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Listeria monocytogenes Infection Reduces the Functionality of Human Choriocarcinoma JEG-3 Cells

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Abstract

Listeria monocytogenes is a facultative intracellular bacterium associated with foodborne disease outbreaks. Pregnant women, and their fetuses, are at particular risk for adverse outcomes to pregnancy. The mechanisms by which *L. monocytogenes* infection disrupts homeostasis in the placenta are incompletely understood. In this study, we tested the ability of *L. monocytogenes* to invade, multiply within, and alter the function of JEG-3 cell monolayers. The effects of *L. monocytogenes* infection on JEG-3 cell monolayer integrity, cell function, cell metabolic activity, and cell death were measured with Transepithelial Electrical Resistance (TEER), Alamar Blue reduction, and LDH release, respectively. *L. monocytogenes* readily infected JEG-3 cells and multiplied to a peak number of intracellular organisms at 10 hours post-infection. *L. monocytogenes* infection decreased TEER of the JEG-3 monolayer as compared to uninfected JEG-3 cell monolayers. Infected JEG-3 cells also displayed decreased invasion through a fibronectin layer. Finally, *L. monocytogenes* infection decreased JEG-3 cell metabolic activity and caused cell death as measured by LDH release. These findings suggest that *L. monocytogenes* infection of extravillous trophoblast cells may compromise trophoblast functions required by the fetoplacental unit for maternal vascular remodeling and pregnancy success, rather than the trophoblast cells simply serving as a point of entry to infect the fetus.

Keywords: *Listeria monocytogenes*; JEG-3; Placenta; Extravillous trophoblast

Introduction

Listeria monocytogenes is a significant food borne pathogen of pregnant women that causes still birth and spontaneous abortion in 20% of cases and 68% of surviving pregnancies result in neonatal infection [1,2]. Increased risk of infection during pregnancy is thought to be due, in part, to alterations in the maternal immune system to facilitate immune tolerance and prevent rejection of the fetal allograft. However, other mechanisms may also be at play [3-6].

Listeria monocytogenes targets the maternal-fetal interface, particularly placental tissues. There are several possible explanations for how *L. monocytogenes* crosses the maternal-fetal barrier. Specific cell types that are targets for listerial invasion into the fetal placenta include syncytiotrophoblast and extravillous trophoblast (EVT) cells [7-9]. Listerial cell entry into mammalian host cells is mediated by interactions between the bacterial cell wall proteins internalin A and internalin B, and E-cadherin and c-Met-tyrosine kinase, respectively, in non-phagocytic mammalian cells [10,11]. These interactions have played key roles in fetoplacental listeriosis, specifically as a mechanism for *L. monocytogenes* to target and cross the placental barrier [12].

Listerial invasion of syncytiotrophoblast and EVT cells could have serious consequences for fetal health and pregnancy success. It has been shown by others that *L. monocytogenes* can infect primary cultures of EVT cells [8,9]. Although these studies identified trophoblast cells as potential sites of bacterial entry into the fetus, they did not examine whether *L. monocytogenes* infection alters the function of these cells, and how this may relate to the development of adverse pregnancy outcomes.

In this study, we used JEG-3 cells as a model for human EVT cells. Although derived from a choriocarcinoma, JEG-3 cells have similar gene expression and present many of the same biological and biochemical characteristics as normal EVT cells [13,14] and have been shown previously to be infected by and sustain intracellular growth of *L. monocytogenes* [7,15]. We tested the hypothesis that *L. monocytogenes* infection of JEG-3 cells decreases cell viability and invasion ability. We demonstrate that JEG-3 cells infected with *L. monocytogenes* display decreased monolayer integrity and metabolic activity *in vitro* and discuss how these changes may influence pregnancy outcome during listeriosis.

