC cell lines . 44

Figure 9. The effects of *Listeria* infection of THP-1s on overall cell translocation and cytotoxicity after two hours of exposure ................................................................. 46

Figure 10. The effects of intracellular and extracellular *Lm* on THP-1 translocation of and adherence to the co-culture model ................................................................. 48

Figure 11. *Lm*-infected THP-1 interaction with co-culture model tight junctions ....... 50

Figure 12. *Lm*-infected THP-1s disrupt cell junctions of cell co-culture model barrier .. 51

Figure 13. Flow cytometry analysis of cell translocation through co-culture model ...... 53

Figure 14. Graphical illustration representing two possible ways *Listeria* uses the Trojan horse mechanism to breach the placental barrier ......................................................... 63

Figure 15. Phase-contrast microscopy images of confluent cell lines at 200X magnification ...................................................................................................................... 71

Figure 16. Standard curve of FITC-dextran concentration to fluorescent intensity at an excitation of 485 nm and an emission of 528 nm ................................................................. 71
Figure 17. Standard curve of human sICAM concentration and absorbance readings at 450 nm. ................................................................................................................................................................................................................................................. 72

Figure 18. Flow cytometry infected cell counts after cell lines were exposed to GFP Lm (MOI 500) for two hours................................................................................................................................................................................................................................................. 72

Figure 19. Bacterial enumeration counts after translocation assay for treatments of extracellular L. monocytogenes and L. innocua................................................................................................................................................................................................................................................. 73
Introduction

Listeria monocytogenes

Listeria monocytogenes is an intracellular and highly invasive foodborne pathogen that is the cause of listeriosis. L. monocytogenes is a gram-positive, facultative anaerobe ubiquitous in environments worldwide. It is an opportunistic pathogen, typically causing disease in immunocompromised individuals, the elderly, pregnant women, and perinatal infants.

L. monocytogenes was first described by E. G. D. Murray in 1926, who discovered the species while investigating an unknown disease killing rabbits (1). This pathogen earned the specific name monocytogenes after Murray observed monocytosis – an abnormally high level of monocytes – in the infected rabbits. Listeria was found to cause disease in livestock animals, cats, dogs, foxes, rabbits, and guinea pigs not long after its initial discovery, although L. monocytogenes was not recognized as a significant cause of human disease until the first report of Listeria-induced meningitis in 1959 (2).

Foodborne listeriosis in humans has been primarily attributed to contaminated food products such as milk, ice cream, cheese, deli meats, and fresh produce (3). While pasteurization kills Listeria, eating uncooked or unpasteurized food products increases the risk of infection. L. monocytogenes can survive in a wide range of environments, form biofilms, and is resistant to sanitizers and antimicrobials, allowing it to persist in food processing environments (4). The Food and Drug Administration (FDA) regulates food processing to combat Listeria contamination, especially in ready-to-eat products. As
a result, *L. monocytogenes* infection is relatively rare in the United States, with sporadic outbreaks occurring as a result of contamination in food-processing facilities.

**Pathogenic Disease**

*L. monocytogenes* is the causative agent of listeriosis, a rare but severe disease. It is an opportunistic pathogen, generally not causing disease in individuals with normal immune function. As a result, most *Listeria* infections are confined to the gut and do not cause manifestations of disease.

There are two categories of *Listeria* infection that cause listeriosis: intestinal and invasive. The standard method of infection includes consumption of contaminated foods, which introduces the pathogen to the host G.I. tract. It is here that *L. monocytogenes* can cause intestinal illness such as gastroenteritis, with mild symptoms including abdominal pain, nausea, and vomiting (5). If *Listeria* is capable of evading host immune responses and breaches the intestinal barrier, it can enter the bloodstream and travel to other parts of the body. This systemic spread is known as invasive infection, which can affect other host organs, namely the brain and placenta. Most people diagnosed with listeriosis have invasive infections that can cause fever, flu-like symptoms, headache, confusion, loss of balance, and miscarriage in pregnant women (6).

The incubation period for *Listeria* varies depending on the nature of the infection – the average incubation period for intestinal listeriosis is 24 hours; in contrast, invasive listeriosis is characterized by an eight-day incubation period (7). The infectious dose (ID) of *L. monocytogenes* also varies among hosts, bacterial strains, and disease manifestation. The exact ID for human infection is unknown – epidemiological investigations of recent listeriosis outbreaks report varying estimates of infectious doses (8).
Epidemiology

Approximately 1,600 cases of listeriosis are reported annually in the U.S. (9). Listeriosis has a high hospitalization rate of 94% (10). The case fatality rate (CFR) for *L. monocytogenes* infection ranges from 20-30%, with an average of 260 deaths per year in the United States (9, 10).

Infection is typically observed in the first 30 days of life or in patients over 60 years of age (11). The risk of developing fatal listeriosis is 10-100 times higher for neonates and the elderly than the general population (12). Pregnant women are an exception, who are up to 18 times more likely to develop listeriosis than other healthy adults, and account for 16-27% of all reported cases (13, 14). More than 83% of maternal listeriosis cases lead to adverse outcomes in the fetus (15). Fetal and neonatal death occur in approximately 20-60% of reported cases (16).

Infectious Dose

The average infectious dose for *L. monocytogenes* colonization in human hosts has not been determined, but is estimated to range from $10^3$–$10^7$ colony forming units (CFU) (8, 17). The World Health Organization (WHO) estimates a median lethal dose ($LD_{50}$) of *L. monocytogenes* in humans to be approximately $10^6$ CFU (12, 17). The data demonstrate lower doses necessary to cause infection and mortality in susceptible individuals than the general population (18). Discrepancies in the dose-response relationship can be attributed to host susceptibility and variance in the virulence factors among *L. monocytogenes* strains (17).
Virulence Factors

*Listeria monocytogenes* is an intracellular pathogen capable of infecting different types of host cells. Invasion of host phagocytes is a passive process, but *Listeria* infection of non-phagocytic cells is mediated by several virulence factors (19, 20). These virulence factors are predominately expressed from gene clusters referred to as pathogenicity islands (21). *Listeria* pathogenicity islands are conserved in virulent species like *Listeria monocytogenes*, but absent from non-pathogenic species like *Listeria innocua* (21–23).

**Internalins (InlA, InlB, and InlP)**

As the name suggests, the bacterial surface proteins of the internalin family are involved with internalization of *Listeria* into host epithelial cells (19, 20). The most significant virulence factors known for *Listeria monocytogenes* are InlA and InlB (24). *Listeria* uses these proteins to adhere to and invade host cells. InlA and InlB bind to host cell surface receptors E-cadherin and c-Met, respectively (25). E-cadherin is critical for cell-to-cell adhesion, particularly in epithelial tissues. On the other hand, c-Met is a tyrosine kinase from the MET (MNNG HOS transforming gene) family that mediates cell-signaling pathways involved in cell migration and survival (26).

When an internalin binds to its receptor, it signals the cell to induce structural reorganization of the membrane and cytoskeleton, allowing the bacterium to become internalized (19, 20, 25, 27). InlP is a novel virulence factor highly conserved in *Listeria monocytogenes* (28). It was recently discovered to play a role in placental infection; however, the mechanism of action is still unknown (28).
Listeriolysin O (LLO)

As *Listeria* is internalized, it enters the cell inside a phagosome (or a vacuole in non-phagocytic cells) (19). Once inside, *L. monocytogenes* produces listeriolysin O (LLO), a pore-forming toxin that lyases the phagosome and frees the bacteria (15, 19, 27, 29). LLO is a crucial virulence factor for *Listeria*, establishing it as one of few pathogens capable of escaping the phagosome and multiplying inside host cells.

Actin (ActA)

Once in the cytoplasm, *Listeria* uses the actin assembly-inducing protein to mediate actin polymerization and formation of actin “comet tails” (15, 27, 30). The actin tail complex provides motility fundamental for cell-to-cell spread of *L. monocytogenes*. The bacterium is propelled to the cell membrane, which then forms a protrusion into the neighboring cell with *Listeria* at its tip (30). The end of the protrusion is phagocytosed by the new cell, internalizing *Listeria* once again, and the cycle repeats.

Listeria Adhesion Protein (LAP)

*L. monocytogenes* uses listeria adhesion protein (LAP) to bind to host cells and disrupt the epithelial cell junctions (31). LAP binds to receptor heat shock protein 60 (Hsp60), a chaperonin involved in protein folding and degradation (32, 33). LAP binding to Hsp60 induces a cell-signaling cascade that results in cellular distribution of the tight junction proteins (20). This promotes opening of the cell junctions, which mediates bacterial translocation between the cells.

Species Specificity of Virulence Factors

*Listeria* virulence factors InlA and InlB demonstrate stringent species specificity for their respective receptors on host cells (25, 27, 34). The interaction between InlA and E-
cadherin is critical for host infection. While E-cadherin is very similar in humans and mice (85% match), a single amino acid substitution inhibits InlA binding in mice (34, 35). InlB demonstrates a similar specificity, unable to recognize or bind to its Met receptor in guinea pigs or rabbits (36). Since the internalin proteins are critical for *Listeria* pathogenesis, disease development in hosts with inappropriate E-cadherin or Met receptors is stunted (Figure 1). Studies investigating *L. monocytogenes* infection with these models would need to either use transgenic model organisms or genetically modified *Listeria* to mimic human infection (34).

*Figure 1. Species specificity of *L. monocytogenes* surface proteins InlA and InlB.* Murine models are not susceptible to *L. monocytogenes* InlA-E-cadherin interaction due to a substitution of proline for glutamate at position 16 of their E-cadherin N-terminal repeat. Sequencing analysis has also revealed that the c-Met receptor for guinea pigs and rabbits are not compatible with InlB. Thus, the two known species susceptible to both *L. monocytogenes* InlA and InlB interaction are humans and gerbil models. Adapted from Bonazzi et al. 2009 (25).
Infection Mechanisms

There are three primary mechanisms employed by *L. monocytogenes* to breach host barriers and facilitate infection – transcytosis, paracellular crossing, and the Trojan horse mechanism. Transepitosis of *L. monocytogenes* is mediated by virulence factors such as InlA, InlB, LLO, and ActA. The internalins mediate *Listeria* attachment to non-phagocytic host cells and subsequent endocytosis. LLO frees the bacterium from the endosome and ActA promotes assembly of *Listeria*’s “actin tail” to facilitate motility inside the host cell (15). Once in the cytoplasm, the pathogen can either exit the host cell or infect the neighboring cell in cell-to-cell spread (Figure 2).

**Figure 2. Listeria monocytogenes intracellular life cycle schematic.** (1) Extracellular *Listeria* enters the host cell inside a vacuole after interaction with surface receptors such as E-cadherin or c-Met. (2) *L. monocytogenes* produces LLO to lyse the vacuole and free the bacterium into the host cytoplasm. (3) *Listeria* replicates inside the host cell. (4) *Listeria* uses ActA to mediate formation of an actin “comet tail” (5) The actin tail propels the bacterium to the neighboring cell, where it phagocytoses the protrusion containing *Listeria*. (6) The bacteria enters the new cell encapsulated in two layers of cell membrane. (7) It then produces LLO to lyse the vacuole, and the cycle continues. Adapted from Biorender template.
Paracellular crossing is accomplished using LAP binding to Hsp60, which triggers redistribution of the host cell junction proteins (20, 31, 33). *Listeria* takes advantage of this mechanism, slipping through the cell junction and translocating the host barrier.

