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Analysis of the capacity of *Salmonella enterica* Typhimurium to infect the human Placenta

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ABSTRACT

Introduction: *Salmonella* species are gram-negative facultative intracellular bacteria that are common causes of foodborne illness in North America. Infections by *Salmonella* during pregnancy are a significant cause of fetal loss in domestic livestock, and fetal and maternal mortality in mice. Furthermore, *Salmonella* infection is associated with miscarriage, stillbirth and preterm birth in pregnant women. Despite these collective associations, the extent to which *Salmonella* can infect the human placenta has not been investigated.

Methods: Human placental villous explants from several gestational ages were exposed to *Salmonella enterica* serovar Typhimurium (STm) *ex vivo*. Infection was assessed by colony forming unit assay and whole mount immunofluorescence (WMIF).

Results: Viable bacteria were recovered from placental villous explants of all gestational ages tested, but the bacterial burden was highest in 1st trimester explants. Bacterial numbers did not change appreciably with time post-infection in explants from any gestational age examined, suggesting that STm does not proliferate in placental villi. Exposure of villous explants to STm strains defective for the type III secretion systems revealed that *Salmonella* pathogenicity island 1 is essential for optimal invasion. In contrast to placental explants, STm infected and proliferated within villous cytotrophoblast cells isolated from term placentas. WMIF demonstrated that STm was restricted primarily to the syncytiotrophoblast layer in infected placentas.

Discussion: Our study demonstrates that STm can invade into the syncytiotrophoblast but does not subsequently proliferate. Thus, the syncytiotrophoblast may function as a barrier to STm infection of the fetus.

1. Introduction

Infections during pregnancy represent a significant threat to the health of both mother and fetus, for they cause numerous adverse outcomes, including miscarriage, birth defects, preterm labor, and maternal/fetal morbidity and mortality [1,2]. TORCH pathogens (*Toxoplasma* gondii, "others" such as *Listeria* monocytogenes, Rubella virus, Cytomegalovirus, Herpes Simplex Virus) are capable of crossing the placenta and are major causes of perinatal morbidity and mortality worldwide [1]. Similar to TORCH pathogens, *Salmonella* infections during pregnancy cause significant fetal loss in domestic livestock, and fetal and maternal mortality in mice [3–8]. Multiple case reports demonstrate an association between *Salmonella* infections and stillbirth, preterm birth, chorioamnionitis and miscarriage in humans [9–14]. However, the extent to which *Salmonella* species can infect the human placenta has not been determined.

Salmonella species are gram-negative, facultative intracellular bacteria transmitted through contaminated food and the oral-fecal route. Non-typhoidal *Salmonella* (NTS) *enteritidis* serovars such as *Salmonella enterica* Typhimurium (STm) generally cause a self-limiting gastrointestinal infection in healthy adults and are most prevalent in North America and sub-Saharan Africa [15–18]. NTS are a major cause of

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Abbreviations

Ab	antibody
CFU	colony forming units
CK7	cytokeratin 7
EVT	extravillous trophoblast
FBS	fetal bovine serum
HSV	Herpes Simplex Virus
hCG	human chorionic gonadotropin
IFN-λ1	interferon-λ1
LPS	lipopolysaccharide
LM	*Listeria* monocytogenes
NTS	Non-typhoidal *Salmonella enteritidis*
PAMPs	pathogen associated molecular patterns
PRRs	pattern recognition receptors
1xPBS	phosphate buffered saline
SCV	*Salmonella* containing vacuole
STm	*Salmonella enterica* serovar Typhimurium
SYN	*Salmonella* pathogenicity island; syncytiotrophoblast
TG	*Toxoplasma* gondii
TBCs	trophoblast cells
T3SS	type three secretion system
vCTBs	villous cytotrophoblast cells
WMIF	whole mount immunofluorescence

foodborne illness in the United States [17]. Moreover, NTS are estimated to account for > 30% of bloodborne infections in sub-Saharan Africa [15]. Thus, *Salmonella* infections constitute a global health concern for at-risk populations, particularly immune-suppressed individuals and pregnant women.

STm can infect diverse cell types, including epithelial cells and macrophages, but the mechanisms of internalization and intracellular life cycles differ in a cell type-dependent manner. Invasion of epithelial cells by STm requires the *Salmonella* pathogenicity island 1 (SPI1)/type three secretion system 1 (T3SS1), which encodes a needle-like complex and associated effectors [19–21]. Once STm is internalized within an epithelial cell, the bacteria localize to a specialized vacuole termed the *Salmonella*-containing vacuole (SCV) [22–24]. STm can survive within the SCV or escape into the cytoplasm of epithelial cells, where it has been shown to undergo "hyper-replication" [25–27]. In contrast, macrophages internalize *Salmonella* primarily through phagocytosis, after which the bacteria also reside in the SCV [28]. Once the bacteria are within the macrophage SCV, SPI2/T3SS2 encoded gene expression is activated, which maintains the SCV and promotes intracellular survival [28,29].

The fetal component of the placenta that directly contacts maternal blood and the uterine wall is composed of tree-like structures called villi [30]. There are two types of placental villi: 1) floating villi, which are completely bathed in maternal blood and 2) anchoring villi, the tips of which secure the placenta to the uterine wall. The outermost layer of placental villi is composed of specialized epithelial cells called trophoblast cells (TBCs). The syncytiotrophoblast (SYN) forms a multinucleated layer around both floating and anchoring villi and directly contacts maternal blood within the intervillous space, facilitating gas, nutrient and waste exchange. The SYN is continuously regenerated by fusion of underlying villous cytotrophoblast cells (vCTBs). Columns of proliferating CTBs on the ends of anchoring villi differentiate into extravillous trophoblast cells (EVTs), which invade the uterine endometrium and function in remodeling the uterine arteries to facilitate maternal blood flow to the intervillous space. Thus, there are two distinct interfaces at which the human placenta contacts maternal blood and tissues: 1) the SYN with maternal blood within the intravillous space and 2) EVTs with both the maternal decidua and blood within the uterine spiral arteries. These two TBC subtypes represent a potential entry point for vertical transmission of bloodborne pathogens across the human placenta to the fetus. However, several lines of evidence suggest that TBCs function as a barrier against congenital infections. The SYN of 1st trimester tissue is resistant to infection by *Listeria* monocytogenes (LM), *Toxoplasma* gondii (TG) and certain viruses, while EVTs appear to be relatively sensitive to these pathogens [31–34]. Likewise, SYN derived by *in vitro* differentiation of term vCTBs are resistant to infection by a wide variety of unrelated viruses [35,36]. Relatively few studies, however, have compared the susceptibility of the human placenta to infections across gestation.

To date, studies investigating the capacity of *Salmonella* to infect the human placenta have been limited to TBC-like cell lines [8,37]. STm replicates rapidly in these cells, suggesting that human TBCs may be susceptible to *Salmonella* infections. In the current study, we utilized an *ex vivo* floating explant model to investigate the capacity of STm to infect human placental villi across multiple gestational ages. The collective results suggest that the SYN of the human placenta functions as a barrier to STm infection.

2. Materials and methods

2.1. Human tissue collections

A total of 62 placentas was collected for this study. First (7–12 weeks; n = 22) and 2nd trimester (14–22 weeks; n = 17) placentas were collected from elective terminations, while term placentas (> 39 weeks; n = 23) were collected from healthy, uncomplicated pregnancies delivered by caesarean section. Placentas from mothers with known comorbid conditions such as chorioamnionitis, sexually transmitted diseases, chronic hypertension, diabetes, autoimmune disease, pre-eclampsia and renal disease, as well as from reported smokers and illicit drug users, were excluded from this study. Informed consent was obtained for all placentas. This study was approved by the Institutional Human Subjects Review Board at the University of Rochester.