Materials and Methods

Preparation of *L. monocytogenes*

L. monocytogenes strain 2203 (serotype 4b) was generously donated by Dr. Sophia Kathariou (Raleigh, NC) [16]. *L. monocytogenes* cells were stored at -20°C on Cryoban Cryobeads (Copan Diagnostics, Inc., Corana, CA). For each experiment, a bead was placed into 5 ml of Brain Heart Infusion (BHI) broth and incubated overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation (3,500×g for 5 minutes) and washed three times in phosphate buffered saline. The bacterial suspensions were diluted to the desired concentration, and numbers of viable *L. monocytogenes* confirmed by plating serial

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dilutions onto tryptic soy agar with 5% sheep blood (BD Biosciences, Franklin Lakes, NJ).

Chemicals and media

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), and trypsin were purchased from Cellgro (Kansas City, MO). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Lauranceville, GA). NP-40 detergent was purchased from USB Corporation (Cleveland, OH). Brain heart infusion (BHI) broth was purchased from Difco (BD Biosciences). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

JEG-3 cells

The human choriocarcinoma-derived JEG-3 cells used in these experiments were obtained from the American Type Culture Collection (Rockville, MD) [17,18]. The cells were cultured in DMEM supplemented with 10% FBS and passaged by brief enzymatic digestion using 0.1% trypsin-EDTA.

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

For invasion and growth experiments, JEG-3 cells were cultured in 24-well plates for 48 hours. The monolayers were incubated with *L. monocytogenes* strain 2203 at a multiplicity of infection (MOI) of 5 for 1 hour. The 24-well plates were then washed extensively with warm HBSS. Some wells were then lysed with 0.1% Triton X-100 for 5 minutes and the lysates serially diluted in phosphate buffered saline and plated to determine the initial invasion by *L. monocytogenes*. The remaining wells received DMEM medium supplemented with 10% FBS and 200 μg/ml gentamicin. At selected time points, medium was removed from wells and the monolayers lysed to estimate numbers of intracellular *L. monocytogenes* as indicated above. The medium was changed daily throughout the experiments.

TEER measurements

JEG-3 cells were seeded onto translucent polyethylene terephthalate (PET) cell culture inserts (3.0 μm average pore size; BD Falcon, San Jose, CA) and incubated at 37°C with daily medium changes (1 ml of medium on the bottom side of the insert and 700 μl on the top side of the insert) for 3 days in DMEM with 10% FBS. Transepithelial electrical resistance was measured using a voltmeter and End-Ohm six-chamber cup electrodes (World Precision Instruments, Sarasota, FL). Initial TEER values for each monolayer were determined prior to the start of each experiment. Subsequent recordings obtained during the course of the experiment were normalized to this initial reading. Only monolayers with initial readings ≥ 80 Ω were used in experiments based on previous published work from our laboratory [19]. JEG-3 cells were originally seeded at 1×10^5 cells per well and incubated until monolayer formation noted by visualization with an indirect light microscope. The cell monolayer was then treated with *L. monocytogenes* (MOI of 5) for 1 hour, washed extensively with HBSS, and then incubated with DMEM supplemented with 10% FBS and 200 μm/ml gentamicin. Resistance measurements were taken at 4,8,12,24,48 and 72 hours post infection and recorded manually.

JEG-3 invasion assay

Cell invasion was performed using PET cell culture inserts (8.0 μm average pore size; BD Falcon, San Jose, CA) pre-coated with fibronectin (BD Biosciences). This was done by adding 200 μl of 20 μg/ml fibronectin solution to the top of the filter, followed by incubation for 1 hour at 37°C. After incubation, all excess liquid was removed.

Cell suspensions (1×10^5 cells/ml in 400 μl) were seeded on top of the filters and the inserts placed into a 24 well plate, whose wells contained 600 μl of medium. Cells were incubated for 4 hours at 37°C and then incubated for 1 hour with *L. monocytogenes* (MOI of 5) added to the top of the filter cup. Monolayers were washed extensively with HBSS, then fresh DMEM supplemented with 10% FBS and 200 ug/ml gentamicin was added. Control cell cultures were not exposed to *L. monocytogenes*. JEG-3 cells were incubated at 37°C for 24 hours to allow invasion though the fibronectin layer and filter pores to the bottom side of the filter. The fluid on the top of the filter was removed and the cells on the upper surface of the filter removed with a sterile cotton swab. Alamar Blue dye (AB) was added to the medium (1:10) and the plate was incubated at 37°C for 24 hours [20]. After 24 hours incubation, 200 μl of conditioned culture media was transferred from each well to a well in a new 96 well plate, and absorbance read at 570 and 600 nm using a plate reader (Synergy HT, Biotek, Winooski, VT). The number of cells that migrate through the fibronectin and across the filter are expressed as a percentage of AB reduction, and compared to a standard curve of uninfected cells to extrapolate percent invasion [20,21].

