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IL-10 Produced by Trophoblast Cells Inhibits Phagosome Maturation Leading to Profound Intracellular Proliferation of *Salmonella enterica* Typhimurium

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Abstract

Introduction—*Salmonella enterica* Typhimurium (ST) is a phagosomal pathogen that can infect placental trophoblast cells leading to abortion and severe maternal illness. It is unclear how the trophoblast cells promote profound bacterial proliferation.

Methods—The mechanism of internalization, intracellular growth and phagosomal biogenesis in ST-infected human epithelial (HeLa), macrophage (THP-1) and trophoblast-derived cell lines (JEG-3, BeWo and HTR-8) was studied. Specific inhibitors were used to block bacterial internalization. Phagosomal maturation was determined by confocal microscopy, western-blotting and release of lysosomal α -galactosidase by infected cells. Bacterial colony forming units were determined by plating infected cell lysates on agar plates.

Results—ST proliferated minimally in macrophages but replicated profoundly within trophoblast cells. The *ST- invA* (a mutant of *Salmonella* pathogenicity island-1 gene effector proteins) was unable to infect epithelial cells, but was internalized by scavenger receptors on trophoblasts and macrophages. However, ST was contrastingly localized in early (Rab5⁺) or late (LAMP1⁺) phagosomes within trophoblast cells and macrophages respectively. Furthermore trophoblast cells (unlike macrophages) did not exhibit phagosome-lysosomal fusion. ST-infected macrophages produced IL-6 whereas trophoblast cells produced IL-10. Neutralizing IL-10 in JEG-3 cells accelerated phagolysosomal fusion and reduced proliferation of ST. Placental bacterial burden was curtailed in vivo in anti-IL-10 antibody treated and IL-10-deficient mice.

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Discussion—Macrophages phagocytose but curtail intracellular replication of ST in late phagosomes. In contrast, phagocytosis by trophoblast cells results in an inappropriate cytokine response and proliferation of ST in early phagosomes.

Conclusion—IL-10 production by trophoblast cells that delays phagosomal maturation may facilitate proliferation of pathogens in placental cells.

Keywords

trophoblast cells; *Salmonella* Typhimurium; pregnancy; interleukin 10; phagosomal maturation; immune response

Introduction

Salmonella serovars are highly virulent re-emerging food-borne pathogens causing huge economic losses worldwide. In humans, *Salmonella enterica* serovar Typhi causes typhoid fever, while *Salmonella enterica* serotype Typhimurium causes gastroenteritis [1]. However, non typhoidal systemic fever caused by *S. Typhimurium* is increasing in prevalence among the immuno-compromised, including HIV-infected individuals [2]. *Salmonella* also cause pregnancy complications such as chorioamnionitis, trans-placental infection, abortions, neonatal and maternal septicemia in humans [3–5] and pregnancy loss in livestock [6;7].

Salmonella are facultative intracellular Gram-negative bacteria that reside within modified phagosomes of a cell known as the *Salmonella* containing vacuoles (SCV) [8]. *Salmonella* encodes two Type III secretion systems (TTSS) within *Salmonella* pathogenicity island 1 and 2 (SPI1 and SPI2) genes [1]. The SPI1-TTSS assembles a needle that injects effector proteins directly into the host cells, causing membrane ruffling allowing *Salmonella* to invade even non-phagocytic cells. In contrast, the SPI2-TTSS effector proteins modify the biogenesis of SCVs to support intracellular growth and proliferation of *Salmonella* [8]. Thus, *Salmonella* virulence mechanisms effectively evade host immunity leading to chronic infection [9].