2.2. Isolation of human term cytotrophoblast

Primary term vCTBs were isolated by Percoll gradient centrifugation and negative immunoselection as previously described [38] with the following exceptions: immunoselection was performed using anti-Mouse IgG MicroBeads (Miltenyi Biotec Inc., San Diego, CA, USA), and "The Big Easy" EasySep Magnet (STEMCELL Technologies Inc., Vancouver, Canada). vCTB purity was determined by immunostaining or flow cytometry with a cytokeratin-7 antibody (clone LP5K, EMD Millipore, Billerica, MA) and ranged from 92 to 98%. Cell viability was assessed using a LIVE/DEAD stain assay (Invitrogen, Carlsbad, CA).

2.3. Bacterial preparation

Non-typhoidal STm strains SL1344, x3339 (an *in vivo* passaged derivative of SL1344 [39]), x3339ΔSPI1, x3339ΔSPI2 and x3339ΔSPI1/ΔSPI2 were grown overnight and subcultured with agitation in LB media containing 50 μg/ml streptomycin until the cultures reached an optical density of approximately 0.8 at 600 nm. Cultures were diluted with glycerol to a final concentration of 20%, aliquoted and frozen at −80 °C. Prior to use, bacterial stocks were thawed, washed once with 1x phosphate buffered saline (1xPBS) and diluted in 1xPBS to 10^8 colony forming units (CFU)/ml.

2.4. Placental villous explant and vCTB infection and quantification by CFU assay

Placentas were processed within an hour after collection and

washed in 1xPBS. Villous explants (~3–10 mg wet weight) were dissected, washed in 1xPBS (Gibco, Grand Island, NY) at room temperature and adapted to *ex vivo* culture by incubation for 1 h at 37 °C in DMEM/F12 (Gibco) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and 25 μg/ml streptomycin (Gibco). Since 1st trimester placental tissue contains a substantially greater proportion of anchoring versus floating villi compared to tissues of later gestational ages (and therefore more EVTs), floating villi were preferentially selected for this study based on morphological criteria. STm is resistant to streptomycin, therefore this antibiotic was included in the culture medium to prevent inadvertant contamination by Streptomycin-susceptible bacteria. Individual explants were subsequently co-incubated with STm (10^7 CFU) for 1 h at 37 °C, after which the explants were washed extensively with 1xPBS, transferred to fresh plates with media containing 100 μg/ml gentamicin (Gibco), and cultured for 2 h to kill extracellular bacteria. Thereafter, the explants were washed with 1xPBS and incubated in media with 10 μg/ml gentamicin. Placental explants were weighed and homogenized in 1xPBS (1 ml) using a handheld tissue homogenizer (Omni International, Kennesaw, GA). Lysates were serially diluted, streaked onto LB agar plates containing 25 μg/ml streptomycin, and plates incubated overnight at 37 °C. CFUs were counted and normalized to explant wet weight. At least three explants from each placenta was used for each experimental condition.

Human vCTBs were infected at a multiplicity of infection (MOI) of 10 as previously described for choriocarcinoma cells [8,37]. Briefly, cells were plated in 24-well plates at 1×10^5 cells/well and infected the following day.

2.5. Hormone measurements

Explant culture medium was collected at the times described and frozen at −80 °C. Human chorionic gonadotropin (hCG), progesterone and 17β-estradiol levels in the medium were measured by electrochemiluminescence using a Roche Cobas e411 automated analyzer system (Roche Diagnostics, Indianapolis, IN, USA) as described by the manufacturer.

2.6. Antibodies

Primary antibodies used were: anti-hCG (Dako, Glostrup, Denmark), FITC anti-human cytokeratin 7 (CK7) (Millipore, Billerica, MA), and anti-*Salmonella* Typhimurium lipopolysaccharide (STm LPS; Thermo Fisher Scientific, Rockford, IL). The following secondary antibodies were used: Cy3 goat anti-rabbit (EMD Millipore) for anti-hCG, and APC goat anti-mouse IgG1 (Jackson ImmunoResearch, West Grove, PA) for STm LPS. Purified mouse IgG1 isotype κ (Biolegend, San Diego, CA) and rabbit polyclonal isotype (Biolegend) antibodies served as negative controls in the immunofluorescence assays.

2.7. WMIF

Explants were fixed for 2 h at 4 °C with 90% methanol, washed with 1xPBS/0.1% triton-X100, and stained overnight at 4 °C with primary antibodies in 1xPBS containing 0.1% triton-X100 and 5% goat serum [40]. Stained samples were washed with 1xPBS/0.1% triton-X100 and incubated with secondary antibodies for 2 h at 4 °C with agitation. Explants were washed and mounted on slides using Mowiol mounting medium and imaged using an Olympus FV1000 laser scanning confocal microscope. Confocal images were taken using PLAPON 60x oil (NA 1.42) and UPLSAPO 100x oil (NA 1.4) objectives. Maximum intensity projections were compiled into Z-stacks of ~10 μm thickness. The microscope settings were defined within the linear range for each antibody at the beginning of the study and were maintained throughout.

2.8. Statistics

To estimate the differences in bacterial growth under different tissue conditions over time, two-way ANOVA models were fit to the measures of $\log_{10}$-bacteria growth. These models were each fit by maximum-likelihood (ML) estimation using the lm function in R version 3.0.2. *Post-hoc* contrasts between treatments were estimated at prespecified time points when interaction effects were significant. The error level on the contrasts was controlled for multiple comparisons by using Tukey HSD p-values. P-values less than the significance level of 0.05 were considered statistically significant.

3. Results

3.1. Salmonella Typhimurium can infect human placental explants from multiple gestational ages

To investigate the extent to which *Salmonella* can infect the human placenta, we established a floating explant model in which we exposed villous explants from 1st trimester (7–12 weeks), 2nd trimester (14–22 weeks) and term (> 39 weeks) placentas to 10^7 CFUs of STm strain SL1344 for 1 h, washed and cultured the explants for 2 h with 100 μg/ml gentamicin to kill extracellular bacteria, and harvested the explants for CFU assays at 2, 6 and 24 h post-infection to enumerate the numbers of viable, intracellular bacteria. First trimester placental tissues contain a substantially greater proportion of anchoring versus floating villi compared to later gestational tissues (and therefore significantly more EVTs), which would complicate the interpretation of the results

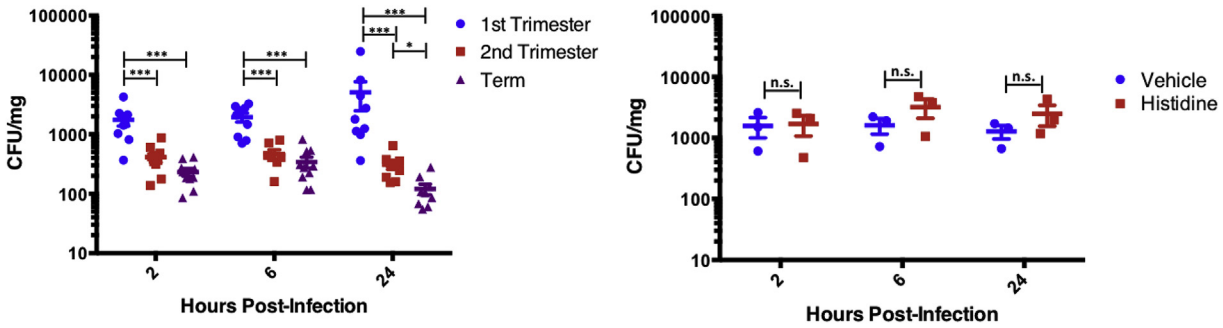

Fig. 1. Analysis of the capacity of STm to infect human placental villi across gestation. **A)** Individual villous explants from 1st trimester (N = 9; ●), 2nd trimester (N = 9; ■) and term (N = 10; ▲) placentas were exposed for 1 h to 10^7 colony forming units (CFU) of STm strain SL1344 and the numbers of intracellular bacteria were enumerated at 2, 6, and 24 h post-infection by CFU assay. The data points represent the average CFU recovered from at least three explants of each placenta examined, normalized to the explant wet weights. The error bars show the standard error in reference to the mean. Statistical significance ($p < 0.001$ and $p < 0.05$) is represented by *** and *, respectively. **B)** Villous explants from 1st and 2nd trimester placentas (N = 3) were exposed to STm strain SL1344, and subsequently cultured in the absence (●) or presence (■) of exogenously added histidine (50 μg/ml). CFU assays were performed at 2, 6 and 24 h post-infection as described for 1A. Results were not statistically significant (ns).

comparing the susceptibility of the different gestational age tissues to STm infection. Therefore, floating villi were selectively chosen from 1st trimester placentas during the dissection process based on morphological differences with anchoring villi. The numbers of STm CFU were normalized to explant weight. No colonies were obtained from uninfected placentas of any gestational age (data not shown). Viable bacteria were recovered from 100% of the placental explants exposed to STm, but the bacterial numbers obtained from 1st trimester tissues were significantly higher compared to 2nd trimester and term (Fig. 1A). The numbers of colonies obtained in these assays were similar to those observed in a previous *ex vivo* study of human intestinal biopsies [41]. Importantly, the CFU numbers recovered did not change appreciably over time post-infection from explants of the same gestational age (Fig. 1A), suggesting that STm does not proliferate significantly within intact human placental villi.