Cell metabolic activity and viability

Cell metabolic activity was measured with AB as previously described [20]. Briefly, JEG-3 cells were seeded into 24 well plates as described above for the invasion assay and incubated at 37°C for 24 hour. AB was added (1:10) to the cell culture medium and incubated for 24 hr incubation at 37°C to allow viable cells to reduce the AB. Samples of conditioned media (200 μl) were transferred to wells of a new 96 well plate and absorbance read at 570 and 600 nm using a plate reader [20,22].

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

Statistical analysis

For invasion and growth experiments, data from at least three experiments were analyzed by ANOVA followed by the Tukey post-test for all groups. The Mann-Whitney test was used for TEER, invasion, cell metabolism, and cell viability experiments (Instat, Graph Pad 6.0). Statistical significance for all comparisons was set at $p < 0.05$.

Results

L. monocytogenes invades and grows within JEG-3 cells

Previous studies suggested that both syncytiotrophoblasts and cytotrophoblasts are targets for *L. monocytogenes* infection [7-9]. Figure 1 demonstrates that JEG-3 cells can be infected with *L. monocytogenes* after only a one-hour incubation and support intracellular listerial multiplication that peaks at 10 hours and then declines by 24 hours post infection. Microscopic inspection of the monolayers revealed cytopathic changes including the accumulation of cellular debris
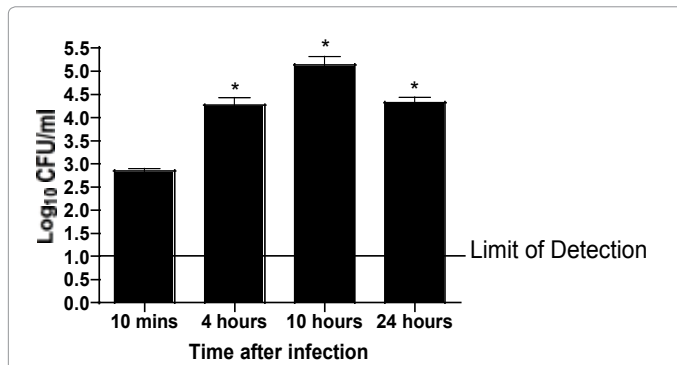
Figure 1: *Listeria monocytogenes* invades JEG-3 cells.

JEG-3 cells were incubated with 10^5 CFU of LM2203 for 1 hour, then extensively washed with HBSS. Gentamicin was then added to prevent extracellular bacterial growth. At selected time points, cells were lysed and intracellular microbial load quantified by diluting and plating lysates on blood agar. Data presented are the mean ± SEM of 6 replicates at each time point from one representative experiment, of 3 separate experiments that were performed. Asterisks indicate $p<0.01$ for intracellular growth time points compared to the initial 10-minute invasion time point.
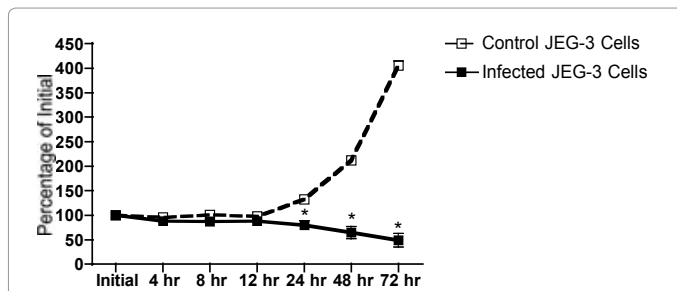
Figure 2: *Listeria monocytogenes* infection decreases JEG-3 cell monolayer TEER.