The Trojan horse mechanism is employed by multiple intracellular pathogens to breach host barriers. This process is not mediated by any *Listeria* virulence factors, as internalization of pathogens is normal behavior for professional phagocytic cells (37). *L. monocytogenes* can subvert this attack and take advantage of the defense mechanism. Some intracellular pathogens such as *Listeria* and *Shigella* spp. can escape the phagosome inside the phagocyte, and multiply within the cytoplasm safe from host immune surveillance (37, 38). In addition, pathogens inside the phagocytes can be transported throughout the host as the phagocytes travel throughout the circulatory system. Thus, these Trojan horse cells can act as both a safe haven and a transportation service for *Listeria*.

*The Human Placenta*

The placenta is a chimeric organ that contains cells of both fetal and maternal origin (39). It acts as the maternal-fetal interface, allowing for exchange of gasses, nutrients, and immunoglobulins between the mother and fetus (40). The placenta is the only temporary organ, only remaining in the mother’s body for the duration of pregnancy before being expelled during the birthing process.

Developing alongside the embryo in the uterus, the placenta undergoes rapid changes during pregnancy. Although it only begins as a few cells, a mature placenta is typically 15–25 cm² in diameter, 2–3 cm² thick, and weighs 500g at the time of birth (40, 41).
Six days post-fertilization, the zygote develops into a hollow ball of cells known as a blastocyst (42). The blastocyst consists of two cell types – the outer trophoblast cells and the inner cell mass (ICM) (42). Placental development begins approximately seven days post-fertilization, after the blastocyst implants in the endometrium (the inner epithelial lining of the uterine mucosa). The trophoblast cells begin to proliferate and differentiate into cytotrophoblast (CYT) and syncytiotrophoblast (SCT) cells, which help anchor the blastocyst into the endometrium (43).

The placenta includes a complex vascular network circulating both maternal and fetal blood. This network is developed through two processes: vasculogenesis (formation of new blood vessels de novo) and angiogenesis (formation of vessels from preexisting vessels) (44). Placental vasculogenesis begins by day 18 post-fertilization, forming primitive fetal capillaries in the developing placenta (45, 46). Angiogenesis begins 21 days post-fertilization, establishing a network of maternal blood vessels towards the trophoblast layer (46). By day 32 of development, the placental blood vessels connect to fetal circulation through the umbilical cord, establishing primitive fetoplacental circulation (46, 47). This vascular network continues to grow and mature throughout the pregnancy.

By the end of gestation, the human placenta has a surface area of 15 m² and an extensive capillary network approximately 550 km in length, which helps facilitate exchange between mother and fetus (45). As the baby grows, the placenta grows with it, allowing the placenta to keep up with its increasing nutrient requirements.
Placental Functions

The placenta is a multifaceted organ that performs several functions to support development of the fetus. The placenta is involved in respiration, nutrition, excretion, endocrine, and protection of the baby. The placenta acts as a transportation system, facilitating exchange of molecules between mother and fetus.

Respiration

*In utero*, the fetal lungs do not partake in gas exchange, so the fetus is entirely dependent on the placenta for its respiratory function (40). This function involves the exchange of oxygen and carbon dioxide gasses between maternal and fetal blood. Both molecules are exchanged through the placenta by passive diffusion, dependent upon the pressure gradient between maternal blood and fetal blood in the placenta (40). Deoxygenated blood is transported from the fetus to the placenta to be replenished by the maternal blood, and the newly oxygenated blood gets circulated back to the fetus (48).

Nutrition

Fetal growth and survival is dependent upon maternal nutrient availability and the placental ability to transport those nutrients to the fetus (49). Since the placental barrier prevents direct contact of fetal and maternal blood, nutrient transport is dependent upon facilitated diffusion and active transport, as simple diffusion with a concentration gradient is insufficient to meet the needs of the fetus (49, 50). Significant nutrients in maternofetal metabolic transfer include glucose, amino acids, fatty acids, and cholesterol (40, 49, 50).
Excretion

The placenta takes on hepatobiliary function while the fetal liver is developing. During this time, the fetus is not capable of discharging waste products including bile or bilirubin on its own, and must depend upon the placenta for excretion (51). This process is accomplished using transporters and ATP-dependent pumps in the membranes of cells comprising the placental barrier (51).

Endocrine

The endocrine functions of the placenta promote fetal growth and facilitate adaptation of maternal physiology to support the fetus. The placenta produces peptide hormones, steroid hormones, and metabolic proteins to maintain a successful pregnancy.

Peptide hormones secreted by the placenta include human chorionic gonadotropin (hCG), human placental lactogen (hPL), and placental growth hormone (pGH) (52, 53). Human chorionic gonadotropin promotes maternal immunotolerance of the fetus during pregnancy (54). Both hPL and pGH influence maternal adaptations and fetal growth through promotion of insulin resistance to adjust the mother’s metabolism to sustain the fetus (53, 55, 56). Similarly, the metabolic proteins adiponectin and lectin also support placental growth, metabolic regulation, and maternal immune tolerance of the fetus (53, 57, 58).

The placenta also produces steroid hormones such as estrogen and progesterone. Estrogen has several functions that are critical to a successful pregnancy – it increases insulin resistance, regulates other hormones, promotes angiogenesis, and promotes growth of the uterus and fetus (53, 59, 60). Progesterone modulates maternal immune responses and suppresses uterine contractions to prevent rejection of the embryo and ensure proper
implantation (53, 60, 61) Both estrogen and progesterone stimulate mammary gland development (60, 62).

Protection

A developing fetus must be protected from harmful substances, pathogens, and the mother’s own immune system. The placenta protects the developing fetus in two ways: by acting as a selective barrier and interacting with the mother’s own immune system. Physical defenses against pathogens include the placental barrier – trophoblast cells and fetal endothelial cells physically separating the maternal and fetal blood supply.

The placenta is also capable of innate immune signaling and releasing antimicrobial effectors including cytokines, exosomes, and antimicrobial peptides (63). These mechanisms can be used to attack pathogens and evade maternal immune defenses that could target the fetus – allowing for a successful pregnancy, while leaving the maternal immune system mostly intact (64).

The immune system produces five classes of immunoglobulins, although only IgG subclasses IgG1 and IgG4 can cross the placental barrier in substantial amounts (65–68). IgG is the most common immunoglobulin, and the predominant class present in the blood (69). IgG is capable of binding to the neonatal fragment crystallizable region (FcRn) receptor on placental cells (70). Upon binding to the FcRn, IgG is endocytosed and released into fetal circulation via exocytosis (40, 65, 69). This passive immunity provides additional protection to the fetus that continues after birth for up to 6 to 12 months (71).
Anatomy

The placenta is comprised of cells originating from both the mother and developing embryo. The embryonic component of the placenta forms the chorion, and the maternal component forms the decidua (72). These two sides are separated by the intervillous space.

The chorion develops from the embryonic trophoblast cells, specifically syncytiotrophoblast and cytotrophoblast cells (73). The chorionic plate includes connective tissue, the amnion, and chorionic arteries and veins (74). The chorionic blood vessels connect to the umbilical cord vessels, which circulate blood between the fetus and placenta. The chorion becomes folded to form multiple villi (finger-like projections), maximizing surface area to allow gas and nutrient exchange between maternal and fetal blood (73).

Upon implantation of the blastocyst into the uterine lining, decidualization occurs in the endometrium. Decidualization is the process in which endometrial stromal fibroblast cells (ESCs) differentiate into decidual cells (DSCs) (75). These changes are the result of gene reprogramming regulated by biochemical, hormonal, and immunological factors (75). Angiogenesis also occurs in the uterus during decidualization, with the new maternal blood vessels extending towards the fetus (76, 77). The decidua includes DSCs, maternal blood vessels, and maternal immune cells (77). The decidua also gets folded into projections called placental septa, which invade the intervillous space (74).

The two sides of the placenta are separated by the intervillous space, a cavern between the two halves where the chorionic villi and placental septa reach toward each other (40). Maternal blood from the uterine arteries fill this space, coming into direct contact with
the syncytiotrophoblast cells of the chorion (40, 74). The chorion and decidua are not completely separated; they are attached where the decidua gets penetrated by anchoring chorionic villi (78).

**Figure 3. Anatomy of the human placenta.** Oxygenated blood flow is indicated with red vessels and a red arrow, deoxygenated blood flow is indicated with dark purple vessels and arrows.

**Placental Barrier**

The placental barrier is the crux of the maternofetal interface, a multi-layered structure separating fetal blood from the maternal blood in the intervillous space. This barrier is comprised of trophoblast cells on the maternal side and fetal endothelial cells on the fetal side (79).
**Trophoblast Cells**

The syncytiotrophoblast is the outermost layer of the placenta that implants into the endometrium and is in direct contact with maternal blood. A syncytium is a multinucleated cell, and a syncytiotrophoblast is formed by the fusion of the underlying cytotrophoblast cells. Since this epithelial covering is bathed in maternal blood, it is the front line of defense against any pathogens or harmful substances present in the mother’s circulatory system.

The fusion of cells forming the syncytium results in a lack of intracellular junctions that pathogens could exploit for entry (80). The syncytiotrophoblast cells are densely covered with microvilli which may inhibit pathogen adhesion to the cell (80, 81). The syncytium also contains a dense actin network that restricts physical deformations used for pathogen entry (80).

The underside of the syncytium is lined with cytotrophoblast cells. They act as progenitor cells for syncytiotrophoblast cells, contributing to the syncytium through fusion as the placenta develops (82).

**Fetal Endothelial Cells**

Endothelial cells line all blood vessels and regulate exchange between the bloodstream and surrounding tissues (83). Fetal endothelial cells comprise the fetal capillaries in the placenta, encapsulating fetal blood circulating to and from the umbilical cord. These placental endothelial cells exhibit remarkable flexibility, as they undergo rapid change through vasculogenesis during placental development (82, 84).
These cells function to regulate blood flow and exchanges between the bloodstream and surrounding environment (83). Endothelial cells sense changes in blood pressure and can modify the diameter of the blood vessels to adapt to the change in blood flow (83). Placental endothelial cells facilitate exchange between the fetal and maternal blood through intracellular or paracellular transport of molecules (79). The necessary gasses and nutrients for fetal development must pass through both the trophoblast cell layer and the endothelial cell layer to reach the fetal blood.

Figure 4. Anatomy of the human placental barrier. The placental barrier is comprised of the maternally derived trophoblasts (purple) and the fetally-derived endothelial cells (pink).

**Listeria Infection of the Human Placenta**

**Maternal Outcomes**

Pregnancy is a major risk factor for invasive *Listeria* infection. Pregnant women are estimated to have a listeriosis incidence 20X higher than the general population (85–87). Additional risk of listeriosis is present for women carrying multiple fetuses (88, 89).
Maternal listeriosis typically develops after the mother ingests food contaminated with *L. monocytogenes*. Most maternal infections occur during the third trimester, when the maternal T-cell immunity is most impaired (86, 87). Maternal illness is generally mild or asymptomatic, but can cause devastating harm to the fetus (85). Maternal infection alone does not guarantee infection of the placenta or fetus (90). Symptoms of maternal listeriosis are often non-specific, such as fever, abdominal pain, and flu-like symptoms (91).