To assess the health and viability of the villous tissues in our *ex vivo* model and to examine potential effects of *Salmonella* infection on placental health, secretion of hCG, progesterone and 17β-estradiol into the culture medium was measured by electrochemiluminescence at each time point postinfection from a subset of experiments (two 1st trimester, two 2nd trimester and six term). Hormone levels for the 24 h time point of the term samples are shown in Supplemental Table 1. Secretion of all three hormones was readily detectable from every villous explant examined, and hormone levels were within previously observed ranges for a given gestational age [42–46]. Furthermore, there were no discernible effects of STm infection on hCG, progesterone or estradiol levels at any time point postinfection. These results indicate that the villous tissues remained viable in our *ex vivo* system, both in the absence and presence of STm infection.

STm strain SL1344 is defective for histidine biosynthesis [47], thus it does not replicate efficiently in human macrophages in the absence of supplemental exogenous histidine [48]. To evaluate whether the inability of STm strain SL1344 to proliferate in human placental explants is due to insufficient histidine levels, 1st trimester and 2nd trimester villous explants were subjected to CFU assays in the absence or presence of supplemental histidine (50 μg/ml). Culturing with a higher histidine concentration had no statistically significant effects on the numbers of CFUs at any time point (Fig. 1B). These results indicate that the lack of STm strain SL1344 proliferation in placental villous explants is not due to its deficiency in histidine biosynthesis.

3.2. STm deficient in SPI1 is significantly compromised in its ability to infect human placental villi

To investigate the roles of the T3SS in STm infection of the human placenta, we exposed 2nd trimester villous explants to a STm double mutant defective for both SPI1 and SPI2 [39], and enumerated bacterial numbers by CFU assay. The bacterial numbers recovered from villous explants infected with the ΔSPI1/SPI2 strain were significantly lower (> 10-fold) compared to the SL1344 and parental X3339 strains (Fig. 2A), suggesting that the T3SS are essential for infection of placental villi. To distinguish whether one or both of the T3SS are necessary for optimal placental infection, 2nd trimester explants were exposed to single mutant strains of SPI1 and SPI2. Similar numbers of colonies were obtained from explants infected with the parental and ΔSPI2 strains (Fig. 2B). In contrast, significantly lower numbers of bacteria were recovered from villous explants exposed to the ΔSPI1 mutant (Fig. 2B). None of the strains proliferated extensively between 2 and 6, or 2 and 24 h post-infection (Fig. 2). These results suggest that SPI1 is required for invasion of human placental villi, but SPI2 is dispensable.

3.3. Intracellular localization of STm is restricted primarily to the SYN in infected placental explants

To identify the specific placental cells infected by STm, we exposed 1st trimester and term villous explants to STm and performed WMIF as previously described [40]. An antibody to *Salmonella* LPS was used to identify intracellular bacteria, and antibodies to hCG and cytokeratin-7 (CK7) to identify the SYN and TBCs, respectively. As expected, staining for STm LPS was not detected in uninfected villous explants from 1st trimester or term placentas at any time point examined (Figs. 3A and 4A, data not shown). However, at 2 and 24 h post infection, individual and small clusters of LPS^+ bacteria were detected in explants from both 1st trimester and term tissues (Figs. 3B and 4B; data not shown). Higher magnification views of the tissues revealed that the bacteria were rod-shaped (Supplemental Fig. 1; data not shown). Exposure of 1st trimester or 2nd trimester villous explants to a recombinant STm strain expressing red fluorescence protein [49] further confirmed that the bacteria were indeed *Salmonella* (Supplemental Fig. 2). Importantly, LPS^+ bacteria were restricted to the outer placental layer (SYN) that stained for hCG at both 2 and 24 h post-infection, as revealed in the orthogonal views of Figs. 3B and 4B. The reproducible observation of small clusters of STm at the same invasion site within the SYN, even after only 2 h postinfection, is consistent with a recent study demonstrating that STm invasion of polarized epithelial cells is cooperative [50]. Furthermore, it is essential to note that in numerous WMIF assays examining STm localization within placental villi, bacteria were never observed in the placental stroma (Figs. 3 and 4; data not shown). Together, these results suggest that STm infection is restricted to the SYN in infected placental villi from both early and late gestation.

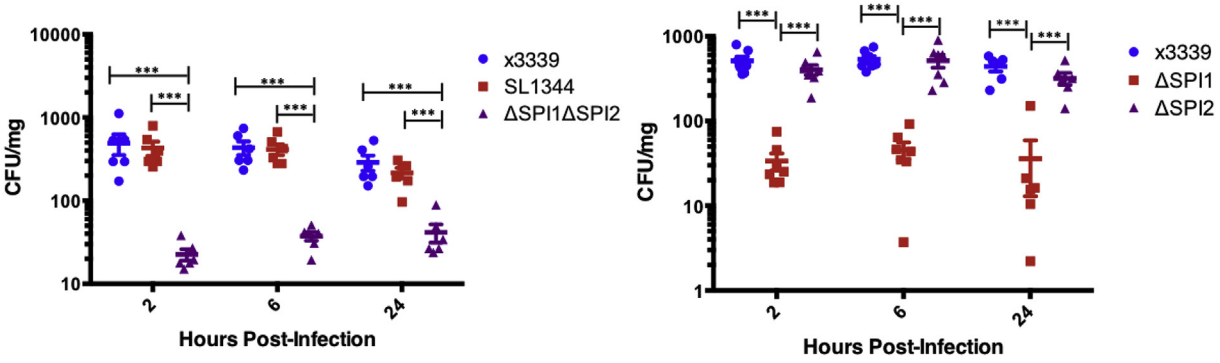

Fig. 2. Examination of the roles of *Salmonella* pathogenicity island 1 (SPI1) and SPI2 in infection of human placental villi. Second trimester human placental villous explants (N = 7) were exposed for 1 h to 10^7 CFU of **A)** STm x3339 parental (●), parental SL1344 (■), or the double mutant ΔSPI1/SPI2 (▲), or **B)** ST x3339 parental (●), a ΔSPI1 mutant (■), or ΔSPI2 mutant (▲). Infection was assessed by colony-forming assay at 2, 6, and 24 h post-infection as described for Fig. 1. Each data point represents the average CFU per mg of tissue of three individual explants infected with a specific strain of STm. Statistical significance ($p < 0.001$ is represented by ***.