JEG-3 cells were seeded (1×10^5 cells) and incubated until formation of a monolayer, which was then infected as described in Figure 1, and TEER measured and calculated as the percentage change from the initial measurement. Data are expressed as mean ± SEM of 3 replicates for control and 9 replicates for infected wells at each time point. Data represents one experiment, which is representative of 3 separate experiments that were performed. Asterisks indicate $p<0.01$ for infected JEG-3 cells.

and cells lifting off the dish in *L. monocytogenes*-infected monolayers starting at 24 hours after inoculation (not shown).

L. monocytogenes infection of JEG-3 cells decreases monolayer integrity

Cytopathic changes in infected monolayers led us to determine if functional alterations in the monolayers also occurred during infection. We assessed the effects of *L. monocytogenes* infection on JEG-3 cell monolayers by measuring TEER across the surface area of the cell monolayer. Total resistance is made up of both transcellular and paracellular electrical resistance. As the integrity of the monolayer diminishes, electrical conductivity across it increases and TEER declines [23]. As shown in Figure 2, TEER values for *L. monocytogenes* infected cells began to decline between 12 and 24 hours of infection and continued to decline through 72 hr. These findings contrast with uninfected monolayers, which exhibited steadily increasing TEER, reflecting the continued proliferation of JEG-3 cells. However, specific cellular JEG-3 cell proliferation was not directly measured.

L. monocytogenes infection of JEG-3 cells diminishes cell invasion

JEG-3 cells are used as a model for EVT cells, which form the trophoblast cell column, attach fetal placental tissue to maternal decidual tissue, and migrate into decidual tissue to remodel the maternal spiral arteries [24,25]. We hypothesized that *L. monocytogenes* infection of JEG-3 cells impairs their invasion ability. We tested invasion of JEG-3 cells through 8 μm pore PET cell culture inserts coated with fibronectin using Alamar Blue reduction to quantify cells that invaded through the insert (Figure 3). As illustrated in Figure 3B, JEG-3 cells incubated with *L. monocytogenes* displayed decreased invasion through fibronectin and across the cell culture insert compared to uninfected JEG-3 cells.

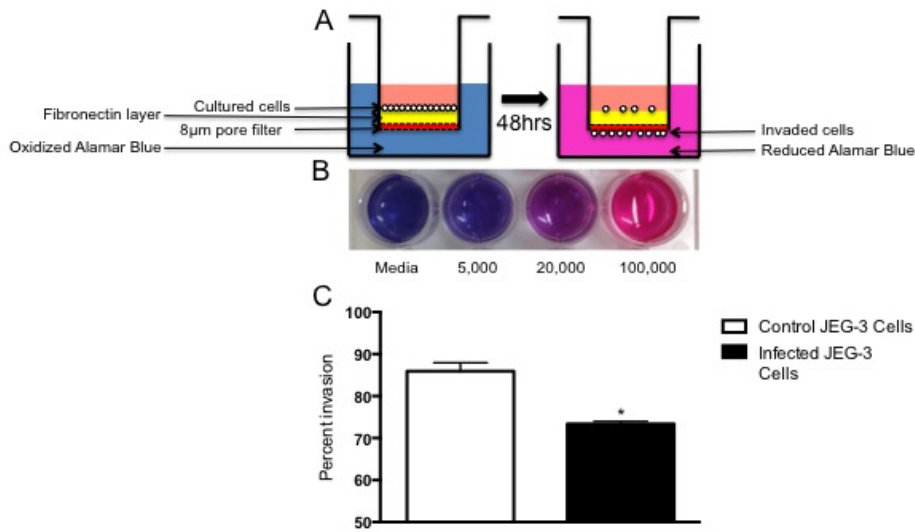## *L. monocytogenes* infection of JEG-3 cells decreases cell metabolism and increases cell death

The above experiments showed reduced function of JEG-3 cells (decreased TEER and invasion). These results suggested that infected JEG-3 cells were not proliferating and experienced cell damage following infection. Next, we determined if infection causes diminished JEG-3 cell metabolic activity or caused cell death. Using AB reduction to quantify JEG-3 cell metabolic activity we show that *L. monocytogenes* infected JEG-3 cells exhibit decreased cell activity within 48 hours of infection, compared to uninfected control cell cultures (Figures 3B and 4A). Furthermore, infection caused significant cell death at 48 and 72 h (as measured by LDH release) compared to uninfected JEG-3 cells (Figure 4B).