Severe maternal illness is rare, but is more likely if the mother has pre-existing conditions such as HIV (91). Pregnant women hospitalized with listeriosis have higher rates of maternal morbidity, acute respiratory distress syndrome, sepsis, and shock (87, 92). They are also at greater risk of having a caesarean section, preterm birth, or stillbirth than pregnant women without listeriosis (87, 92).

If the placenta becomes colonized by *L. monocytogenes*, it can amplify bacterial load and become a reservoir from which the bacteria can continuously re-infect the mother (88, 90). A *Listeria* breach of the placental barrier can result in chorioamnionitis – infection of the placenta and amniotic fluid. The placenta, umbilical cord, and amniotic fluid yield significantly higher bacterial loads than nonreproductive tissues during maternal infection with *Listeria* (93). Infected placentas can be observed with abscess formation, acute inflammation, necrosis, villitis, and hemorrhages during histological evaluation (88, 93–96). Additionally, the umbilical cord can develop abscess formation, acute inflammation, necrosis, vasculitis, and thrombosis when infected (96). Damage caused by infection compromises the integrity of the placenta and its effectiveness, endangering the fetus.
Fetal Outcomes

In contrast to maternal listeriosis, neonatal infection with *L. monocytogenes* is often severe, resulting in death of the neonate or long-term sequelae in surviving infants (91). The mortality rate for reported cases of neonatal listeriosis has an average of about 25% (85, 91, 94, 97, 98). The lethal dose of *L. monocytogenes* for human fetuses is estimated to be $1.9 \times 10^6$ CFU (93, 99).

Risk factors include maternal consumption of unpasteurized dairy products, inadequate prenatal care, an immunodeficient mother, and co-infection with other pathogens such as HIV or CMV (63). Additionally, a fetus is at an increased risk of infection if the mother has increased vaginal pH values during pregnancy (100). Although rare, *L. monocytogenes* can colonize the vaginal tract (91, 101). Pregnancy does not affect the vaginal carriage rate of *Listeria*, though its presence may lead to infection of the baby during vaginal delivery and subsequent late-onset neonatal listeriosis (91, 100, 102).

Development of neonatal listeriosis can occur after vertical transmission of *L. monocytogenes* through translocation of the placental barrier, inhalation of infected amniotic fluid, or ascending colonization from the vagina (93, 94). Clinical manifestations of neonatal infection vary, though the most frequent consequences of infection include sepsis, meningitis, pneumonia, lesions, rash, jaundice, and respiratory distress (91, 93–95, 97, 98).

Most recorded cases occur as a result of placental translocation during the latter half of pregnancy. Approximately two-thirds of neonatal listeriosis cases occur during the third trimester, while one-third occur during the second trimester (95, 98, 103). The underlying cause is poorly understood, though it is speculated that it could be the result of increased
blood exchange between mother and fetus, decreased cell-mediated immunity, and increasing physiological burden on the mother as pregnancy progresses (88, 93, 94). An additional explanation for the lack of cases in the first trimester could be that early fetal death is more likely to go unreported, especially if the mother was unaware of pregnancy (88, 94). Although neonatal listeriosis is more likely to occur in the third trimester, adverse effects are less likely to develop at an older gestational age (103).

There are two main forms of neonatal listeriosis: early-onset and late-onset. Early-onset listeriosis is caused by intrauterine infection and takes place within the first week after birth (104). Newborns with early onset listeriosis are associated with more severe manifestations of disease and a bleak prognosis (94). An infected fetus may develop granulomatosis infantiseptica – pathognomonic of neonatal _Listeria_ infection – characterized by granuloma-like lesions on internal organs (97). These microabscesses are most often observed on the lungs, liver, and spleen of the fetus (96, 97). The lesions contain cell debris, necrotic material, leukocytes, and growth of _L. monocytogenes_ (96, 97).

Late-onset neonatal listeriosis develops two weeks or more postpartum, likely as a result of intrauterine infection or passage through a birth canal harboring _Listeria_ (104). Clinical manifestations of disease can be non-specific, although sepsis and meningitis are common (94).

Discordant perinatal listeriosis has been observed in women carrying multiple fetuses, with one twin being infected with _Listeria_ and the other remains uninfected (94, 95). It is speculated that chorionicity in twin pregnancies is responsible for this inconsistency (95). A monochorionic twin pregnancy occurs when both twins share the same placenta; if that
placenta becomes infected, it can pass along the pathogen to both fetuses. Whereas in a dichorionic twin pregnancy, each fetus has its own placenta – neonatal listeriosis would only occur in both twins if Listeria infects both placentas.

Treatment and Prevention

The United States FDA has implemented guidelines and recommendations for preventing Listeria contamination in the food industry. These include regulations of food handling, processing, transport, storage, and sanitation (105, 106). In addition, the Center for Disease Control (CDC) has recommended that pregnant women avoid at-risk foods, such as unpasteurized dairy products, deli meats, and unwashed fruits and vegetables (6, 91, 106).

Diagnosis of maternal listeriosis is accomplished predominately using blood culture (86, 106). If listeriosis is suspected, the mother can be started on antimicrobial therapy before definitive diagnosis by laboratory testing (86).

Due to the intracellular nature of this pathogen, bacteriostatic antibiotics are largely ineffective (94, 103, 106). Penicillin, gentamicin, and ampicillin are the most frequently used antibiotics to treat listeriosis (91, 98, 104, 106, 107). Patients allergic to penicillin-class antibiotics can be prescribed trimethoprim/sulfamethoxazole as an alternative (91, 94, 103, 106). The recommended treatment for maternal listeriosis is 6 g ampicillin daily for a minimum of 14 consecutive days (91, 94, 103, 106, 108). If the fetus survives this period, treatment duration can be extended (91).

While erythromycin is effective against L. monocytogenes, it has demonstrated poor placental transmission, resulting in sub-therapeutic levels in the fetus and amniotic fluid
(86, 94). Additionally, both gentamicin and trimethoprim/sulfamethoxazole are contraindicated due to toxicity and potential teratogenic effects on the fetus (91, 94, 103, 106, 107).

Placental Tropism

*L. monocytogenes* exhibits strong placental tropism (85). Studies have observed significantly higher bacterial loads in the placenta, umbilical cord, fetus, and amniotic fluid than non-reproductive tissues in cases of maternal listeriosis (88). *Listeria* is trafficked from the maternal organs to the placenta in small numbers – a single bacterium can cause placental infection (109). Once in the placenta, the bacteria are able to replicate in large numbers and get trafficked back into maternal circulation – a possible explanation for why maternal listeriosis differs from other forms of listeriosis (109). Thus, maternal immunosuppression of cell-mediated immunity may not be solely responsible for maternal infection, as high numbers of *Listeria* continue to re-infect the maternal organs.

Recent studies have found a new virulence factor involved in *Listeria* infection of the placenta, named InlP (28). InlP promotes placental infection and facilitates placental tropism (28, 110). InlP is highly conserved in *Listeria* species, indicating that it has a critical role in infection and survival of the pathogen (28).

Known Mechanisms

*InlA and InlB*

Both InlA receptor E-cadherin and InlB receptor Met are expressed on trophoblast cells of the placenta (16). Thus, both internalins can cause invasion of the placental barrier. However, the importance of InlA and InlB for placental invasion is controversial.
Although both have been observed infecting placental cells, there have been studies reporting no significant difference between infection with or without the presence of InlA or InlB (16, 81). Alternatively, it has been proposed that a synergistic effect occurs in the presence of both InlA and InlB, and that both functional proteins must be present to infect the placenta (25).

**InlP**

InlP is a novel virulence factor for *L. monocytogenes* that strongly promotes placental infection (28). InlP binds to host cell receptor afadin, an adherens junction protein critical for embryogenesis and present on syncytiotrophoblast cells comprising part of the placental barrier (111, 112). The presence of InlP can significantly increase the bacterial load in a placental infection, but has minimal impact on infection of other maternal organs (28).

InlP is strongly conserved in virulent strains of *L. monocytogenes*, with additional homologs found in other members of the genus including *L. ivanovii*, *L. innocua*, and *L. seeligeri* (110). All identified homologs lack the full-length leucine-rich regions found in *L. monocytogenes* InlP, which could explain why they are not implicated in placental infections (110).

**Unknown Mechanisms**

**LAP**

LAP receptor Hsp60 is abundant in endometrial and uterine cells – with a critical role in hormone synthesis and blastocyst development (113). While it is possible that LAP could have a role in *Listeria* placental infection, at the time of this writing, there have been no investigations into this possibility in the published literature.
Trojan Horses

Another unknown mechanism of *Listeria* infection is the Trojan horse mechanism. Like its namesake, this method consists of pathogen infection of a host cell (typically a leukocyte) that transports it throughout the body undetected by host immune defenses. *L. monocytogenes* has been observed using this mechanism to breach the blood-brain barrier, however, its use of this method at the placental barrier has not been investigated.

**The Trojan Horse Mechanism**

**Role of Phagocytes**

The human body has a myriad of defenses against potential pathogens. One such defender is the phagocyte – a leukocyte that ingests harmful materials in the body. The main groups of phagocytes include monocytes, macrophages, granulocytes, and dendritic cells. These cells are an integral part of innate immunity, destroying harmful particles, pathogens, as well as dead and dying host cells.

Phagocytes are the first line of cell-mediated defense in the host. Responsible for internalizing harmful agents in the body, they are often the first immune cells that pathogens contact upon infection. This process – called phagocytosis – is crucial for maintaining a healthy host.

Phagocytosis begins once the phagocyte detects a pathogen – the immune cells “recognize” pathogen surface receptors, which triggers internalization. The phagocyte’s plasma membrane begins to cover the pathogen and pull it inward, eventually sealing the pathogen inside a phagosome. The phagosome is the vesicle containing the pathogen inside the phagocyte. After lysosome fusion, the phagosome becomes highly acidic and
contains degradative enzymes that can digest the pathogen. Once the pathogen is destroyed, its debris is released from the phagocyte through exocytosis (114).

Since phagocytes can usually recognize and ingest pathogens on their own, these microbes do not need to facilitate internalization. Rather, they take advantage of normal phagocytic behaviors to create their own “Trojan horse” (37). If a pathogen can escape the phagosome-lysosome or endure its corrosive qualities, it can remain in the cell and be shuttled through the bloodstream safe from any other immune defenses.

**Trojan Horse Overview**

*Definition*

While they are critical for healthy hosts, phagocytes can also be inadvertently used as Trojan horses themselves, ingesting pathogens that can then replicate and travel throughout the body while inside them.

Phagocytes are white blood cells that travel through the blood stream and can migrate to different tissues. By infecting one of these cells, a pathogen can be inadvertently ferried throughout the body. Although this can be a passive process for a pathogen, many have developed strategies to manipulate phagocyte behavior to their benefit.