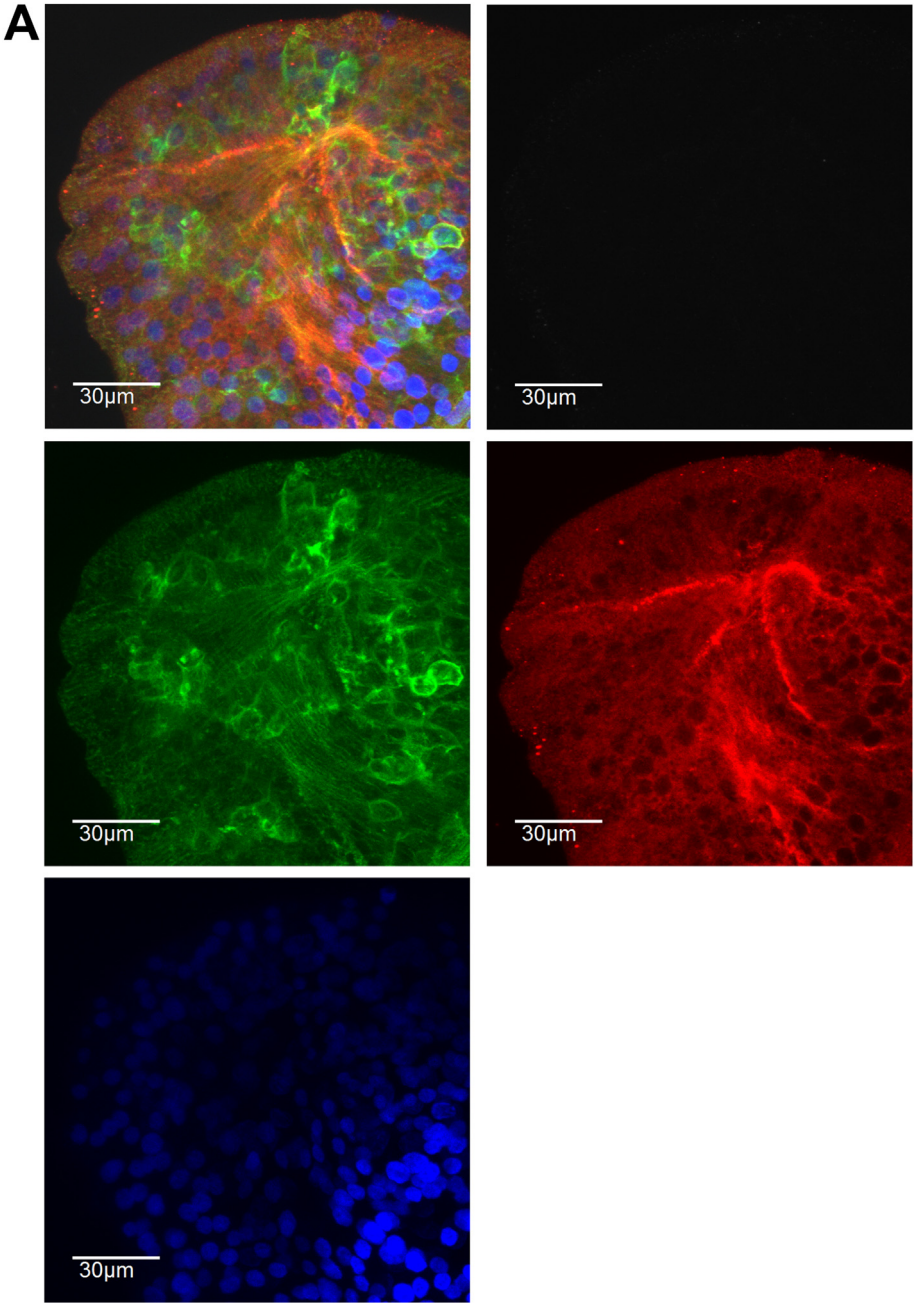

Fig. 3. WMIF analysis of STm intracellular localization in infected 1st trimester placental explants. First trimester placental explants were either: **A**) mock infected or **B**) infected with STm strain SL1344 for 24 h and subjected to WMIF using antibodies to STm LPS (grey; top right panel), CK7 (green; middle left panel) and hCG (red; middle right panel). DNA was stained with DAPI (blue; lower left panel). The upper left panels represent the merged images and include the orthogonal views (shown by black arrowheads on the bottom and right sides) revealing the localization of the STm within the tissue. The white arrowheads indicate LPS^+ STm. Images are representative Z-stacks of approximately 10 μm thickness. Scale bars represent 30 μM. The results are representative of 11 experiments.

3.4. STm actively proliferates within isolated human vCTBs, but SPI1 is dispensable for invasion

In contrast with the lack of proliferation observed within placental villous explants, STm infects and rapidly proliferates within human trophoblast-derived choriocarcinoma cells [8,37]. To investigate the capacity of STm to productively infect primary TBCs, we isolated vCTBs from term placentas and performed CFU assays using the parental and STm ΔSPI strains. Significantly higher numbers of CFUs were recovered from term vCTBs infected with the parental strain at 6 and 24 h compared to 2 h post-infection (Fig. 5), which demonstrates that STm can productively infect and replicate within human term vCTBs. Similar numbers of CFUs were obtained with the STm ΔSPI1 versus the parental strain at all time points post infection, suggesting that SPI1 is not essential for invading or replicating within human vCTBs (Fig. 5). In contrast, the CFU numbers obtained from vCTBs infected with the ΔSPI2 strain did not change significantly between 2 and 6 or 2 and 24 h, suggesting that SPI2 is critical for replication within these cells.

4. Discussion

Although *Salmonella* infections during pregnancy have been linked to adverse outcomes, the extent to which *Salmonella* species can infect the human placenta has not been investigated. In this study, we utilized

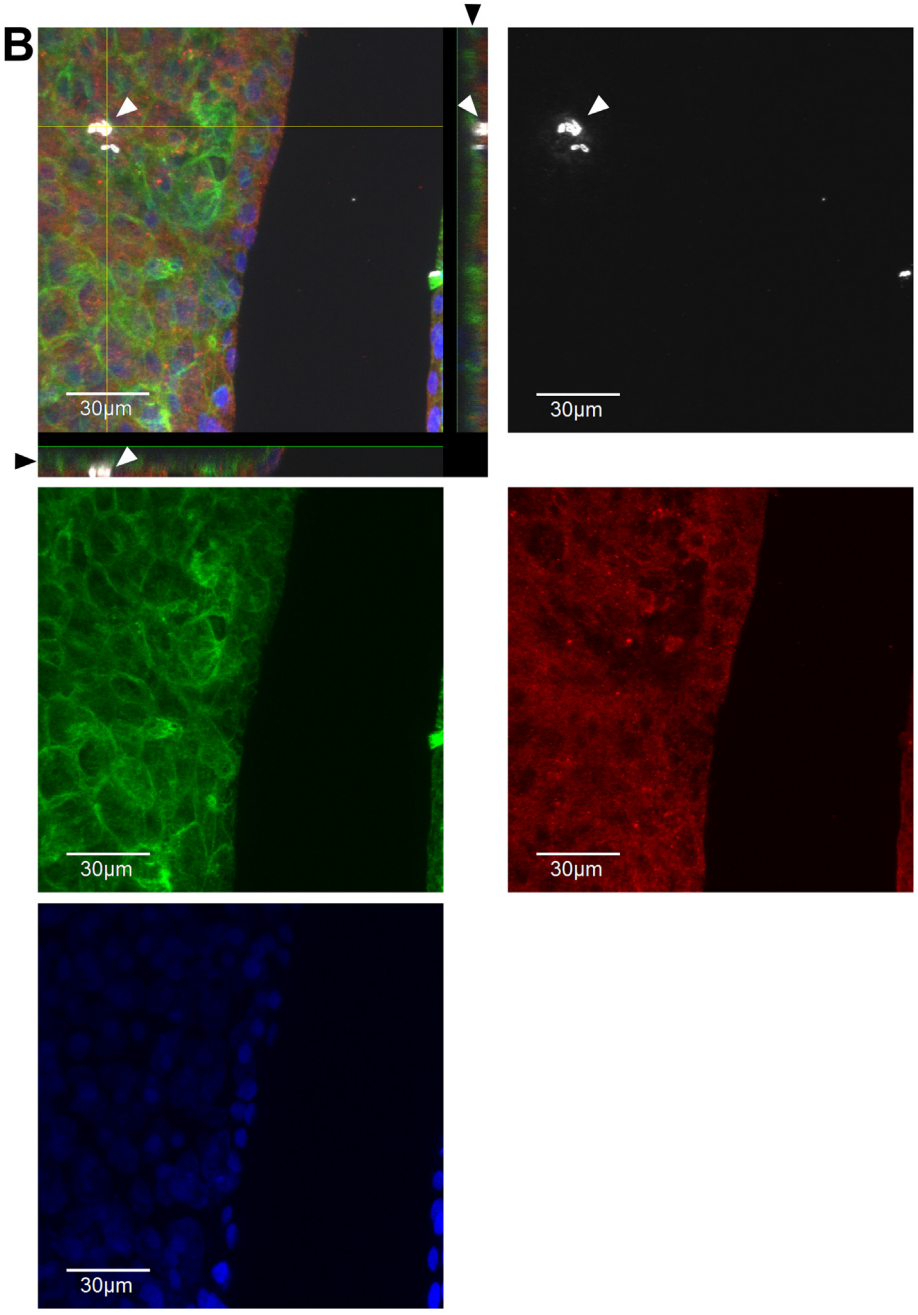

Fig. 3. (*continued*)