The placenta acts as a physical and immunological barrier to many invading pathogens [10]. The two potential sites of pathogen entry are the syncytiotrophoblast-maternal blood interface, and extravillous-trophoblast-uterine junction [10]. In human organ cultures, the syncytiotrophoblast is often resistant to infection with diverse pathogens such as *Listeria monocytogenes*, *Trypanosoma gondii*, *Herpes simplex* virus and *Cytomegalovirus* [10–13]. Lack of internalization receptors for some pathogens may provide a molecular basis for syncytiotrophoblast resistance [10]. In contrast, the cytotrophoblasts are often more susceptible to infection by pathogens such as *Listeria*, *Toxoplasma* and *cytomegalovirus* [14;15]. The second potential site of pathogen exposure, the extravillous trophoblast is juxtaposed with maternal immune cells within the decidua capable of providing protection. Furthermore, the expression of Toll-like receptors (TLR) by the human placenta is regulated spatially and temporally limited to inner cytotrophoblast layers and extravillous trophoblasts, thus conferring host defence properties to these cells [16]. Overall, the placenta provides an efficient barrier, and only pathogens that breach the outer TLR-negative syncytiotrophoblast are able to evoke inflammatory damage [17].

Phagocytic cells such as macrophages actively internalize pathogens and particles by phagocytosis. Subsequently, the newly formed phagosome matures by a series of interactions with endocytic vesicles, eventually fusing with lysosomes. Phagolysosome formation is essential for the degradation of intracellular pathogens [18]. Phagocytosis is also exhibited by placental cells, and facilitates uterine invasion and uptake of pathogens [19]. Previously, we showed that the placental cells allow profound intracellular

proliferation of *S. Typhimurium* [9]. Herein, we demonstrate that trophoblast cells actively uptake *Salmonella* by receptor-mediated endocytosis. However, IL-10 production by trophoblasts prevents maturation of the SCV culminating in profound bacterial proliferation with early phagosomes.

Materials and Methods

Bacterial strains

S. enterica serotype Typhimurium strain SL1344 was used throughout (abbreviated ST). Virulent wild-type ST (ST-WT) strain and the mutant strains *invA* were a gift from Dr. Brett Finlay (University of British Columbia, Vancouver, Canada). The deletion of *rck* in wild-type SL1344 (*rck*) or *invA*:kan (BKC1–5) backgrounds (*invArck*) was performed as described by [20]. Briefly, primers *rck-F* and *rck-R* were used with Vent polymerase to amplify from pKD3. The resulting PCR product was concentrated and transformed into wild-type SL1344 or *invA*:kan strains containing pKD46. Deletion strains generated were confirmed by PCR using the primers listed in supplementary Table 1. Bacterial strains were grown in liquid culture in brain-heart infusion (BHI) medium (Difco Laboratories). At mid-log phase (OD₆₀₀ = 0.8), bacteria were harvested and frozen at –80°C (in 20% glycerol). Colony forming units (CFU) were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI-agar plates.

Cell lines

HeLa, human epithelial cells; THP-1, human monocytic cells; JEG-3 and BeWo, human choriocarcinoma cells were obtained from American Type culture collection (ATCC). HTR-8, human trophoblast cell line was a gift from Dr. Andre Gruslin (Ottawa Health Research Institute, Ottawa, Canada). All cells were grown in RPMI-1640 supplemented with 8 % fetal bovine serum (FBS) and gentamycin (10 µg/ml) at 37°C in 5% CO₂. THP-1 (5 × 10⁵/ml) cells were differentiated to macrophages following exposure to 1 nM phorbol-12-myristate-13-acetate (PMA) for 24 hours. JEG-3 cells were also similarly exposed to PMA in some experiments.

In vitro bacterial infection for intracellular entry and proliferation

Cells were seeded in 24-well plate at a density of 5 × 10⁵ cells/well in RPMI + 8% FBS medium. After 24 hours, cells were infected for 30 min with ST strains at a multiplicity of infection (MOI) of 10. In some studies cells were pre-incubated with inhibitors or antibodies for 1.5 h prior to, and during the 30 min infection pulse. The inhibitors used included; actin polymerization inhibitors cytochalasin B and D (Sigma, St Louis, MO), PI3K inhibitors LY294002 and Wortmannin (Sigma, St Louis, MO), the pan-scavenger receptor inhibitor fucoidan (Sigma, St Louis, MO), mannose receptor inhibitor soluble mannan (Sigma, St Louis, MO). Rat anti-human IL-10 antibody or rat IgG2a isotype antibody was purchased from eBioscience (San Deigo, CA).