*Examples*

There are several pathogens capable of utilizing the Trojan horse mechanism, including species of bacteria, fungi, protists, and viruses (Table 1).
Table 1. Pathogens capable of utilizing host lymphocytes as Trojan horses during pathogenesis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Trojan Horses</th>
<th>Barriers Affected</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucella abortus</em></td>
<td>Bacteria</td>
<td>Neutrophils, Macrophages (115)</td>
<td>BBB, Placenta, Intestinal</td>
<td>Brucellosis (typical symptoms, miscarriage, endocarditis, liver abscesses (116)</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>Bacteria</td>
<td>Macrophages (117, 118)</td>
<td>Alveolar-capillary barrier (119)</td>
<td>Legionnaires’ disease (120)</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Bacteria</td>
<td>Monocytes (121)</td>
<td>BBB, Placenta, intestinal</td>
<td>Listeriosis (typical symptoms, miscarriage, meningitis, etc.)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Bacteria</td>
<td>Neutrophils</td>
<td>BBB</td>
<td>Tuberculosis (lung infection), TB meningitidis</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>Fungi</td>
<td>Macrophages (122)</td>
<td>BBB, Placenta</td>
<td>Candidiasis, yeast infection, oral thrush, congenital cutaneous candidiasis (123)</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Fungi</td>
<td>Macrophages (37, 124, 125)</td>
<td>BBB, Placenta</td>
<td>Lethal meningoencephalitis (126)</td>
</tr>
<tr>
<td><em>Paracoccidioides spp.</em></td>
<td>Fungi</td>
<td>Dendritic cells (127)</td>
<td>BBB</td>
<td>Paracoccidioidomycosis (PCM)</td>
</tr>
<tr>
<td><em>Leishmania major</em></td>
<td>Parasite</td>
<td>Neutrophils, Macrophages* (128–130)</td>
<td>BBB</td>
<td>Leishmaniasis has three forms: cutaneous, mucocutaneous, or visceral</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>Parasite</td>
<td>Dendritic cells (131)</td>
<td>BBB</td>
<td>Malaria</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Parasite</td>
<td>Dendritic cells, Monocytes (132)</td>
<td>BBB, Placenta, Intestinal</td>
<td>Toxoplasmosis, congenital toxoplasmosis</td>
</tr>
<tr>
<td>HIV</td>
<td>Virus</td>
<td>Dendritic cells (133–135)</td>
<td>BBB, Placenta</td>
<td>AIDS, congenital infection</td>
</tr>
<tr>
<td><em>SARS-CoV-2</em></td>
<td>Virus</td>
<td>Monocytes and Macrophages (136)</td>
<td>BBB</td>
<td>COVID-19, meningitis</td>
</tr>
<tr>
<td>West Nile Virus</td>
<td>Virus</td>
<td>Neutrophil (137)</td>
<td>BBB</td>
<td>Encephalitis, meningitis</td>
</tr>
<tr>
<td>Zika Virus</td>
<td>Virus</td>
<td>Monocytes (138)</td>
<td>BBB, Placenta</td>
<td>Congenital syndrome (139–141)</td>
</tr>
</tbody>
</table>

*Leishmania* mainly utilize neutrophils to infect macrophages (their definitive host cells) rather than to breach any barriers
*L. monocytogenes* and *Brucella abortus* are both examples of bacteria that use phagocytes to further infection in a host (115). Both are gram-negative, intracellular foodborne pathogens that can breach the placenta and blood brain barrier (BBB). Interestingly, both species are associated with maternal infections and miscarriage (86, 116, 121). While both species utilize phagocytes to translocate host barriers, they are also capable of invasion without phagocytes present – indicating that the Trojan horse mechanism is not required for invasive infections.

The Trojan horse mechanism has been thoroughly investigated in fungi such as *Cryptococci neoformans* and *Candida albicans*. *C. albicans* is a normal commensal in the gastrointestinal tract and vagina, while *C. neoformans* is acquired by host inhalation of fungal spores (124). These fungi can survive inside a phagosome long enough to be transported into the bloodstream and through both the BBB and placental barriers (37, 142). Both species can exit phagocytes through non-lytic exocytosis and continue infection throughout the body (122, 125). However, these fungi employ multiple strategies to cause infection and are not dependent upon the Trojan horse method.

Protozoa that utilize the Trojan horse mechanism include the parasite *Toxoplasma gondii* and species from the genus *Leishmania*; infections by these parasites result in toxoplasmosis and leishmaniasis, respectively. Both species have animal reservoirs and typically do not have human-to-human transmission. Once inside human hosts, both groups can utilize host phagocytes to further infection. Although *Leishmania major* can use this method to breach the BBB, it predominantly utilizes neutrophils as Trojan horses in order to infect macrophages, which are the definitive host cells of *Leishmania spp.* (128). *Leishmania* is capable of delaying apoptosis in infected neutrophils, giving more
time for macrophages (whose major function is clearance of apoptotic cells) time to
ingest the infected neutrophil (128, 129). On the other hand, T. gondii is capable of
infecting almost any nucleated cell in the human body, including immune cells. The
parasitized phagocytic cells exhibit hypermigratory functions and are manipulated by T.
gondii to transport them from the gut mucosa to blood circulation (132).

The Trojan horse mechanism is most understood in human immunodeficiency virus
(HIV) pathogenesis. The main target of HIV is the host T helper cells (CD4); however,
they are also capable of infecting other immune cells (133). Dendritic cells are the first
immune cells to encounter HIV in the body. Upon phagocytosis by dendritic cells, HIV
can use them as a means of transportation to the lymph nodes, where it can infect a high
density of T helper cells quickly (134). The Trojan horse mechanism has also been
observed during Zika virus pathogenesis; once in the bloodstream, the virus can be
phagocytosed by host monocytes and transported through host barriers including the
blood-brain barrier and placenta (143). Zika-infected monocytes demonstrate higher
expression of adhesion molecules, which assist in attachment and transmigration through
host barriers (138). Additionally, this mechanism has recently been observed in the
pathogenesis of the SARS-CoV-2 virus, which was recently discovered to also use
phagocytes as Trojan horses during infection (136).

**Immune System Evasion**

There are several benefits of using phagocytes as Trojan horses. Once inside the cell, a
pathogen usually will not be detected and targeted by additional immune defenses such as
other lymphocytes, antibodies, or complement. Some pathogens are also capable of
replicating inside phagocytes, so they can establish a greater population without immune
system interference. They also get a free ride through the host’s circulatory system, spreading through the body at a much faster rate (37).

However, there are disadvantages to this mechanism. The Trojan horse method can only work if the pathogen is intracellular and can withstand the hostile environment inside the phagocyte. Professional phagocytes have evolved to target and destroy host invaders, and they employ multiple weapons (such as acidity and degradative enzymes) to accomplish this.

*L. monocytogenes* Trojan Horses

**General**

*L. monocytogenes* utilizes the Trojan horse mechanism during systemic infections. *Listeria*-infected leukocytes have been found in peripheral blood and ventricular system in patients with CNS infections (144–147). While it can infect the majority of blood cells, more than 90% of *Listeria*-infected leukocytes are monocytes – mononuclear phagocytes that are recruited to sites of infection or injury (145, 147).

Typical infection occurs after *Listeria* breaches the intestinal barrier and enters the bloodstream. There they are exposed to circulating monocytes, subsequently infecting these cells and then using the Trojan horse mechanism. In comparison to other intracellular bacteria, *L. monocytogenes* can survive within monocytes for a long time (148). Interestingly, while *Listeria* can replicate within host cells, very few replicate inside monocytes during intestinal infection (149).

Once inside the Trojan horse, *Listeria* is protected from external defenses of the host immune system as well as antibiotics. Although antibiotics like gentamicin are highly
bactericidal against *L. monocytogenes*, it poorly permeates host cells. Infected individuals treated with gentamicin have significantly lower counts of extracellular *Listeria*; however, cell-associated *L. monocytogenes* are still present and capable of causing CNS infection (150).

Additionally, *L. monocytogenes* has demonstrated the ability to modify host monocyte behaviors. Monocytes developing in the bone marrow during lethal and sublethal *Listeria* infections experienced alterations inhibiting their ability to produce reactive oxygen species (ROS) (146). Monocytes and macrophages produce ROS molecules as an antimicrobial defense during infection (151). Inhibition of this antimicrobial activity benefits the invading *Listeria*, characterizing these phagocytes as ideal Trojan horses rather than effective bactericidal cells.

The importance of monocytes in *Listeria* infection is demonstrated in studies using monocyte-deficient murine models. Those lacking monocytes were less susceptible to CNS infection than those with monocytes – despite these phagocytes being critical for host defense against pathogenic invasion (148).

*Breaching Host Barriers*

Monocytes circulating through the blood stream encounter host barriers such as the BBB and placental barrier. Infected phagocytes can adhere to endothelial cells, allowing *Listeria* to spread from cell-to-cell, effectively breaching the host barrier (144, 150). Once inside the cells comprising these barriers, *L. monocytogenes* can disseminate into the surrounding tissues. Additionally, these infected monocytes can facilitate cell-to-cell spread of *Listeria* to neurons more easily than direct invasion by extracellular *Listeria* (144).
During pregnancy, maternal monocytes are recruited to the uterus and placenta (152). *L. monocytogenes* has demonstrated the ability to infect placental tissues using maternal leukocytes as Trojan horses (121). Future studies are needed to investigate the relationship between these Trojan horses and the placental barrier.
Table 2. Pathogens capable of breaching the placental barrier and causing congenital infection.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Mode of Infection</th>
<th>Trojan Horse</th>
<th>Fetal Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Bacteria</td>
<td>Foodborne</td>
<td>Y</td>
<td>Granulomatosis infantiseptica, death, preterm delivery, neonatal sepsis (153)</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>Bacteria</td>
<td>Foodborne, zoonotic</td>
<td>Y</td>
<td>Miscarriage, congenital brucellosis, growth and developmental delay (116)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Bacteria</td>
<td>Commensal</td>
<td>N</td>
<td>Neonatal sepsis, bullous pustules, skin lesions (154)</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>Bacteria</td>
<td>Commensal</td>
<td>N</td>
<td>Neonatal sepsis (153)</td>
</tr>
<tr>
<td><em>Treponema pallidum</em></td>
<td>Bacteria</td>
<td>Sexual</td>
<td>Y</td>
<td>Death, rhinitis, chorioretinitis, anemia, periostitis (153)</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>Protozoa</td>
<td>Zoonotic, Fecal-Oral</td>
<td>Y</td>
<td>Microcephaly, jaundice, cerebral calcifications, chorioretinitis (153, 155)</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>Protozoa</td>
<td>Vector</td>
<td>N</td>
<td>IUGR, preterm delivery, severe hypoglycaemia (153)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>Protozoa</td>
<td>Vector</td>
<td>N</td>
<td>IUGR, respiratory failure, meningitis, heart failure, heart disease, hydrops (153)</td>
</tr>
<tr>
<td>Zika Virus</td>
<td>Virus</td>
<td>Vector</td>
<td>Y</td>
<td>Microcephaly, death, IUGR (140)</td>
</tr>
<tr>
<td>CMV</td>
<td>Virus</td>
<td>Fecal-Oral</td>
<td>N</td>
<td>Chorioretinitis, death, anemia, calcifications, developmental delay (153, 155)</td>
</tr>
<tr>
<td>HSV</td>
<td>Virus</td>
<td>Sexual, oral</td>
<td>N</td>
<td>Neonatal meningitis, dermatological lesions, microcephaly, chorioretinitis (153, 155)</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>Virus</td>
<td>Respiratory</td>
<td>N</td>
<td>Death, anemia, hydrops (153, 155)</td>
</tr>
<tr>
<td>Rubella Virus</td>
<td>Virus</td>
<td>Respiratory</td>
<td>N</td>
<td>Conjunctivitis, deafness, rhinitis, heart defects, developmental delay (153, 155)</td>
</tr>
<tr>
<td>West Nile Virus</td>
<td>Virus</td>
<td>Vector</td>
<td>N</td>
<td>Miscarriage, microcephaly (156)</td>
</tr>
</tbody>
</table>

IUGR = Intrauterine growth restriction
Further Investigation

The use of Trojan horses by *L. monocytogenes* has been extensively studied at the blood-brain barrier. However, little is known about the exact nature of *Listeria* invasion of the placenta using these monocytes (121, 149). The placenta itself is partially responsible for this lack of data, as it is difficult to study because it is a temporary organ that changes rapidly during its time in the uterus. The placenta is also very species-specific, meaning that there are several differences between the placentas of different mammalian species. Differences include shape, structure, and location of the placenta within the uterus.