a floating explant model to demonstrate that STm can infect human placental villi from multiple gestational ages, but the bacteria do not proliferate significantly. Our results suggest that: 1) 1st trimester placental villi are more susceptible to STm invasion compared with 2nd trimester or term tissues, 2) SPI1 is required for optimal invasion of placental villous explants and 3) STm is restricted to the SYN in infected villi.

The strengths of our study include using human placental villous explants from all three trimesters, which enabled us to directly compare the relative susceptibility of floating villi from different gestational ages. To avoid potential long term culture-mediated changes in cytokine production and other factors that could impact the extent of infection, tissues were adapted to *ex vivo* culture for a relatively short time. Additionally, the use of WMIF made it possible to visualize the relatively small numbers of bacteria present within the infected explants, which would have been challenging had we used immunohistochemistry due to the necessity to section the tissues. One limitation of our study is that neither the role(s) of infiltrating immune cells nor placental blood flow on the progression of STm infection could be evaluated using the villous explant model. Furthermore, the relative susceptibility of the SYN versus EVTs in 1st trimester tissues could not be rigorously compared using the floating explant model due to the limited numbers of EVTs within anchoring villi in the absence of *in vitro* outgrowth [45,51].

Constant maternal exposure to diverse pathogens surely resulted in strong selective pressure for evolution of a placental barrier that would prevent transmission to the fetus. The surface area of the human SYN is estimated to reach approximately 11–12 m^2 by the 3rd trimester in order to meet the increasing nutrient and gas demands of the growing fetus [52], providing a large surface for potential contact with blood-borne pathogens. WMIF revealed that STm infection of human placental villi was restricted to the SYN. Importantly, the CFU assays

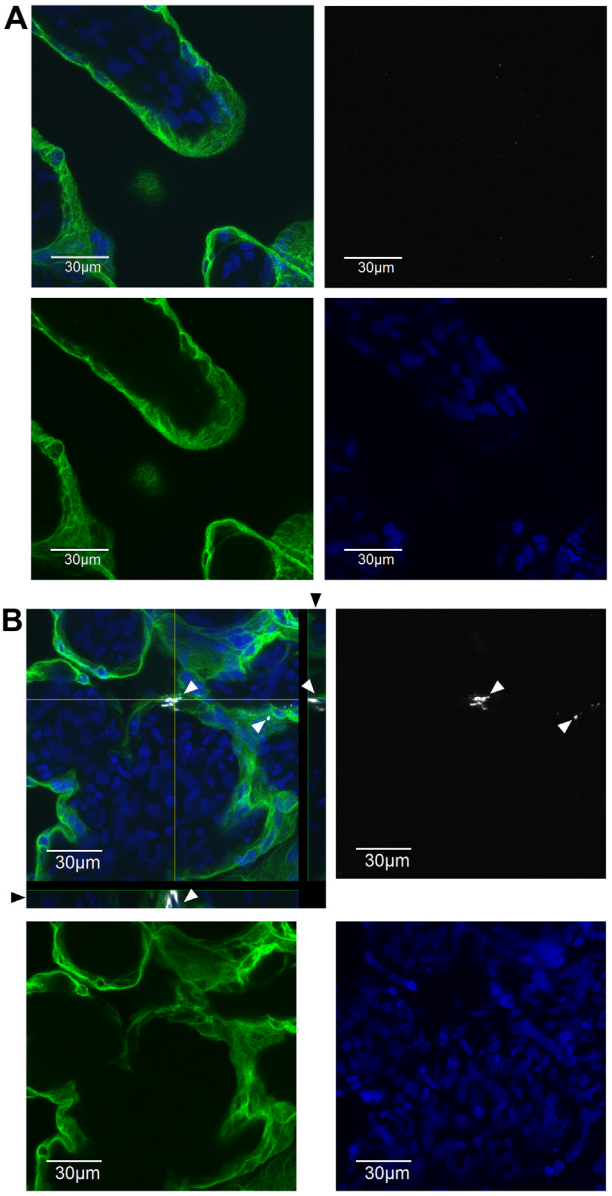

Fig. 4. STm is restricted to the syncytiotrophoblast of infected term placental explants. Term placental explants were **A**) uninfected or **B**) infected for 24 h with STm strain SL1344 and subjected to WMIF using antibodies to STm LPS (grey; upper right panel) and CK7 (green; lower left panel), and DAPI to identify the DNA (blue; lower right panel) as described for Fig. 3. The upper left panels represent the merged images, and this panel in **B** includes the orthogonal view of STm within the tissue (shown by black arrowheads on the bottom and right sides). The LPS^+ bacteria are indicated by the white arrowheads. Images are representative Z-stacks that are ~10 μm thick. Scale bars represent 30 μM. This experiment was repeated 7 times with similar results.

demonstrated that the bacteria did not proliferate significantly over 24 h in placental villi from any gestational age tested. In contrast, STm proliferated extensively in monolayers of term vCTBs. The numbers of bacteria detected in the CFU assays of placental villous explants per milligram of tissue were comparable to what was previously observed in human intestinal biopsies, which function as a barrier to pathogenic bacteria [41]. Based on these collective observations, we propose that the SYN functions as a barrier to *Salmonella* infection, in part by preventing bacterial proliferation. In the event that the SYN layer is compromised, STm could infect and subsequently replicate within the underlying vCTBs and cause placental pathology.

Comparison of the STm parental and T3SS mutant strains revealed

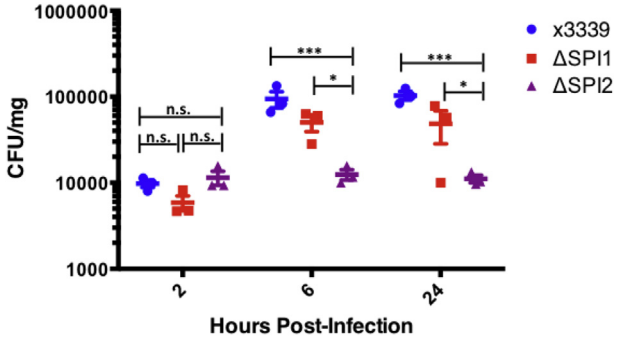

Fig. 5. Analysis of the ability of STm to infect human vCTBs. Primary vCTBs were isolated from term placentas (N = 3) and infected at an MOI of 10 with ST x3339 parental (●), an STm mutant lacking SPI1 (■), or a mutant lacking SPI2 (▲). Infection was assessed by CFU assay at 2, 6, and 24 h post-infection. Each dot represents the average STm CFU recovered from three replicates in a single experiment. Statistical significance ($p < 0.001$ and $p < 0.05$) is represented by *** and *, respectively.