After infection, cells were washed thrice with RPMI medium and then incubated for 2 h in RPMI containing 8% FBS and gentamicin (50 µg/ml) to remove extracellular ST. Representative cell culture wells were lysed with 0.1% Triton X-100 + 0.01% SDS in phosphate buffered saline (PBS) and serial dilutions were spread onto BHI agar plates to enumerate number of internalized intracellular bacteria. The remaining culture wells were transferred to RPMI medium containing a lower dose of gentamycin (10 µg/ml). To determine bacterial proliferation, cell lysates were plated on BHI-agar plates at various times after infection. Doubling time was calculated using the formula $G = t(3.3 \times \log b/B)$, where G is the generation time, t is the time elapsed, B is the CFU at the start time, and b is the CFU at the end time.

Immunofluorescence assays for phagosome maturation markers

THP-1 and JEG-3 (10^4 cells) were seeded onto glass coverslips and were infected with ST as described above. Cells were fixed at early (5 minutes) and late (60 minutes) time points post-infection with freshly prepared 4% paraformaldehyde for 15 min and then washed three times in PBS. The cells were blocked and permeabilized with PBS containing 5% normal goat serum (NGS) with 0.3% Triton X-100 for 1 h. The cells were then incubated with primary antibodies overnight at 4°C. Primary antibodies used were mouse anti-*Salmonella* Typhimurium lipopolysaccharide (LPS) and anti-LAMP-1 (Pierce Thermo Scientific, Rockford, IL); rabbit anti-Rab5, anti-Rab7 (Cell Signaling Technologies, Danvers MA) all diluted 1:100. Cells were washed with PBS three times and incubated with Alexa Fluor 488 (Rab5, Rab7, and LAMP) and Alexa Fluor 594 (*Salmonella* Typhimurium LPS)-coupled goat anti-rabbit secondary antibodies (1:200) for 1 h. Cells were washed three times with PBS + 0.03% Triton X100, stained with Hoechst, and imaged using a confocal microscope (Olympus Fluoview FV1000).

Western Blot Analysis

THP-1, JEG-3 or BeWO cells (3×10^6) were seeded onto 60 mm culture dishes. Tosylactivated magnetic beads (Invitrogen, Grand Island, NY) or ST were added to the cells for 30 min. Then extracellular beads were removed by washing the cells 3 times with RPMI. Phagosomes were isolated by swelling the cells, disrupting the cell membrane followed by magnetic isolation as described previously [21]. An equal amount of phagosomal protein sample was separated on 10% SDS-polyacrylamide gel and the proteins were transferred to a polyvinyl difluoride (PVDF) membrane (BioRad, Mississauga, ON). The membrane was blocked in TRIS-buffered saline (TBS) containing 5% dried milk powder (w/v) and 0.1% Tween-20, for 1 h at room temperature. After three washes with TBS-0.1% Tween-20, the PVDF membranes were incubated with primary antibodies against Rab5, Rab7 (Cell Signaling Technologies), and or cathepsin D (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was used at 1:2000 dilution overnight at 4°C. The membranes were washed thoroughly for 30 min with TBS-0.1% Tween before incubation with 1:5000 dilution of the secondary HRP-conjugated immunoglobulin for 1 h at room temperature and further washing for 30 min with TBS-0.1% Tween-20 followed by development.

Cytokine analysis and RT-PCR

Supernatants were collected after 24 h of infection and concentrated using Amicon Ultra-0.5 centrifugal filter 3K devices (Millipore, Billerica, MA). Secretion of IL-6 was determined by a sandwich ELISA, using antibody pairs (purified anti-human IL-6 clone MQ2-13A5 and MQ2-39C3) purchased from BD Pharmingen™. IL-10 expression was determined by q-RT PCR. Total RNA was isolated from non-infected or infected cells using the RNeasy mini kit (Qiagen). 500 ng of RNA was reversed transcribed with 0.2 µg oligo(dT). The prepared complementary DNA was subjected to quantitative RT-PCR with Fast SYBR Green Mastermix (Applied Biosystems) and with hominid-specific primers for β -actin, and IL-10 (Supplementary Table 1). Transcription levels of target genes were assayed in triplicate, expression levels was normalized β -actin and the mRNA quantified by C_t method.