Infection studies of the placenta using *Listeria* have mostly used it in its extracellular state. Future studies and improved models are required to determine the full impact of Trojan horses during *Listeria* infection of the placental barrier.

Materials and Methods

Bacterial Growth Conditions

Three cultures of *Listeria* were used in this study: wild-type *L. innocua* (F4248), *L. monocytogenes* F4244 (serotype 4b, clinical isolate), and *L. monocytogenes* F4244 containing a plasmid coding for green fluorescent protein (GFP). The wild-type *L. innocua* and *L. monocytogenes* were cultivated in tryptic soy broth with 5% yeast extract. *L. monocytogenes* expressing GFP were cultivated in Luria broth (LB) supplemented with 2 µg/ml erythromycin. All cultures were grown for 14 – 18 hours on a shaker at 120 rpm at 37°C. Details of all bacterial strains and reagents used are listed in the Key Resources Table (Table 3).
Cell Line Cultivation

The epithelial placental origin BeWo cells were obtained from ATCC and were cultured and propagated in F-12K medium containing fetal bovine serum (FBS) to a final concentration of 10%.

The human-monocytic leukemic THP-1 cell lines were obtained from ATCC and cultivated and propagated in RPMI-1640 medium containing 2-mercaptoethanol to a final concentration of 0.05 mM and FBS to a final concentration of 10%.

The primary human placental vascular endothelial cells (HPVEC) were obtained from ScienCell and cultivated in F-12K media containing 10% fetal bovine serum (FBS), 1% endothelial cell growth supplement (ECGS), and 1% penicillin/streptomycin.

All cell lines were incubated at 37°C in a 5% CO₂ atmosphere.

Establishing a Co-culture Experimental Model

The co-culture model was established using transwell inserts with 4 µm pores. A working stock of fibronectin solution was prepared by combining 150 µL of 1mg/ml Bovine Plasma Fibronectin with 10 mL Dulbecco’s phosphate buffered saline (DPBS). The underside of the transwell inserts was coated with the fibronectin solution for at least 2 hours. HPVECs were seeded on the underside of the inserts at densities of approximately 2.8 x 10⁴ cells for 12-well inserts or 8.25 x 10³ cells for 24-well inserts. The HPVEC cells were given 1 hour to attach to the insert before they were inverted. BeWo cells were seeded on the inside of the inserts at seeding densities of approximately 2.8 x 10⁴ cells for 12-well inserts or 8.25 x 10³ cells for 24-well inserts. The co-culture model was incubated in F-12K media containing 10% FBS and 1% ECGS at 37°C with 5% CO₂.
Figure 5. The co-culture model of the human placental barrier. BeWo epithelial cells are seeded on the apical side of the transwell filter, while the HPVECs are seeded on the underside of the filter. The apical side of the 24-well transwell filter received 100 µL of cell culture media, while the well itself received 600 µL.

Preparation of Lm-infected THP-1 cells (Trojan horses)

THP-1s were stained using the Cytopainter Cell Tracking Staining Kit © with red fluorescence in accordance with the manufacturer’s protocol. The cells were then incubated with GFP L. monocytogenes at an MOI of 100 for 1 hour. After infection, the cells were treated with gentamicin solution for 30 minutes to kill any extracellular Listeria. Staining and infection were confirmed using the Zeiss confocal laser-scanning microscope.
**FITC-Dextran Permeability Assay**

**FITC-Dextran Standard Curve**

The FITC-dextran working solution was prepared using 1 mg/1 mL of non-metabolizable 4 kDa FITC-dextran powder (FD4) in F-12K media. A serial dilution was performed using the working solution and F-12K media with a dilution factor of 2. Fluorescence was recorded using a Tecan Genios microplate reader at an excitation of 485 nm and emission of 528 nm.

**FITC-Dextran Permeability Assay with Placental Barrier**

Three groups of transwell inserts were seeded with the following cells: HPVECS, BeWos, or both to create the co-culture model. A 1mg/mL FD4 working solution was prepared in F-12K media. The solution was added to the apical side of the transwell inserts containing the cell cultures and incubated at 37°C with 5% CO₂. At each time point (0, 0.25, 1, 2, 4 hours), 50 µL samples were taken from the basal media under the filter and added to a black 96-well plate. The basal media was replenished with 50 µL of fresh media. After all samples were retrieved, the fluorescence was measured using a Tecan Genios microplate reader at an excitation of 485 nm and an emission of 528 nm.

**Permeability Assay with Trojan Horses**

Three groups of transwell inserts were seeded with the following cells: HPVECS, BeWos, or both to create the co-culture model.

The FD4 working solution was prepared using 1 mg/1 mL FD4 in F-12K media containing 50 µg/ml gentamicin. Trojan horses were prepared using THP-1s and *L. monocytogenes* strain 4244 (serotype 4b) as described above. The Trojan horses were
combined with the FD4 working solution and added to the apical side of the inserts. Each transwell insert received 100 µL of the FD4 working solution containing approximately 1 x 10⁶ THP-1 cells/mL. Samples were taken at the same time points and read at an excitation of 485 nm and an emission of 528 nm.

**FITC-Dextran Concentration and Permeability Coefficient Calculations**

The concentration of FITC-dextran in the samples was determined using the equation derived from a FITC dextran standard curve. The permeability coefficient of each sample was determined using the following calculation:

\[
P_c = \frac{(V_r \times C_f)}{(C_i \times A \times t)}
\]

- \(P_c\) = Permeability coefficient
- \(V_r\) = Receiver volume (mL)
- \(C_f\) = Final receiver concentration (mg/mL)
- \(C_i\) = Initial receiver concentration (mg/mL)
- \(A\) = Membrane growth area (cm²)
- \(t\) = time (seconds)

**Translocation Assay**

**Treatments**

After cytopainter staining and *Listeria* infection of the THP-1 cells, they were resuspended in fresh F12 media. Any treatments without extracellular bacteria were suspended in F12 media with 50µg/ml of gentamicin. Additional treatments, such as extracellular *L. monocytogenes* and *L. innocua* were established and centrifuged at 13,000 rpm before the pellets were resuspended in fresh media.
Exposure

The co-culture cell models received treatments on the apical side of the transwell inserts. Those treated with THP-1 cells received ~1 x 10⁵ cells on the apical side of the insert. Any treatments that contained gentamicin also had gentamicin (50µg/ml) added to the basal media beneath the inserts. Those cells with extracellular bacteria were infected at an MOI of 100. Exposure lasted for 2 hours at 37°C.

Visualization and Quantification of Infection

Bacterial Enumeration

*Extracellular* Listeria

Extracellular bacteria were enumerated by serial dilutions of media collected from the basolateral chamber of transwell setup on TSA plates.

*Intracellular* Listeria

Basal media had been removed from the transwell setup and centrifuged at 800 rpm for 5 minutes. The supernatant was discarded, and the bacterial cells were washed with PBS and centrifuged again. After three washes, the cells were resuspended in a media containing 0.1% Triton-X for 5 minutes. The samples were then serially diluted before plating on TSA plates.

The plates were incubated at 37°C for approximately 48 hours. The number of colonies were counted following incubation. Colony-forming units were determined using dilutions that resulted in the growth of 30 – 300 colonies.
Microscopy for Enumeration of Trojan Horses

Samples of host cells were obtained from the basal media and used for viable cell counts. Each sample was combined with Trypan blue in a 1:1 ratio before counting using a hemocytometer.

Additional samples were obtained from basal media and observed using the Zeiss confocal laser-scanning microscope. Cells were observed at 63X/1.40 NA oil immersion objective equipped with 405nm/Argon/561nm lasers. The X-Z and Y-Z cross-sections were produced by orthogonal reconstructions from z-stack scanning at 0.25mm intervals using a 63X objective in 10 mm thick co-cultures.

Flow Cytometry

Samples of basal media were centrifuged at 800 rpm for 5 minutes before being washed with PBS. The pellets were resuspended in a 1:1 solution of PBS and 4% formaldehyde for 20 minutes at room temperature. The cells were centrifuged again and washed with PBS three times. The suspended cells were passed through a 70 µm filter into new eppendorf tubes before flow cytometry analysis by using a BD Accuri C6 Flow Cytometer. Fluidics were set to 30,000 events. Data was acquired with fluorescent detector channels FL1 (emission 533/30 nm) and FL2 (emission 585/40 nm).

LDH Cytotoxicity Assay

The LDH cytotoxicity assay was performed using the Cayman LDH Cytotoxicity Assay Kit ©. Reagents and assays were prepared in accordance with manufacturer guidelines.
Figure 6. **LDH cytotoxicity assay reaction.** When a cell membrane is damaged, LDH is released from the cytoplasm to the surrounding cell media. Samples of this media were treated with an LDH reaction solution including lactate, NAD+, and tetrazolium salt (INT). First, LDH will catalyze NAD+ reduction to NADH using the H+ from the lactate. The loss of the hydrogen ion oxidizes lactate into pyruvate. The NADH produced in the process is then used as a catalyst for the reduction of INT to formazan, a brightly colored substance that is absorbed strongly at 490 nm. The level of formazan produced is proportionate to the amount of LDH released by cells in the co-culture model.

Cells treated with 10 % Triton-X solution served as a maximum release and the supernatants from untreated cells served as spontaneous release. 100 µL of experimental supernatants served as experimental release samples.

A black 96-well plate containing 100 µL of each sample was incubated for 30 minutes at 37°C with gentle shaking. Absorbance was recorded at 495 nm by a BioTek Epoch 2 microplate spectrophotometer with BioTek Gen5 microplate data analysis software. The cytotoxicity for each treatment was determined using the following equation:

\[
\% \text{ cytotoxicity} = \frac{(\text{Experimental value} - \text{spontaneous release})}{(\text{Maximum release} - \text{spontaneous release})} \times 100
\]
ICAM-1 ELISA

Soluble intracellular adhesion molecule 1 (sICAM-1) levels were measured using the RayBio Human sICAM-1 ELISA kit ©. Reagents were prepared in accordance with manufacturer guidelines. Assay diluent B was used to dilute cell culture samples as instructed. Standards were made by performing a serial dilution with a positive control in the kit. 100 µL of experimental supernatant samples from translocation assays were added to individual wells of a 96-well coated with anti-human sICAM-1. After completion of the ELISA assay, absorbance was immediately read at 450 nm. The human sICAM-1 concentrations (mg/mL) of each sample were calculated using the equation produced from the standard curve.