intriguing differences in the mechanisms required for STm internalization into different TBC subtypes and the subsequent intracellular life cycles. Specifically, deletion of SPI1 decreased STm invasion into human placental villi by ~10-fold, indicating that SPI1-mediated internalization is the primary mechanism by which STm enters the human placenta. This is similar to the internalization mechanism into other epithelial cell types [19–21]. Moreover, multiple bacteria were commonly detected in infectious foci within the SYN in villous explants at 2 h post-infection. This is consistent with a previous study demonstrating that STm invasion of polarized epithelial cells is cooperative, such that SPI1-mediated alteration of the host cell actin network during invasion of the first bacterium enables internalization of additional bacterial cells [53]. Furthermore, the STm proliferation rate in polarized versus non-polarized epithelial cells was substantially lower, similar to the human placental villi [53]. In marked contrast, SPI1 is dispensable for STm invasion of human choriocarcinoma cells [37] and vCTBs (this study). Similar to macrophages, choriocarcinoma cells utilize phagocytotic-like mechanisms involving scavenger receptors for STm uptake [37]. *Salmonella* also employs SPI1-independent pathways of invasion into other types of epithelial cells, including the outer membrane protein Rck encoded by the *Salmonella* large virulence plasmid [54,55]. However, Rck is dispensable for STm invasion of human TBC lines [37]. The observation that SPI1 deletion results in 10-fold lower invasion rates into human placental villi indicates that SPI1 is responsible for > 90% of internalization events, which suggests that Rck (and other non-SPI1 mechanisms) plays only a minor (< 10%) or negligible role in placental internalization of STm. Lastly, intracellular proliferation of the ΔSPI2 strain in vCTBs is significantly lower compared to the parental strain. This is consistent with previous studies demonstrating that SPI2 plays an essential role in maintaining SCV integrity in macrophages and certain epithelial cells, which facilitates intracellular proliferation [23,24,28,29,37]. However, while the SCV matures and fuses with lysozymes in macrophages, leading to bacterial destruction, it fails to mature in choriocarcinoma cells, allowing STm to proliferate rapidly and ultimately kill the infected host cells [37]. Neither the parental nor the ΔSPI strains proliferated in placental villous explants. It is currently unclear whether the inability of STm to proliferate in the SYN is associated with a novel intracellular localization (i.e., vacuolar or cytoplasmic).

Previous studies suggest that the human SYN is resistant to infections by numerous pathogens, but the precise molecular mechanisms remain incompletely understood [32,34–36,56,57]. However, available evidence suggests that the SYN utilizes multiple, distinct mechanisms that are pathogen-dependent. For example, human placental resistance to Herpes Simplex Virus (HSV) infection correlates with the absence of

HSV receptors on vCTBs and the SYN [34]. Furthermore, SYN derived by *in vitro* fusion of term vCTBs express a family of microRNAs encoded by chromosome 19, which appear to confer protection against infection by a wide variety of unrelated viruses, but not *Listeria* monocytogenes (LM) or *Toxoplasma* gondii (TG) [36,57]. Moreover, interferon-λ1 expression is induced when term vCTBs fuse into SYN *in vitro*, which protects against Zika virus [35]. The SYN of 1st trimester placentas was also shown to be relatively resistant to *ex vivo* infections by TG and LM [31–33]. Moreover, SYN derived by *in vitro* fusion of term vCTBs is resistant to TG attachment and replication [58]. Disruption of the syncytial actin network moderately enhanced the ability of LM to invade, suggesting that the syncytiotrophoblast forms a biophysical barrier to this bacterium [33], but the precise molecular mechanism responsible has not yet been defined. Examining the potential role of trophoblastic fusion status on the capacity of pathogenic bacteria to invade and proliferate may shed light on these mechanisms.

Although our observations provide evidence that STm can invade the SYN, there are several other routes by which blood-borne pathogens may infect the placenta and affect pregnancy outcomes, such as infection of invasive EVTs by pathogens that reach the uterine decidua by ascending through the genital tract, migration of immune cells that were infected at other sites and antibody-mediated transcytosis [1]. Like STm, LM is a facultative intracellular bacterial species, but it escapes from the vacuole in a productive infection and proliferates in the cytosol [59]. It is well established that LM can be transmitted across the placenta to infect the fetus, leading to fetal loss, stillbirth and neurological defects [60–62]. LM was previously detected within the SYN and vCTBs in term placentas from pregnant women with *Listeriosis* [61]. Similarly, LM was restricted predominantly to the SYN after *ex vivo* infection of term villous explants [61]. Based on these results, the authors proposed that the SYN is compromised in the course of congenital LM infection. However, a different group subsequently examined LM infection of 1st trimester villous explants using an *in vitro* matrigel outgrowth model, and observed that LM infection was primarily localized to the EVTs, whereas the SYN was relatively resistant to infection [31]. Likewise, although EVTs only represent ~5% of the surface area of 1st trimester explants, over 80% of placental TG vacuoles were detected in EVTs [32]. Based on these results and studies in a guinea pig model, this group proposed that the principle route of pathogen transmission across the placenta is through initial infection of the decidua and subsequent spread to EVTs. However, to the best of our knowledge, few studies have used the same experimental model to quantitatively examine the susceptibility of the human placenta from multiple gestational ages to infection by the same pathogen. The current study utilized a floating villous explant model to focus on the potential barrier function of the syncytiotrophoblast to STm infection across gestation. Thus, future *ex vivo* studies of anchoring villi from 1st trimester placentas cultured on Matrigel and decidual explants are necessary to examine the capacity of *Salmonella* to infect and replicate within EVTs and decidual cells.

While our studies suggest that human SYN can function as a barrier to STm infection, the underlying bases for *Salmonella*-mediated pregnancy complications such as miscarriage and preterm birth remain to be identified. *Salmonella* expresses multiple pathogen associated molecular patterns (PAMPs), including LPS and flagellin, which are recognized by mammalian pattern recognition receptors (PRRs) and induce inflammatory responses [63]. Comparable bacterial burdens were observed in the placenta when pregnant mice were infected with the STm auxotrophic mutant ΔaroA versus the parental SL1344 strain [8]. Intriguingly, infection with the parental SL1344 but not the ΔaroA strain resulted in fetal resorption and maternal death that was associated with increased systemic and placental production of multiple pro-inflammatory cytokines and chemokines [8]. Interestingly, PRRs are differentially expressed by human TBC subtypes across gestation, which implies that the specific consequences of placental exposure to PAMPs may be gestational age-dependent [64–68]. Exposure of both human placental explants and TBCs to LPS results in upregulation of pro-inflammatory cytokine expression [66,69,70]. Moreover, it is well established that altered pro-inflammatory cytokine production, particularly TNF-α, is associated with pregnancy complications [71]. Thus, although the human SYN may block *Salmonella* proliferation, infection-induced production of pro-inflammatory cytokines may result in placental damage and subsequent pregnancy complications. Future studies are required to address this possibility.

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Declaration of interest

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Appendix A. Supplementary data

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References

[1] N. Arora, Y. Sadovsky, T.S. Dermody, C.B. Coyne, Microbial vertical transmission during human pregnancy, Cell Host Microbe 21 (5) (2017) 561–567.
[2] A.P. Kourtis, J.S. Read, D.J. Jamieson, Pregnancy and infection, N. Engl. J. Med. 370 (23) (2014) 2211–2218.
[3] K. Linde, V. Bondarenko, V. Sviridenko, Prophylaxis of Salmonella abortus ovis-induced abortion of sheep by a Salmonella typhimurium live vaccine, Vaccine 10 (5) (1992) 337–340.
[4] M. Cagiola, G. Severi, K. Forti, M. Menichelli, P. Papa, A. De Giuseppe, P. Pasquali, Abortion due to Salmonella enterica serovar Abortusovis (S. Abortusovis) in ewes is associated to a lack of production of IFN-gamma and can be prevented by immunization with inactivated S. Abortusovis vaccine, Vet. Microbiol. 121 (3–4) (2007) 330–337.
[5] I. Luque, A. Echeita, J. Leon, S. Herrera-Leon, C. Tarradas, R. Gonzalez-Sanz, B. Huerta, R.J. Astorga, Salmonella Indiana as a cause of abortion in ewes: genetic diversity and resistance patterns, Vet. Microbiol. 134 (3–4) (2009) 396–399.
[6] L.F. Costa, T.A. Paixao, R.M. Tsolis, A.J. Baumler, R.L. Santos, Salmonellosis in cattle: advantages of being an experimental model, Res. Vet. Sci. 93 (1) (2012) 1–6.
[7] B. Pejcic-Karapetrovic, K. Gurnani, M.S. Russell, B.B. Finlay, S. Sad, L. Krishnan, Pregnancy impairs the innate immune resistance to Salmonella typhimurium leading to rapid fatal infection, J. Immunol. 179 (9) (2007) 6088–6096.
[8] A. Chattopadhyay, N. Robinson, J.K. Sandhu, B.B. Finlay, S. Sad, L. Krishnan, Salmonella enterica serovar Typhimurium-induced placental inflammation and not bacterial burden correlates with pathology and fatal maternal disease, Infect. Immun. 78 (5) (2010) 2292–2301.
[9] A.R. Scialli, T.L. Rarick, Salmonella sepsis and second-trimester pregnancy loss, Obstet. Gynecol. 79 (1992) 820–821 5 (Pt 2).
[10] L.B. Coughlin, J. McGuigan, N.G. Haddad, P. Mannion, Salmonella sepsis and