The expression of macrophage scavenger receptor 1 (MSR1) was evaluated by semiquantitative PCR. Briefly, for cDNA production, equal amounts of RNA were reverse-transcribed with the following primer pairs for MSR1 (supplementary Table 1) and expression at thermocycles 20–30 were compared with β -actin. Either a water control for the PCR, or an RT reaction using RNA without the reverse transcriptase (to check for genomic DNA contamination), were performed as controls.

β-Galactosidase assay by flow cytometry for phagosome maturation kinetics

The phagosome maturation kinetics was performed as described previously [21]. Briefly, tosylactivated magnetic beads or ST were coated with a lipophilic (C12) derivative of fluorescein-di-beta-D-galactopyranoside (FDG) for 1 hour at 37°C with sonication every 10 minutes to prevent clumping. C12FDG coated beads or C12FDG-ST was added to the cells for 30 mins and extracellular beads or bacteria were removed with cold RPMI wash. Cells were fixed at various times and acquired using BD FACSCanto and data were analysed using FlowJo® software. Phagosome maturation kinetics was determined based on increased mean fluorescence intensity (MFI) due to cleavage of FDG by lysosomal α -galactosidase.

In vivo ST infection

C57BL/6J and homozygous IL-10-deficient (B6.129P2-IL10tm1Cgn/J) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Non-pregnant and age-matched pregnant mice (days 11–12 of gestation) were infected intravenously with 1000 CFU of ST-WT suspended in 200 μ l of 0.9% NaCl. Anti-IL10 antibody (eBioscience, San Diego, CA) was administered to the wild-type mice intraperitoneally twice; a day prior to, and on the day of infection (100 μ g/injection). Mice were euthanized 24 h later and bacterial burden in infected placenta determined. Animals were maintained in accordance with the approved NRC animal use protocol 2010.04 and guidelines of the Canadian Council of Animal Care.

Statistical analysis

Data were analyzed by two-way ANOVA or student *t* test as indicated in the figure legends using PRISM® software.

Results

ST proliferates profoundly in epithelial cells but not in macrophages

Fig. 1a shows the intracellular bacterial growth of wild-type *Salmonella Typhimurium* (ST-WT) and mutant strains (ST- *invA*, *rck*, *rck invA*) in HeLa (uterine epithelial cells) and THP-1 (monocytes) cells. The first data point at 2 h shows internalization of ST-WT and ST- *rck* (a ST outer membrane protein invasion mutant) efficiently by both HeLa and THP-1 cells. In contrast, ST- *invA* (a SPI-1 mutant) or the double mutant ST- *rck invA* lacked the ability to invade epithelial HeLa cells (but not macrophages). This suggested that type III-dependent mechanisms primarily aid infection of epithelial cells whereas macrophages may engulf the bacteria by alternate mechanisms such as phagocytosis. Fig. 1a also shows that a greater number of ST WT gained entry into the THP-1 cells upon activation with PMA (that causes differentiation of monocytes to macrophages). Fig. 1b shows the doubling time of ST-WT in HeLa cells was rapid whereas it was much slower (5–13 h) in THP-1 cells, indicating the ability of these cells to curtail intracellular bacterial expansion particularly upon activation. Figure 1c demonstrates that ST-WT was efficiently internalized by JEG-3, BeWo and HTR-8 trophoblast-derived cell lines (Fig. 1c), and exhibited profound intracellular proliferation, with a doubling time of <2 h (Fig. 1d). Unexpectedly, ST- *invA* and ST- *rck* mutants also efficiently entered trophoblast cell lines (Fig. 1c), suggesting that trophoblast cells similar to macrophages engulf the bacteria by alternate mechanisms.