Immunostaining of Cell-Cell Junctions

Cells were fixed on the transwell insert membranes using 4% formaldehyde. After a 20-minute incubation at room temperature, the inserts were washed with PBS three times for 5 minutes each. Inserts were incubated with a blocking buffer for 1 hour at room temperature.

All antibodies used are listed in the Key Resource Table (Table 3). Primary mouse antibodies for occludin were diluted at a ratio of 1:100 in antibody dilution buffer (ABDB). The secondary goat anti-mouse antibodies with 488 fluorescent markers were diluted at a ratio of 1:500 in the same ADBD. The blocking solution was replaced with a mix of both primary and secondary antibodies and incubated for 18 hours at 4°C. After incubation, the occludin antibody solution was removed, and the cells were washed with PBS five times for five minutes each.
Primary mouse antibodies for claudin-1 were diluted in ABDB at a ratio of 1:100. Goat anti-mouse antibodies with conjugated 555 fluorescent markers were diluted in the same ABDB at a ratio of 1:500. The co-culture model was incubated with the claudin-1 antibody solution for 18 hours at 4°C.

The cells were washed with PBS five more times. A sterile 1% DAPI stain solution was prepared using DAPI nucleic acid stain and PBS. The PBS was removed from the transwell inserts and replaced with DAPI solution. The cells were incubated for 10 minutes in the dark at room temperature.

DAPI was removed from the cells before the insert filters were removed using razor blades. The filters were placed on microscope slides and mounted in ProLong Antifade Gold. The inserts were covered with a coverslip and allowed to dry for at least one hour before confocal microscopy. Cells were observed using a 63X/1.40 NA oil immersion objective equipped with 405nm/Argon/561nm lasers. The X-Z and Y-Z cross-sections were produced by orthogonal reconstructions from z-stack scanning at 0.25mm intervals with 63X objective in 10 µm thick co-cultures.
Figure 7. Distribution of cell media and seeded cells after translocation assay. (A) Apical media was reserved and used to perform the LDH cytotoxicity assay and the human sICAM-1 ELISA. (B) Basal media was distributed for multiple tests including FITC-dextran permeability reading, bacterial enumeration plating, hemocytometer counts of translocated THP-1 cells, fluorescent microscopy of translocated cells, and flow cytometry. (C) The cell co-culture model was stained for the tight junction molecules occludin and claudin 1 and observed using the fluorescent microscope.

Quantification And Statistical Analysis

Experimental data were analyzed using GraphPad Prism (La Jolla, CA) software. Comparisons between more than two datasets were performed using a one-way ANOVA analysis of variance with Tukey’s multiple-comparison test were performed. All data are representative of at least 3 independent experiments. Unless otherwise indicated, data for all experiments are presented as the mean ± standard error of the mean (SEM).
Results

Establishment and permeability characterization of a human placental co-culture model using BeWo and HPVEC cell lines

A previous study by Aengenheister et. al. (2018) established and determined the efficiency of a co-culture model of the placental barrier using monolayers of trophoblast cells and human placental endothelial cells (157). The same model was employed for this study, seeding BeWos and HPVECs on the apical and basal side of transwell inserts, respectively. The co-culture model was monitored for five-days post-seeding (Figure 8 A).

To determine permeability of this barrier, a FITC-dextran permeability assay was performed using the individual cell lines and the co-culture model. The co-culture model consistently demonstrated the lowest permeability compared to the individual BeWo and HPVEC cell monolayers (Figure 8 B). After two hours of exposure, the co-culture model had a significantly lower level of permeance compared to the BeWo and HPVECs by themselves (Figure 8 C).

Additionally, fluorescent microscopy imaging of this model show confluent monolayers of both cell lines on the transwell insert filter. The tight junction immunostaining of this model also demonstrates typical tight junction formation for both occludin and claudin-1 (Figure 8 D).
Figure 8. Establishment of a cell co-culture model using BeWo and HPVEC cell lines.
(A) Phase-contrast microscopy of the co-culture model at 200X magnification. The co-culture model is imaged approximately 24, 48, and 120 hours after initial cell seeding. (B) The permeability coefficient ± SEM of the cell lines exposed to a 4 kDa FITC-dextran solution over a 2-hour period. (C) The permeability coefficient ± SEM of the cell lines exposed to a 4 kDa FITC-dextran solution at 2 hours. (D) Fluorescent microscopy
imaging of a co-culture model after tight junction immunostaining. Cell nuclei were stained with DAPI (blue), claudin-1 was tagged with fluorescent marker 555 (red), and occludin was tagged with fluorescent marker 488 (green). Separated fluorescent channels are shown below the merged image. Statistical significance is reported as follows: ns indicates P>0.05, * indicates P ≤ 0.05, ** indicates P ≤ 0.01, *** indicates P ≤ 0.001, and **** indicates P ≤ 0.0001.

Lm-infected THP-1 cells increase translocation and promote infection of the placental barrier model

Hemocytometer counts demonstrate that Lm-infected THP-1 cells translocated the barrier at a significantly higher rate than THP-1s and Li-infected THP-1s (Figure 9 A). Translocation of intracellular bacteria was determined through enumeration plating, in which exposure to Lm-infected THP-1s resulted in significantly higher levels of translocated Listeria (Figure 9 B). This corresponds with levels of LDH cytotoxicity reported for these treatments, as Lm-infected THP-1s also demonstrate significantly higher cytotoxicity than Li-infected THP-1s (Figure 9 C).

Exposure to THP-1 treatments resulted in greater production of soluble ICAM-1 than those without exposure to THP-1s. However, only THP-1s and Lm-infected THP-1s demonstrated a significant increase in sICAM-1 concentrations (Figure 9 D). Changes in overall barrier permeability were determined by simultaneous exposure to FITC-dextran and the respective treatments. Barrier permeability increased significantly after exposure to THP-1s and Lm-infected THP-1s (Figure 9 E). Upon exposure to Lm-infected THP-1s, barrier permeability coefficient increased by 2.4 x 10^{-5} \text{ cm}^2/\text{sec} more than barriers exposed to Li-infected THP-1s, a trend consistent with the extracellular treatments of Lm and Li (Figure 9 F).
Figure 9. The effects of Listeria infection of THP-1s on overall cell translocation and cytotoxicity after two hours of exposure. (A) Number of viable cells translocated per mL of basal chamber media. Samples were stained with trypan blue, and the number of living cells were counted using a hemocytometer. (B) The number of Listeria colony-forming units (CFU) per mL of basal chamber media. (C) The percentage of cytotoxicity calculated after performing an LDH cytotoxicity assay and recording absorbance levels at 490 nm. (D) Human sICAM-1 concentrations in apical media determined using a human sICAM-1 ELISA and recording results at 450 nm. (E) The permeability coefficient of the co-culture model barriers after exposure to treatments. Overall permeability is determined by comparing the levels of 4 kDa FITC-dextran translocation with each treatment. Basal media samples were read at an excitation of 485 nm and an emission of 528 nm for FITC. The control includes readings from both the 2-hour mark and the initial reading (0 hours). (F) The permeability coefficient of the co-culture model barriers after exposure to
extracellular *L. monocytogenes* and *L. innocua*. Basal media samples were read at an excitation of 485 nm and an emission of 528 nm for FITC. The control includes readings from both the 2-hour mark and the initial reading (0 hours). Statistical significance is reported as follows: ns indicates $P > 0.05$, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$, and **** indicates $P \leq 0.0001$.

The presence of extracellular Lm with THP-1s increases translocation of monocytes

Exposure to extracellular Lm in addition to infected and uninfected THP-1s demonstrated significantly higher translocation of THP-1s (Figure 10 A). Although uninfected THP-1s with EC Lm had higher numbers of translocated cells than THP-1s previously infected with Lm. Similarly, the THP-1s with extracellular Lm yielded higher translocation of both extracellular and intracellular Lm than the Lm-infected THP-1s with extracellular Lm (Figure 10 B). Likewise, THP-1s with extracellular Lm generated a higher percentage of cell cytotoxicity than Lm-infected THP-1s with extracellular Lm (Figure 10 C).

Exposure to THP-1s and Lm-infected THP-1s with extracellular Lm resulted in a significant increase of human sICAM production (Figure 10 D). Although THP-1s with extracellular Lm had a higher level of sICAM-1 than THP-1s with intracellular and extracellular Lm. While both THP-1 and Lm-infected THP-1 treatments with extracellular Lm resulted in increased sICAM-1 production, both yielded significantly less than the treatments of THP-1s and Lm-infected THP-1s without any extracellular bacteria.

Overall permeability of the co-culture model was increased after exposure to these treatments, however, only Lm-infected THP-1s with extracellular Lm caused significantly greater permeability than the control (Figure 10 E).
Figure 10. The effects of intracellular and extracellular Lm on THP-1 translocation of and adherence to the co-culture model. (A) The number of viable translocated THP-1s per mL of basal chamber media. The number of living cells were counted in trypan blue stained samples using a hemocytometer. (B) The number of *Listeria* colony-forming units (CFU) per mL of basal chamber media. The solid color bars represent CFU counts of
extracellular *Lm*, and the striped bars represent CFU counts of both extracellular and intracellular *Listeria* (taken after intracellular bacteria has been released from infected cells). (C) The percentage of cytotoxicity calculated after performing an LDH cytotoxicity assay and recording absorbance levels at 490 nm. (D) Human sICAM-1 concentrations in apical media determined using a human sICAM-1 ELISA and recording results at 450 nm. (E) The permeability coefficient of the co-culture model barriers after exposure to treatments. Overall permeability is determined by comparing the levels of 4 kDa FITC-dextran translocation with each treatment. Basal media samples were read at an excitation of 485 nm and an emission of 528 nm for FITC. The control includes readings from both the 2-hour mark and the initial reading (0 hours). Statistical significance is reported as follows: ns indicates *P* > 0.05, * indicates *P* ≤ 0.05, ** indicates *P* ≤ 0.01, *** indicates *P* ≤ 0.001, and **** indicates *P* ≤ 0.0001.

**Lm-infected THP-1s disrupt tight junctions and increase permeability of placental barrier model**

Tight junction immunostaining imaging revealed THP-1 interaction with claudin-1 tight junctions of the co-culture model (Figure 11). The relative size and characteristics of the nuclei present allow determination of monocyte and placental barrier cells.
Figure 11. Lm-infected THP-1 interaction with co-culture model tight junctions. (A) Fluorescent microscopy imaging of co-culture model occludin tight junctions after exposure to Lm-infected THP-1s. Cell nuclei were stained with DAPI (blue) and occludin was tagged with fluorescent marker 555 (red). Interaction between monocytes and occludin is indicated with a white arrow. Lm not shown. (B) Fluorescent microscopy imaging of co-culture model claudin-1 tight junctions after exposure to Lm-infected THP-1s. Cell nuclei were stained with DAPI (blue), claudin-1 was tagged with fluorescent marker 555 (red), and Lm had the GFP plasmid (green). An orange arrow indicates a Lm-infected THP-1.
While exposure to THP-1s alone did result in some alteration of tight junctions, most junctions remained intact akin to the typical co-culture model barriers (Figure 12 A-B). However, severely disrupted occludin and claudin tight junctions were observed in co-culture model barriers exposed to *Lm*-infected THP-1s (Figure 12 C). Despite being in cell media containing gentamicin, intracellular *Lm* from the infected THP-1s are seen infecting the cells of the co-culture model and directly interfering with tight junction proteins.