miscarriage, Clin. Microbiol. Infect. 9 (8) (2003) 866–868.
[11] R.L. Schloesser, V. Schaefer, A.H. Groll, Fatal transplacental infection with non-typhoidal Salmonella, Scand. J. Infect. Dis. 36 (10) (2004) 773–774.
[12] A. Gyang, M. Saunders, Salmonella Mississippi: a rare cause of second trimester miscarriage, Arch. Gynecol. Obstet. 277 (5) (2008) 437–438.
[13] M.B. Vigliani, A.I. Bakardjiev, First trimester typhoid Fever with vertical transmission of salmonella typhi, an intracellular organism, Case Rep. Med. 2013 (2013) 973297.
[14] B. Rai, T. Utekar, R. Ray, Preterm delivery and neonatal meningitis due to transplacental acquisition of non-typhoidal Salmonella serovar montevideo, BMJ Case Rep. 2014 (2014).
[15] E.A. Reddy, A.V. Shaw, J.A. Crump, Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis, Lancet Infect. Dis. 10 (6) (2010) 417–432.
[16] N.A. Feasey, G. Dougan, R.A. Kingsley, R.S. Heyderman, M.A. Gordon, Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa, Lancet 379 (9835) (2012) 2489–2499.
[17] E. Scallan, R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones, P.M. Griffin, Foodborne illness acquired in the United States–major pathogens, Emerg. Infect. Dis. 17 (1) (2011) 7–15.
[18] O. Gal-Mor, E.C. Boyle, G.A. Grassl, Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ, Front. Microbiol. 5 (2014) 391.
[19] J.E. Galan, A. Collmer, Type III secretion machines: bacterial devices for protein delivery into host cells, Science 284 (5418) (1999) 1322–1328.
[20] S.L. Marcus, J.H. Brumell, C.G. Pfeifer, B.B. Finlay, Salmonella pathogenicity islands: big virulence in small packages, Microb. Infect. 2 (2) (2000) 145–156.
[21] J.E. Galan, H. Wolf-Watz, Protein delivery into eukaryotic cells by type III secretion machines, Nature 444 (7119) (2006) 567–573.
[22] F. Garcia-del Portillo, J.W. Foster, M.E. Maguire, B.B. Finlay, Characterization of the micro-environment of Salmonella typhimurium-containing vacuoles within MDCK epithelial cells, Mol. Microbiol. 6 (22) (1992) 3289–3297.
[23] M.A. Bakowski, V. Braun, J.H. Brumell, Salmonella-containing vacuoles: directing traffic and nesting to grow, Traffic 9 (12) (2008) 2022–2031.
[24] O. Steele-Mortimer, The Salmonella-containing vacuole: moving with the times, Curr. Opin. Microbiol. 11 (1) (2008) 38–45.
[25] C.R. Beuzon, S.P. Salcedo, D.W. Holden, Growth and killing of a Salmonella enterica serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines, Microbiology 148 (Pt 9) (2002) 2705–2715.
[26] J.H. Brumell, C.M. Rosenberger, G.T. Gotto, S.L. Marcus, B.B. Finlay, SifA permits survival and replication of Salmonella typhimurium in murine macrophages, Cell Microbiol. 3 (2) (2001) 75–84.
[27] L.A. Knodler, B.A. Vallance, J. Celli, S. Winfree, B. Hansen, M. Montero, O. Steele-Mortimer, Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia, Proc. Natl. Acad. Sci. U. S. A. 107 (41) (2010) 17733–17738.
[28] S.R. Waterman, D.W. Holden, Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system, Cell Microbiol. 5 (8) (2003) 501–511.
[29] J.A. Ibarra, O. Steele-Mortimer, Salmonella–the ultimate insider. Salmonella virulence factors that modulate intracellular survival, Cell Microbiol. 11 (11) (2009) 1579–1586.
[30] A. Moffett, C. Loke, Immunology of placentation in eutherian mammals, Nat. Rev. Immunol. 6 (8) (2006) 584–594.
[31] J.R. Robbins, K.M. Skrzypczynska, V.B. Zeldovich, M. Kapidzic, A.I. Bakardjiev, Placental syncytiotrophoblast constitutes a major barrier to vertical transmission of Listeria monocytogenes, PLoS Pathog. 6 (1) (2010) e1000732.
[32] J.R. Robbins, V.B. Zeldovich, A. Poukchanski, J.C. Boothroyd, A.I. Bakardjiev, Tissue barriers of the human placenta to infection with Toxoplasma gondii, Infect. Immun. 80 (1) (2012) 418–428.
[33] V.B. Zeldovich, C.H. Clausen, E. Bradford, D.A. Fletcher, E. Maltepe, J.R. Robbins, A.I. Bakardjiev, Placental syncytium forms a biophysical barrier against pathogen invasion, PLoS Pathog. 9 (12) (2013) e1003821.
[34] H. Koi, J. Zhang, A. Makrigiannakis, S. Getsios, C.D. MacCalman, J.F. Strauss 3rd, S. Parry, Syncytiotrophoblast is a barrier to maternal-fetal transmission of herpes simplex virus, Biol. Reprod. 67 (5) (2002) 1572–1579.
[35] A. Bayer, N.J. Lennemann, Y. Ouyang, J.C. Bramley, S. Morosky, E.T. Marques Jr., S. Cherry, Y. Sadovsky, C.B. Coyne, Type III interferons produced by human placental trophoblasts confer protection against Zika virus infection, Cell Host Microbe 19 (5) (2016) 705–712.
[36] E. Delorme-Axford, R.B. Donker, J.F. Mouillet, T. Chu, A. Bayer, Y. Ouyang, T. Wang, D.B. Stolz, S.N. Sarkar, A.E. Morelli, Y. Sadovsky, C.B. Coyne, Human placental trophoblasts confer viral resistance to recipient cells, Proc. Natl. Acad. Sci. U. S. A. 110 (29) (2013) 12048–12053.
[37] T. Nguyen, N. Robinson, S.E. Allison, B.K. Coombes, S. Sad, L. Krishnan, IL-10 produced by trophoblast cells inhibits phagosome maturation leading to profound intracellular proliferation of Salmonella enterica Typhimurium, Placenta 34 (9) (2013) 765–774.
[38] J.C. Choi, R. Holtz, M.G. Petroff, N. Alfaidy, S.P. Murphy, Dampening of IFN-gamma-inducible gene expression in human choriocarcinoma cells is due to phosphatase-mediated inhibition of the JAK/STAT-1 pathway, J. Immunol. 178 (3) (2007) 1598–1607.
[39] A.L. Radtke, J.W. Wilson, S. Sarker, C.A. Nickerson, Analysis of interactions of Salmonella type three secretion mutants with 3-D intestinal epithelial cells, PLoS One 5 (12) (2010) e15750.
[40] M.E. Bushway, S.A. Gerber, B.M. Fenton, R.K. Miller, E.M. Lord, S.P. Murphy, Morphological and phenotypic analyses of the human placenta using whole mount immunofluorescence, Biol. Reprod. 90 (5) (2014) 110.
[41] A. Haque, F. Bowe, R.J. Fitzhenry, G. Frankel, M. Thomson, R. Heuschkel, S. Murch, M.P. Stevens, T.S. Wallis, A.D. Phillips, G. Dougan, Early interactions of Salmonella enterica serovar typhimurium with human small intestinal epithelial explants, Gut 53 (10) (2004) 1424–1430.