Trophoblast cells internalize ST by scavenger receptor-mediated phagocytosis

Next, we deciphered the relative percentage of ST that entered into various cell types in the presence of inhibitors (that block specific processes involved in phagocytosis) relative to control untreated (cells in DMSO medium used to solubilize inhibitors) cultures. We also confirmed that the viability of cells (by MTT assay) in the presence of the highest tested

dose of inhibitor in DMSO was not compromised (Supplementary Fig. 1). Firstly, cytochalasin B and cytochalasin D significantly inhibited the entry of ST into THP-1 and JEG-3 cells (Fig. 2a and b) relative to entry into HeLa cells. Cytochalasin inhibits actin filament re-arrangement, a process that is required for early events of phagocytosis. Actin filament re-arrangement is also modulated by bacterial effector proteins as a late event following injection of the TTSS needle apparatus by *Salmonella*. Thus, cytochalasin may inhibit late events of TTSS-mediated entry of ST. Next, Wortmanin and LY294002, inhibitors of PI3K which is essential for the uptake by phagocytosis and maturation of phagosomes blocked entry of ST into JEG-3 cells (Fig. 2c and d). Finally, fucoidan (a pan scavenger receptor inhibitor) but not mannan (mannose receptor inhibitor) significantly blocked entry of ST into THP-1 and JEG-3 cells (Fig. 2e and f). From these data we deduced that JEG-3 cells internalized ST through scavenger receptor-mediated endocytosis. Similar results were observed for BeWo trophoblast cells (data not shown).

ST thrives within contrasting vacuolar niche in macrophages and trophoblasts

Next, cells were infected with ST-WT, and fixed after either 5 min (early) or 60 mins (late) time points for assessment of intracellular co-localization of ST with markers of phagosomal maturation by immunofluorescence. In THP-1 macrophages, at 1 h post-infection ST containing vacuoles exhibited weak Rab5 staining, a marker of the early endosome, whereas co-localization with Rab7 and Lysosomal activating membrane protein (LAMP-1, a late endosomal marker) was clearly evident (Fig. 3a). In contrast, in JEG-3 cells, ST-containing vesicles exhibited strong Rab5 staining at 1h post-infection and poor LAMP-1 staining (Fig. 3b). By enumerating 100 fluorescent intracellular bacteria and their co-association with various markers, we quantified the percentage of ST containing vesicles that also expressed specific endosomal proteins. The composite analysis of such images from 3 independent experiments indicates contrasting state of phagosomal maturation in ST-infected THP-1 (late phagosomes) and JEG-3 (early phagosomes) cells (Fig. 3c).

ST inhibits phagosomal maturation in trophoblasts

We next characterized the expression of proteins in isolated phagosomes by Western blotting. Fig. 4a is representative western blot of phagosomes isolated from ST infected cells. The data from three different experiments were quantified and are presented in graphic format in Fig 4b. Collectively, the data indicate that phagosomes of JEG-3 cells exhibited strong Rab5 expression and relatively reduced Rab7 expression at 5 and 60 minutes after ST infection. In contrast, phagosomes from ST-infected THP-1 cells exhibited stronger expression of the intermediate endosomal protein Rab7 but reduced expression of Rab5. Next, the phagosomes of ST-infected JEG-3 cells exhibited primarily procathepsin D, even at 60 minutes post-infection. Furthermore, activation of JEG-3 cells with PMA did not result in cleavage of procathepsin D to its active form. In contrast, phagosomes of THP-1 cells showed cleavage of procathepsin D to its mature form, suggesting progression to mature phagosomes (Fig. 4a). When THP-1 cells were differentiated into macrophages with PMA prior to infection, although diminished expression of procathepsin D (in comparison to JEG-3) was evident, mature cathepsin D could not be detected (Fig. 4a). However, ST can manipulate the phagosome maturation process by sequestering cathepsin D from mature phagosomes [8]. Overall these results also indicate that phagosomal maturation is curtailed in ST-infected trophoblast cells and this could not be rescued by activation with PMA.