**Figure 12.** *Lm*-infected THP-1s disrupt cell junctions of cell co-culture model barrier. (A) Fluorescent microscopy imaging of a co-culture model that received no treatment (NT). (B) Fluorescent microscopy imaging of a co-culture model after exposure to THP-1s. A white arrow indicates altered tight junctions of both claudin-1 and occludin. (C) Fluorescent microscopy imaging of a co-culture model after exposure to *Lm*-infected THP-1s. *Lm* contain the GFP plasmid (green). White arrows indicate disrupted junctions. Yellow arrows indicate direct *Lm* interaction with tight junctions. Cell nuclei were stained with DAPI (blue), claudin-1 was tagged with fluorescent marker 555 (red), and occludin was tagged with fluorescent marker 488 (green). Separated fluorescent channels are shown below the merged images for clarity.
Flow cytometry quantitative measure of host cell Lm infection and translocation through co-culture model barrier

Flow cytometry analysis of basal media samples reveal that the presence of extracellular Lm enhances translocation of the co-culture model. The number of translocated THP-1s is similar for both the THP-1 treatment and Lm-infected THP-1s, although higher counts were recorded for both treatments with extracellular Lm (Figure 13 B). Of the THP-1s that did breach the barrier, only 11% of Lm-infected THP-1s still had intracellular Listeria, whereas 20.8% of translocated THP-1s from the Lm-infected THP-1s with extracellular Lm contained intracellular Listeria. Interestingly, the uninfected THP-1s with extracellular Lm had the highest percentage of infected THP-1s translocate the co-culture model at 35.1% (Figure 13 C).

A separate population of infected cells were detected during flow cytometry. This population became prominent after treatment with extracellular Lm. Of this population, most cells demonstrated infection with Listeria, with 89.2% and 95.3% for Lm-infected THP-1s and THP-1s with extracellular Lm, respectively (Figure 13 D). Of this population, virtually none of the cells expressed detectable fluorescence using the red channel, indicating that none of the cells in this population were the stained THP-1s (Figure 13 E).
Figure 13. Flow cytometry analysis of cell translocation through co-culture model. (A) Fluorescent microscopy of stained THP-1 cells (red) and L. monocytogenes (green). (B)
Flow cytometry detection of cell populations after translocation with different treatments using forward scatter (FSC-A) and side scatter (SSC-A) signals. (C) Number of infected cells in the THP-1 population determined by the presence of green fluorescence signal from Lm. (D) Number of infected cells in the placental cell population determined by the presence of green fluorescence signal from Lm. (E) Number of THP-1s in the infected cell population determined by the presence of red fluorescent signal from the THP-1s after staining with cytopainter red. Percentages are listed in the upper corners; the left side of the plots indicate uninfected cells, whereas the right indicates cells with GFP Lm (C – E). Orange coloring indicates the THP-1 population, blue coloring indicates placental cell populations.

**Discussion**

*The cell co-culture model is an effective in vitro model system for the human placental barrier*

The efficacy of this model was verified using translocation assays of 4 kDa FITC-dextran permeants and fluorescent microscopy imaging of the tight junction formations. Both permeability and tight junction formation of the barrier were comparable to those of the model established by Aengenheister et. al. (157). Although the aforementioned study tested membrane permeance with 40 kDa FITC-dextran while this study utilized 4 kDa FITC-dextran, the overall permeance trends of the cell barriers remain consistent. The co-culture model demonstrated significantly less permeability than the BeWo and HPVEC cell monolayers, even after two hours of exposure (Figure 8 C). This was to be expected, as the co-culture barrier consisted of two cell monolayers (therefore approximately twice as many cells). Tight junction immunostaining results also agree with the Aengenheister et. al. (2018) study as well as normal occludin and claudin-1 tight junction formation in the human placental barrier (157–159).
*Lm*-infected THP-1s translocate the placental barrier at higher levels than THP-1s.

Unlike *Li*-infected THP-1s, exposure to *Lm*-infected THP-1s resulted in a significant increase of co-culture model permeability (Figure 9 E). This is consistent with barrier permeability after exposure to extracellular *L. monocytogenes* and *L. innocua*, in which treatment with *Lm* resulted in a significantly higher barrier permeability than *Li*.

Similarly, bacterial enumeration revealed greater levels of intracellular *Lm* breaching the cell barrier than intracellular *L. innocua* (Figure 9 F). This was expected, as *L. innocua* does not contain the *Listeria* pathogenicity islands encoding for virulence factors like LLO or ActA, which are critical for intracellular survival (21, 23). Thus, nearly all treatments resulted in increased permeability of the co-culture model barrier, indicating that the increase in membrane permeability is not specific to THP-1s.

*Lm*-infected THP-1s yielded the highest level of cytotoxicity compared to the other treatments. This is consistent with previous literature, where *L. monocytogenes* generated significantly higher cytotoxic effects than *L. innocua*. Another study investigating the cytotoxic effects of *Listeria* spp. tested the same strains used in this study (*L. monocytogenes* F4244 (4b) and *L. innocua* F4248), and observed high levels of LDH cytotoxicity for *Lm* and no cytotoxicity for *Li* (160). This is consistent with our findings, as *L. innocua* caused no detectable LDH cytotoxicity after 2 hours of exposure to the co-culture model (Figure 9 C). These results are consistent with previous literature, indicating that this is a pathogen-specific mechanism for *L. monocytogenes*. 
Placental barrier exposure to THP-1s correlates with increases in sICAM-1 expression

ICAM-1 is a cell-surface protein receptor known to regulate leukocyte recruitment and adhesion to endothelial cells during inflammation (161). Normally expressed at low levels, this glycoprotein is upregulated in response to inflammatory stimuli. The main function of ICAM-1 has been identified as mediating transendothelial migration of leukocytes, particularly in the mediation of rolling adhesion. Rolling adhesion occurs on endothelial cell surfaces, and is considered the first step in the recruitment of circulating leukocytes to organs or sites of infection (162). ICAM-1 binds to lymphocyte function-associated antigen-1 (LFA-1), an integrin present on the surface of all leukocytes (163).

ICAM-1 can be released from endothelial cell membranes via proteolytic splicing to create a soluble form known as sICAM-1 (161, 164, 165). There are several proteases that have demonstrated the ability to mediate cleavage of ICAM-1, including elastases, cathespins, and bacteria-derived enzymes (161, 166, 167). Despite cleavage, sICAM-1 is still functional and capable of binding to LFA-1 on leukocytes. After binding LFA-1, the sICAM-1 prevents attachment of the lymphocyte to the endothelium, acting as an inhibitor for transendothelial migration (168). Increased levels of soluble ICAMs result in decreased levels of leukocyte accumulation and transmigration at endothelial barriers (169, 170). Increased levels of sICAM-1 have been observed in patients with increased inflammation, sepsis, and shock (161, 168, 171). Previous studies have demonstrated a strong correlation between levels of sICAM-1 and the severity of shock or sepsis in a patient (171).
As expected, there is also a positive trend between expression of both soluble and membrane-bound forms of ICAM (169, 171). Therefore, sICAM-1 can be used as a biomarker that reflects the levels of ICAM-1 expression on endothelial cell surfaces.

With this knowledge, we can use the human sICAM-1 concentrations derived from the sICAM-1 ELISA to make inferences about ICAM-1 expression on the cell co-culture model. Upon the addition of THP-1s to the model, there was a significant increase in sICAM-1 concentration. Lm-infected THP-1s generated similar levels of sICAM-1. However, Li-infected THP-1s induced lower concentrations of sICAM, which were not significant compared to the control (Figure 9 D). In contrast, while the presence of extracellular L. monocytogenes did cause a slight increase in sICAM-1 concentration, the difference was not significant compared to the control. Interestingly, THP-1 treatments with extracellular Lm demonstrated lower levels of sICAM-1 compared to those without the extracellular bacteria. While both treatments of THP-1s with EC Lm demonstrated significantly higher levels of sICAM-1 than the control, the treatment with uninfected THP-1s showed significantly more sICAM-1 than the treatment with Lm-infected THP-1s (Figure 10 D).

These results suggest that the presence of THP-1s lead to an increase in expression of ICAM-1, not Listeria. This conflicts with previous literature, which described an increase in ICAM-1 expression in the presence of Lm-infected hepatic cells (172). Upregulated expression of ICAM-1 resulted in the migration and accumulation of monocytes in areas with high levels of Listeria infection.
While the expression of membrane-bound ICAM-1 would be helpful for promoting infection of the barrier by infected monocytes, increased expression of soluble ICAM-1 would not. A recent study investigating sICAM-1 levels during *Streptococcus pneumoniae* infection demonstrated that more virulent strains of *S. pneumoniae* induced lower levels of sICAM-1 than other strains (173). The researchers of this study suggested that lower concentrations of sICAM-1 were important for the facilitation of BBB invasion by the pathogen. Since *S. pneumoniae* is an extracellular pathogen, it combats lymphocytes like THP-1 monocytes/macrophages during infection. Decreased levels of sICAM-1 would lead to less inhibition of monocyte LFA-1 binding to the brain endothelial cells. As a result, the lymphocytes would migrate to the endothelium while *S. pneumoniae* could move more freely through the bloodstream to reach the BBB and facilitate infection.

In addition, a new mechanism has been proposed for placenta-specific regulation of ICAM-1 expression on the placental endothelial cells. It is speculated that the placenta implements post-transcriptional regulation of ICAM expression to prevent overflowing leukocyte migration and attachment at the placental barrier (174). This mechanism should be investigated *in-vivo* to determine if the placenta employs this method to combat infection by intracellular bacteria.

**Lm-infected THP-1s disrupt tight junctions to promote infection of the placental barrier**

Barriers exposed to uninfected THP-1s did demonstrate some alteration of tight junctions, although most remained intact. This is consistent with previous literature on the
interaction between leukocytes and tight junction proteins during transendothelial migration (175). Other studies have reported that monocytes exclusively use paracellular transmigration through a barrier of human umbilical vascular endothelial cells (176).

Tight junction immunostaining images from this study support the prior findings that leukocytes such as THP-1 cells do not degrade or disrupt overall formation of the tight junction structures. As a matter of fact, most transmigration of leukocytes occurs at tricellular corners (the point where the borders of three cells intersect), where tight junctions are inherently discontinuous (176, 177). Preferential migration at these tricellular corners allows the leukocyte to migrate through the barrier while preserving the integrity of the placental barrier. The discontinuity of both occludin and tight junction barriers observed with the THP-1 treatment appears to occur at a tricellular corner (indicated with a white arrow), supporting the hypothesis that leukocytes tend to migrate through host barriers at these locations (Figure 11 B).