Discussion

This study demonstrates that *L. monocytogenes* infection of the human JEG-3 choriocarcinoma cell line reduces monolayer TEER, decreases invasion through a filter or fibronectin, decreases cell metabolic activity, and causes cell death. Our finding that *L. monocytogenes* readily infects JEG-3 cells, with peak numbers of intracellular bacteria at 10 hours after infection, is similar to a previous report that used BeWo cells and primary human EVT cells [8]. Zeldovich et al. also showed that primary human EVT cells restricted intracellular *L. monocytogenes* growth and suggested that EVT cells inhibit the growth of *L. monocytogenes* better than choriocarcinoma-derived cell lines because the former produces high levels of progesterone. However, we do not believe this explains the intracellular growth of *L. monocytogenes* in JEG-3 cells because they are robust producers of progesterone [14,26]. *L. monocytogenes* is not the only intracellular pathogen that invades fetal trophoblast cells. Human cytomegalovirus is the most common viral pathogen in the pregnant uterus and readily invades all types of trophoblast cells [27,28]. Among bacterial pathogens, *Chlamydia abortus* causes abortion in women exposed to infected ruminant and rodent (species that are endemically infected) fecal material and abortive tissues [29]. It was reported that intracellular growth of *C. abortus* in EVT cells is related to expression of an innate immunity molecule, SLPI, which modulates tissue inflammation and pathology [30]. *Campylobacter rectus* is a Gram-negative anaerobe that can disseminate to the fetus and cause abortion or pre-term delivery and has been shown to invade EVT cells. The pathophysiology behind *C. rectus's* ability to cause abortion has been suggested to include up-regulation of IL-6 and TNF in EVT cell layers [30].

We found that JEG-3 cells do not sustain *L. monocytogenes* intracellular growth for more than 24 hours post infection. This could be due to disruption of the JEG-3 monolayer, as demonstrated in our TEER experiments, which diminished direct contact among JEG-3 cells and thus reduced the opportunities for listerial cells to

Figure 3: *Listeria monocytogenes* infection diminishes JEG-3 cell invasion and illustration of the cell invasion assay.

(A) JEG-3 cells (1×10^5 cells) were added on top of 8 μm PET cell culture inserts that had a fibronectin layer on their upper surface, and placed into wells of a 24 well plate containing cell culture media with 10% FBS. After 24 hrs incubation at 37°C, the medium was removed and cells on top of the filter were removed with a sterile swab. AB was added to the medium in the bottom well, and wells were incubated at 37°C for a further 24 hrs. (B) Examples of color change after 24 hrs incubation with AB in wells with increasing numbers of JEG-3 cells. Percent AB reduction was compared between cells in the transwell (A) and cells grown in a well without transwell system (B) to establish percent invasion. Percent of invasion is shown for control non-infected transwells and *L. monocytogenes* infected transwells. Data are expressed as mean ± SEM of 4 replicates at each time point from 3 separate experiments. Asterisk indicates p<0.05 compared to uninfected JEG-3 cell invasion.
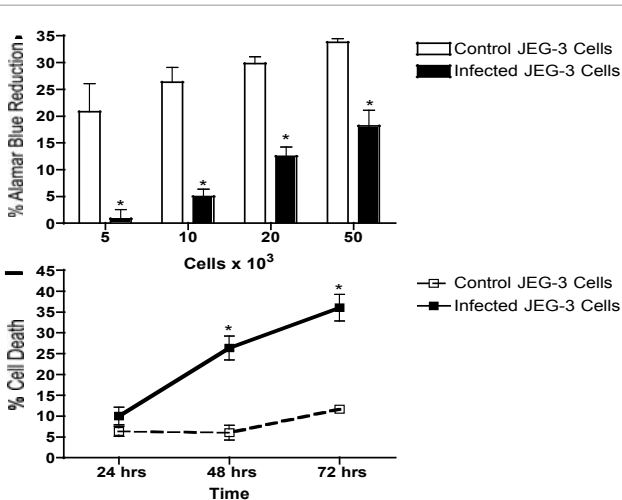
Figure 4: *Listeria monocytogenes* infection decreases cell metabolism and increases cell death in JEG-3 cells