Next we assessed the phagosomal maturation process following internalization of inert beads. Fig. 4c demonstrates the expression of Rab5 in the phagosomes isolated from JEG-3 cells, whereas THP-1 cell phagosomes lacked expression of this early marker. In contrast, the mature form of cathepsin D was observed in the phagosomes of THP-1 cells but not in JEG-3 cells (Fig. 4c). However, when JEG-3 cells were activated with PMA prior to uptake

of beads, cleavage of procathepsin D to its active form was evident. The quantification of the western blot data from 3 independent experiments is shown in Fig. 4d. Thus, trophoblast cells were inherently impaired in activating the phagosomal maturation machinery. Early phagosomes were also observed in BeWo trophoblast cells after uptake of ST or beads (data not shown).

Trophoblasts fail to achieve phagosomal-lysosomal fusion

Phagosomal-lysosomal fusion was evaluated following incubation with tosyl-activated beads-coated C₁₂-di-beta-D-galactopyranoside (FDG) or infection with ST-coated C₁₂FDG (Fig 5). FDG is a self-quenched non-fluorescent galactopyranoside that is hydrolyzed by α -galactosidase found in the lysosomes, to release a fluorescent product. As a result, fluorescence is observed only upon phagosomal-lysosomal fusion, the ultimate event in phagosomal maturation. The flowcytometric profile of cells at various time points after internalization of C₁₂FDG coated-ST or -beads are shown in Fig. 5a and 5b respectively. Mean fluorescent intensity (MFI), increased with phagosomal maturation in THP-1 macrophages following uptake of ST or inert beads (Fig. 5c and 5d). In contrast, JEG-3 cells did not exhibit increased MFI even after 1 hour of ST infection (Fig. 5c) or uptake of beads (5d), suggesting delayed phagolysosomal fusion.

IL-10 produced by trophoblasts inhibits phagosomal maturation and promotes intracellular proliferation of ST

Trophoblasts secrete type 2 cytokines such as IL-10 known to have anti-inflammatory effects. In contrast, macrophages upon activation produce inflammatory cytokines such as IL-6. THP-1 macrophages infected with ST produced copious amounts of IL-6, whereas JEG-3 cells failed to produce this inflammatory cytokine (Fig. 6a). In contrast, JEG-3 cells infected with ST exhibited increase in IL-10 mRNA expression (Fig. 6b). Treatment of JEG-3 cells with anti-IL-10 antibody increased phagosomal maturation (based on FDG cleavage) following ST infection (Fig. 6c and 6d). Additionally, anti-IL-10 antibody pre-treated JEG-3 cells were more able to curtail ST growth (Fig. 6e) as evidenced by increased doubling time (Fig. 6f). Finally, following in vivo infection, the bacterial burden was significantly higher in the placenta of wild-type mice in comparison to tissues from anti-IL-10 depleted wild-type or IL-10-deficient mice. Thus, IL-10 production by trophoblast cells in response to ST infection appeared to contribute to delayed phagosomal maturation and increased bacterial growth.

Discussion

This study indicates the potential for trophoblast cells to act as a reservoir for *Salmonella* dissemination in the pregnant host. Trophoblastic cell lines have been routinely used as surrogates for human villous and extravillous trophoblast primary cultures [22]. Each cell line represents distinct trophoblast characteristics; JEG-3 resembles the undifferentiated cytotrophoblasts, BeWo exhibits characteristics of villous trophoblast including ability to syncytialize, and HTR-8 is a virus transformed first trimester trophoblast cell line. Many intracellular pathogens productively infect trophoblast cell lines. For example, BeWo cells are more permissive than HeLa cells to *Toxoplasma gondii* and *Neospora caninum* infections [23;24]. The abortive pathogen *Coxiella burnetii* causative agent of Q-fever infects and replicates substantially in BeWo trophoblasts, despite its inability to replicate in macrophages [25]. Similarly, JEG-3 cells were shown to be infected by *Listeria*, *Chlamydia*, Cytomegalovirus and HIV [26–29]. Our study provides a mechanistic insight that delayed phagosomal maturation may support replication of intracellular pathogens within trophoblast cell lines. However, studies with human primary placental cells can further elucidate the specific interaction of ST with specific trophoblast cell types.