Compared to the typical tight junction formation observed in the co-culture model, those exposed to Lm-infected THP-1s demonstrated decreased fluorescent intensity and spoiling of both occludin and claudin-1 tight junctions (Figure 12 C). Tight junction formation is critical for barrier formation and function – damaged or decreased expression of these proteins can lead to increased permeability of the membrane (178). Previous studies have demonstrated that L. monocytogenes infection can result in decreased expression of tight junction proteins occludin and claudin-5, which subsequently lead to increased permeability of the BBB (179).
The relative size and characteristics of the nuclei observed with fluorescent microscopy allow us to infer that there were monocytes adhering to the placental barrier cells and interacting with the tight junctions. THP-1 monocytes have an average cell diameter of approximately 10 –15 µm, BeWo 20 – 30 µm, and endothelial cells such as HPVECs average 10 – 30 µm wide and 50 – 70 µm long (180–183). The nuclear size difference between cell types corresponds with these reports and is consistent with the cell lines viewed with phase contrast microscopy (Figure 14). Therefore, it can be inferred that the smaller, brighter nuclei belong to THP-1s and the larger, grainier nuclei belong to the placental cells. With this knowledge, we can observe THP-1 cells adhering to the placental cell barrier and interacting with the occludin tight junctions (Figure 11).

Despite being suspended in cell media containing gentamicin, intracellular Lm was able to infect placental cells – suggesting that THP-1s containing Lm directly transferred the pathogen to the placental barrier cells via lateral transfer. Since monocytes adhere to host barriers like the BBB, they facilitate direct cell-to-cell transfer of Lm to endothelial cells, promoting infection (144, 150). This has also been observed during infection with Cryptococcus neoformans, which can also utilize monocyte Trojan horses to employ lateral transfer of the pathogen to the target cells (184).

The presence of extracellular Lm supports infection of the co-culture model

While THP-1s containing intracellular Lm exhibit the ability to translocate and disrupt the co-culture model barrier, the effects of these infected monocytes are increased when coupled with extracellular Lm. The addition of extracellular Listeria increased the number of cells migrating through the barrier (Figure 10). This data is consistent with the
flow cytometry findings, as the presence of extracellular *Lm* resulted in higher cell counts translocating the barrier. Subsequently, it also increased the number of colony-forming units to breach the barrier, which is also reflected in the flow data.

Interestingly, uninfected THP-1s and extracellular *Lm* demonstrated the greatest capacity for translocation, not *Lm*-infected THP-1s with extracellular *Lm*. This defied expectations, as it was understood that *Lm*-infected THP-1s with extracellular *Lm* would have more bacteria present in total (including intracellular) and thus demonstrating greater infection. This trend is consistent throughout all experiments performed in this study: that is, THP-1s that had not been infected prior to translocation demonstrated higher levels of translocation, cytotoxicity, sICAM-1 expression, and barrier permeability with extracellular *Lm* than THP-1s that had been exposed to *Lm* previously (Figure 10 D). These findings are supported by the flow cytometry results, as exposure to THP-1s with extracellular *Lm* yielded the highest level of THP-1 translocation as well as the highest percentage of infected cells (Figure 13 B-D).

Ironically, the treatment with uninfected THP-1s had a higher percentage of THP-1s infected with *Lm* translocate than the treatments with previously infected THP-1s. Although those THP-1s had less exposure to the bacteria, they still had enough time to be infected during the translocation assay. There are two possible explanations for this phenomenon: firstly, the infected THP-1 could be lysed or undergo cell-death after infection (Figure 14). This would explain why there are less infected THP-1s in treatments that had been infected prior to the translocation assay – there was a greater duration of infection time, leading to higher accumulation of lysed or dead THP-1s. *L.*
*monocytogenes* has been observed causing cell death in mononuclear lymphocytes after conducting intracellular replication for multiple hours (185).

Secondly, the infected THP-1s could be adhering to the co-culture barrier and subsequently allowing the intracellular bacteria to infect the placental cells via cell-to-cell spread (Figure 14). Previous studies have observed *Lm*-infected phagocytes transferring the pathogen to the blood-brain barrier in a similar fashion (186). The significance of *Listeria* cell-to-cell spread for placental infection is a source of debate. While researchers agree that cell-to-cell spread facilitates crossing of the placental barrier, there is a discrepancy between reports as to whether this method is required for placental translocation (88, 109, 187). An increase in sICAM-1 expression for these treatments suggests that there would be an increase in monocyte adhesion to the placental barrier, supporting the possibility. A similar study investigated Nipah virus infection and observed an increase in transendothelial migration and cell-to-cell spread of the pathogen from infected lymphocytes (188). Researchers observed THP-1s infected with the Nipah virus facilitated the spread of the virus to BBB endothelial cells. Ultimately, additional studies should be conducted to investigate the synergistic effects of extracellular and intracellular *L. monocytogenes* at host barriers.
Future Directions

Flow cytometry can be used to further investigate the infection and translocation of monocytes and the placental barrier cells. Samples can be acquired from the basal media after a translocation assay can be treated with cell-specific antibody markers. For example, an anti-human CD14 antibody would recognize the CD14 antigen present on the surface of human monocytes such as THP-1s. These antibodies can be conjugated with fluorescent markers that can be detected by the flow cytometer. Additional antibodies with different fluorescent markers could be selected to bind cell-specific surface molecules on BeWos and HPVECs, allowing for more definitive characterization of the migrating cells using flow cytometry.
Additional tight-junction immunostaining should also be performed to visualize junction disruption after exposure to different treatments, such as \textit{Lm}-infected THP-1s with extracellular \textit{L. monocytogenes}. Other junctions that could be stained such as E-cadherin, zonula occludens, and desmoglein. Observation of \textit{Lm}-infected THP-1 interaction with these barrier junctions could provide a more comprehensive understanding of how \textit{L. monocytogenes} uses the Trojan horse mechanism to bypass host barriers.

Fluorescent microscopy could also be used to visualize the THP-1s and \textit{Listeria} that breach the co-culture model barrier. Cytocentrifugation of samples acquired from basal media after a translocation assay to concentrate the cells and apply a thin monolayer onto a microscope slide. The precision of this technique would allow additional visual observation of translocation of both THP-1s and \textit{L. monocytogenes}.

ICAM-1 expression on placental barrier cells should be compared to sICAM-1 expression after exposure to \textit{Lm}-infected THP-1s. While sICAM-1 production can be quantified using an ELISA assay (as performed in this study), membrane-bound ICAM-1 can be analyzed using a Western blot. Previous studies have demonstrated expression of ICAM and VCAM on HPVECs using this method (189). Comparison between levels of sICAM-1 and ICAM-1 would provide clarity on how \textit{Lm}-infected monocytes could alter placental cell expression of adhesion molecules.

While the placenta acts as a barrier protecting the fetus, it also has an important endocrine function during pregnancy. Exposure of \textit{Lm} treatments to the co-culture model alongside normal levels of placental hormones could provide a more accurate portrayal of how the Trojan horse method works \textit{in vivo}. Treatments like \textit{Lm}-infected THP-1s could be
coupled with normal levels of placenta-derived hormones like β-estradiol or human chorionic gonadotropin (HCG) during a translocation assay (60, 190).

Future studies should also include *in vivo* models. The human placenta is difficult to study, as there are no model organisms that have identical placentas to humans. However, *in vitro* models alone are insufficient in determining how infection would naturally occur in a host. Thus, animal models with placental structure similar to humans would be ideal. Rodent models such as guinea pigs or primate models like the common marmoset or macaque would be ideal candidates, as they have placentas that are discoid and monochorial (88, 191–193). In this case, both the placenta co-culture model and animal models should be used to establish a complete picture of how *L. monocytogenes* utilizes the Trojan horse mechanism to breach the placental barrier.
Appendices
Appendix A: Supplementary Table
### Appendix A: Supplementary Table

**Table 3. Key resources used over the duration of this study.**

<table>
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<tr>
<th>Reagent or Resource</th>
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<tr>
<td><strong>Antibodies</strong></td>
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<tr>
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<td>Santa Cruz Biotechnology</td>
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<td>Mouse monoclonal anti-occludin</td>
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<td><strong>Bacterial Strains</strong></td>
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<td><em>L. monocytogenes</em> F4244 (WT)</td>
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<td><em>L. monocytogenes</em> with GFP</td>
<td>Drolia et al. 2020 (194)</td>
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<td><em>L. innocua</em> F4248</td>
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<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
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<td>Gibco</td>
<td>11875-093</td>
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<tr>
<td>Fetal Bovine Serum</td>
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<td>Bovine Plasma Fibronectin</td>
<td>Science Cell</td>
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<td>Endothelial Cell Growth Supplement</td>
<td>ScienCell</td>
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<td>Penicillin-Streptomycin Solution</td>
<td>Cytiva</td>
<td>SV30010</td>
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<td>S-002-D</td>
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<td>Trypsin</td>
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<td>Dulbecco’s Phosphate Buffered Saline</td>
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<td>Glycerol</td>
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<td>Tryptic Soy Broth Medium</td>
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Table 3 continued

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<td>Dimethyl Sulfide</td>
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Critical Commercial Assays

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<td>LDH Cytotoxicity Assay</td>
<td>Cayman Chemical</td>
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<td>Human sICAM-1 ELISA Kit</td>
<td>Ray Biotech</td>
<td>ELH-ICAM1</td>
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Cell Lines

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</tr>
<tr>
<td>Human Placental Vascular Endothelial Cells</td>
<td>ScienCell</td>
<td>7100</td>
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<td>THP-1s</td>
<td>ATCC</td>
<td>TIB-202</td>
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</tbody>
</table>

Software

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<th>Website</th>
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<td>GraphPad Prism 10</td>
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<td>XFluor4 Software v.4.3</td>
<td>Tecan Systems</td>
<td><a href="http://www.lifesciences.tec">www.lifesciences.tec</a> an.com/software-overview</td>
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</table>
Appendix B: Supplementary Figures
Appendix B: Supplementary Figures

Phase-Contrast Microscopy of Cell Lines

Figure 15. Phase-contrast microscopy images of confluent cell lines at 200X magnification. THP-1s (A), HPVECs (B) and BeWos (C) were observed using a Nikon Eclipse TS 100.

FITC-Dextran Standard Curve

Figure 16. Standard curve of FITC-dextran concentration to fluorescent intensity at an excitation of 485 nm and an emission of 528 nm. The equation for the line of best fit and the R-squared value were calculated using graphpad prism software.
sICAM-1 ELISA Standard Curve

![Standard Curve of human sICAM concentration and absorbance readings at 450 nm.](image)

Figure 17. Standard curve of human sICAM concentration and absorbance readings at 450 nm. Absorbance was determined after performing a sandwich ELISA for sICAM-1. The line of best fit equation and R-squared value were both calculated using Graphpad Prism software.

Flow Cytometry Results

![Flow Cytometry Results](image)

Figure 18. Flow cytometry infected cell counts after cell lines were exposed to GFP Lm (MOI 500) for two hours. The supernatant from both cell cultures were fixed using 4% formaldehyde before flow cytometry. The cell monolayers were treated with trypsin to detach the cells before fixing with 4% formaldehyde and filtration using a 70 µm cell strainer.
Bacterial Enumeration Results for Extracellular *Listeria*

*Figure 19. Bacterial enumeration counts after translocation assay for treatments of extracellular L. monocytogenes and L. innocua.*
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