[42] O. Genbacev, K.D. Jensen, S.S. Powlin, R.K. Miller, In vitro differentiation and ultrastructure of human extravillous trophoblast (EVT) cells, Placenta 14 (4) (1993) 463–475.
[43] O. Genbacev, T.E. White, C.E. Gavin, R.K. Miller, Human trophoblast cultures: models for implantation and peri-implantation toxicology, Reprod. Toxicol. 7 (Suppl 1) (1993) 75–94.
[44] O. Genbacev, S.S. Powlin, R.K. Miller, Regulation of human extravillus trophoblast (EVT) cell differentiation and proliferation in vitro-role of epidermal growth factor (EGF), Placenta 15 (Supplement 1) (1994) 427–442.
[45] R.K. Miller, O. Genbacev, M.A. Turner, J.D. Aplin, I. Caniggia, B. Huppertz, Human placental explants in culture: approaches and assessments, Placenta 26 (6) (2005) 439–448.
[46] S. Yacobi, A. Ornoy, Z. Blumenfeld, R.K. Miller, Effect of sera from women with systemic lupus erythematosus or antiphospholipid syndrome and recurrent abortions on human placental explants in culture, Teratology 66 (6) (2002) 300–308.
[47] S.K. Hoiseth, B.A. Stocker, Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines, Nature 291 (5812) (1981) 238–239.
[48] S.K. Lathrop, K.A. Binder, T. Starr, K.G. Cooper, A. Chong, A.B. Carmody, O. Steele-Mortimer, Replication of Salmonella enterica serovar typhimurium in human monocyte-derived macrophages, Infect. Immun. 83 (7) (2015) 2661–2671.
[49] C.L. Birmingham, A.C. Smith, M.A. Bakowski, T. Yoshimori, J.H. Brumell, Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole, J. Biol. Chem. 281 (16) (2006) 11374–11383.
[50] M. Lorkowski, A. Felipe-Lopez, C.A. Danzer, N. Hansmeier, M. Hensel, Salmonella enterica invasion of polarized epithelial cells is a highly cooperative effort, Infect. Immun. 82 (6) (2014) 2657–2667.
[51] O. Genbacev, S.A. Schubach, R.K. Miller, Villous culture of first trimester human placenta–model to study extravillous trophoblast (EVT) differentiation, Placenta 13 (5) (1992) 439–461.
[52] T.M. Mayhew, C. Ohadike, P.N. Baker, I.P. Crocker, C. Mitchell, S.S. Ong, Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction, Placenta 24 (2–3) (2003) 219–226.
[53] S.U. Holzer, M. Hensel, Divergent roles of Salmonella pathogenicity island 2 and metabolic traits during interaction of S. enterica serovar typhimurium with host cells, PLoS One 7 (3) (2012) e33220.
[54] M. Rosselin, I. Virlogeux-Payant, C. Roy, E. Bottreau, P.Y. Sizaret, L. Mijouin, P. Germon, E. Caron, P. Velge, A. Wiedemann, Rck of Salmonella enterica, subspecies enterica serovar enteritidis, mediates zipper-like internalization, Cell Res. 20 (6) (2010) 647–664.
[55] A. Wiedemann, L. Mijouin, M.A. Ayoub, E. Barilleau, S. Canepa, A.P. Teixeira-Gomes, Y. Le Vern, M. Rosselin, E. Reiter, P. Velge, Identification of the epidermal growth factor receptor as the receptor for Salmonella Rck-dependent invasion, FASEB J. 30 (12) (2016) 4180–4191.
[56] V.B. Zeldovich, A.I. Bakardjiev, Host defense and tolerance: unique challenges in the placenta, PLoS Pathog. 8 (8) (2012) e1002804.
[57] A. Bayer, E. Delorme-Axford, C. Sleigher, T.K. Frey, D.W. Trobaugh, W.B. Klimstra, L.A. Emert-Sedlak, T.E. Smithgall, P.R. Kinchington, S. Vadia, S. Seveau, J.P. Boyle, C.B. Coyne, Y. Sadovsky, Human trophoblasts confer resistance to viruses implicated in perinatal infection, Am. J. Obstet. Gynecol. 212 (1) (2015) 71 e1–71 e8.
[58] S.E. Ander, E.N. Rudzki, N. Arora, Y. Sadovsky, C.B. Coyne, J.P. Boyle, Human placental syncytiotrophoblasts restrict Toxoplasma gondii attachment and replication and respond to infection by producing immunomodulatory chemokines, mBio 9 (1) (2018).
[59] D.A. Portnoy, V. Auerbuch, I.J. Glomski, The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity, J. Cell Biol. 158 (3) (2002) 409–414.
[60] E. Mylonakis, M. Paliou, E.L. Hohmann, S.B. Calderwood, E.J. Wing, Listeriosis during pregnancy: a case series and review of 222 cases, Medicine (Baltim.) 81 (4) (2002) 260–269.
[61] M. Lecuit, D.M. Nelson, S.D. Smith, H. Khun, M. Huerre, M.C. Vacher-Lavenu, J.I. Gordon, P. Cossart, Targeting and crossing of the human maternofetal barrier by Listeria monocytogenes: role of internalin interaction with trophoblast E-cadherin, Proc. Natl. Acad. Sci. U. S. A. 101 (16) (2004) 6152–6157.
[62] H. Elinav, A. Hershko-Klement, L. Valinsky, J. Jaffe, A. Wiseman, H. Shimon, E. Braun, Y. Paitan, C. Block, R. Sorek, R. Nir-Paz, G. Israeli Listeria Study, Pregnancy-associated listeriosis: clinical characteristics and geospatial analysis of a 10-year period in Israel, Clin. Infect. Dis. 59 (7) (2014) 953–961.
[63] L.A. O'Neill, D. Golenbock, A.G. Bowie, The history of Toll-like receptors - redefining innate immunity, Nat. Rev. Immunol. 13 (6) (2013) 453–460.
[64] K. Kumazaki, M. Nakayama, I. Yanagihara, N. Suehara, Y. Wada, Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis, Hum. Pathol. 35 (1) (2004) 47–54.
[65] V.M. Abrahams, G. Mor, Toll-like receptors and their role in the trophoblast, Placenta 26 (7) (2005) 540–547.
[66] G. Mor, Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction, Ann. N. Y. Acad. Sci. 1127 (2008) 121–128.
[67] K. Koga, P.B. Aldo, G. Mor, Toll-like receptors and pregnancy: trophoblast as modulators of the immune response, J. Obstet. Gynaecol. Res. 35 (2) (2009) 191–202.
[68] P. Lye, E. Bloise, M. Javam, W. Gibb, S.J. Lye, S.G. Matthews, Impact of bacterial

and viral challenge on multidrug resistance in first- and third-trimester human placenta, Am. J. Pathol. 185 (6) (2015) 1666–1675.
[69] V.M. Abrahams, P. Bole-Aldo, Y.M. Kim, S.L. Straszewski-Chavez, T. Chaiworapongsa, R. Romero, G. Mor, Divergent trophoblast responses to bacterial products mediated by TLRs, J. Immunol. 173 (7) (2004) 4286–4296.
[70] L. Li, J. Tu, Y. Jiang, J. Zhou, S. Yabe, D.J. Schust, Effects of lipopolysaccharide on human first trimester villous cytotrophoblast cell function in vitro, Biol. Reprod. 94 (2) (2016) 33.
[71] R. Raghupathy, Pregnancy: success and failure within the Th1/Th2/Th3 paradigm, Semin. Immunol. 13 (4) (2001) 219–227.