JEG-3 cells were seeded (1×10^5 cells) and incubated at 37°C until formation of a monolayer, which was then infected as described in Figure 1. (A) Metabolic activity was detected with AB assay. Data represent percent AB reduction of infected cells compared to uninfected control cells. (B) LDH release from *L. monocytogenes* infected cells, compared to uninfected control cells. Data are expressed as percentage maximum LDH activity in wells treated with 1% NP-40 detergent. Data are expressed as mean ± SEM of 6 replicates at each time point for one representative experiment from 3 separate experiments that were performed. Asterisks indicate p<0.01 compared to uninfected JEG-3 cell Alamar Blue reduction and LDH release.

move from one cell to the next. We cannot exclude the possibility that the diminished metabolic activity of infected JEG-3 cells creates a cytoplasmic environment that is not suitable for sustained proliferation of *L. monocytogenes*. JEG-3 cells are also known to produce multiple immunomodulating molecules (e.g. B7 proteins, maltooligosaccharides,

and other unknown factors) [31-33]. However, to the best of our knowledge, no specific antimicrobial compounds have been previously reported in JEG-3 cells.

The decreased TEER of *L. monocytogenes* infected JEG-3 cell monolayers could reflect extracellular (e.g. damage to tight and gap junctions), or transcellular events (damaged or dead cells). If *L. monocytogenes* infection of EVT cells in the cell columns caused similar negative effects, it would be anticipated that they would compromise integrity of the implantation site and would have negative consequences for the fetus because trophoblast cells are an important component of the innate immune barrier during pregnancy [34]. We also cannot rule out that infected EVT cells could potentially still invade and propagate *L. monocytogenes* infection at the maternal-fetal interface. To the author's knowledge, the effect of bacterial infection on trophoblast TEER has not been previously reported. However, TEER has been used to show that different types of growth medium alter electrical resistance across a JEG-3 monolayer [23]. Ikeda et al. also showed that different growth media alter expression of gap junctional and adhesion proteins (including E-cadherins) on the surface of JEG-3 cells. InlA expressed by *L. monocytogenes* binds to E cadherin on host cells, which has been shown to be an important interaction for infection of EVT cells [7,11,23]. We hypothesize that paracellular damage from *L. monocytogenes* infection may alter gap junctional and adhesion proteins in human trophoblasts [31,35,36]. As a result, immune cells in the maternal bloodstream could enter the fetal placental villi and the fetal blood circulation to infect and initiate inflammation in fetal parenchymal tissues. Coupled with instability of the cell columns and compromised invasion, infection-related adverse pregnancy outcomes are a predictable sequel to trophoblast infection.

The results of the invasion and metabolic experiments show that *L. monocytogenes* infection diminishes the functional capacity of JEG-3 cells. We believe that reduced cell invasion was not solely a result of cell death because the former was detected within 24 hr, but significant

cell death was not observed until 48 to 72 hours after *L. monocytogenes* infection (Figure 4B). Similar results have been shown previously with human cytomegalovirus infection diminishing invasion of primary human EVT cells [37]. Extrapolating from our results to an *in vivo* scenario, we hypothesize that *L. monocytogenes* infection could decrease the ability of EVT cells to migrate from the cell column into the maternal decidual tissue.

It has been reported that subsyncytial cytotrophoblasts have bacteriocidal capacity against *L. monocytogenes*, which may prevent the pathogen from crossing the basement membrane in the fetal placental villi and infecting the fetus [8,9]. The results of the present study suggest that attention should also be paid to the functionality of EVT following *L. monocytogenes* infection, rather than simply focusing on the trophoblast cells as a point of entry to infect the fetus. Future investigations are needed to define the mechanisms by which *L. monocytogenes* infection affects attachment and invasion of fetal EVT to the decidual tissue.

Acknowledgement

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