The ability of ST to invade specific cell types is attributed to TTSS needle apparatus under the control of the SPI-1 locus [1]. More recently SPI-1 independent invasins such as Rck and PagN have been reported [30]. We however observed that only the ST- *invA* strains were unable to infect HeLa epithelial cells suggesting primarily TTSS-dependent invasion. Trophoblast cells actively internalized ST-WT, ST- *invA* and ST- *rck* strains suggesting a highly active TTSS-independent mode of entry in these cells. Indeed, the initial uptake of ST at 2 h into JEG-3, BeWo and HTR-8 was 2-fold higher than in HeLa cells. Thus, we speculate that active phagocytosis by trophoblasts may negate the need for bacterial invasins.

In rodent and humans with hemochorial placentation, phagocytic ability is a hall-mark of trophoblast differentiation and imparts invasive capacity for tissue remodeling during implantation [31]. The giant trophoblast cells in particular, demonstrate profound phagocytic activity [19]. Trophoblast phagocytic function also facilitates internalization of pathogens such as *Brucella abortus* and *Listeria monocytogenes* [32]. Our collective observations with various inhibitors implicate scavenger receptor-mediated endocytosis as a mechanism of *Salmonella* internalization into JEG-3 cells. Trophoblasts are known to express Class A and B scavenger receptors that are mainly involved in cholesterol transport [33]. Macrophages mediate clearance of many pathogens by utilizing Scavenger Receptor A-mediated phagocytosis [34]. Furthermore, scavenger receptor mediated endocytosis is implicated in the internalization of *C. sordellii* by decidual macrophages [35]; *B. abortus* and *L. monocytogenes* by trophoblast giant cells [36]. We observed that THP-1 and JEG-3 cells strongly expressed MSR1 (Supplementary figure 2) whereas showed weak expression of other scavenger receptors such as MARCO and scavenger receptor B (data not shown) suggesting MSRI-mediated ST internalization. However, as fucoidan induced only ~50 % inhibition, other host cell receptors may also modulate ST entry. Addressing the interaction of scavenger receptors on primary human placental cells with pathogens may reveal mechanism of pathogen tropism.

Various endosome proteins are gained and lost on the way to phagolysosomal maturation and constitute efficient trackers of the vacuolar biogenesis [37]. Previous studies have mapped the biogenesis of SCVs in epithelial cells and macrophages [8]. In general, SCVs associate early on with proteins such as Rab5, indicative of fusion with early endosomes. Later on, SCVs acquire and retain Rab7 and late lysosomal membrane proteins such as LAMP-1, but exclude lysosomal hydrolytic enzymes such as Cathepsins L and D. We characterized phagosomal maturation pathway in the trophoblast cells using three different techniques; immunofluorescence, Western blotting and an assay for phagolysosomal fusion. Similar to other studies, we demonstrate that in THP-1 macrophages, SCVs rapidly recruited late endosomal proteins Rab7, and LAMP-1, yet sequestered cathepsin D which is consistent with *Salmonella* induced evasion of fusion with trans-golgi-recycled endosomes [8]. The key difference in trophoblast cells was; internalized ST remained co-localized with early endocytic proteins Rab5, failed to recruit LAMP-1 and exhibited lack of lysosomal - galactosidase activity. Thus, *Salmonella* are maintained in early phagosomes within trophoblast cells, and phagolysosomal fusion is delayed.

Salmonella expend substantial energy to regulate various virulence genes of the SPI-2 locus to survive within the hostile milieu of phagosomes in macrophages [1]. Indeed, the intracellular replication rate of *Salmonella* is significantly lower (in hours) relative to their rapid extracellular doubling time of minutes [9;38]. A recent study demonstrated that the ability of extravillous trophoblast and BeWo cells to prevent cytosolic escape of *Listeria monocytogenes* from LAMP-1 positive vesicles results in curtailment of bacterial replication [27]. However, *Listeria* is an intracellular cytosolic pathogen which activates the toxin Listeriolysin O in the phagosomes, restricting its ability to survive within these vesicles [39].

In contrast, *Salmonella* are capable of surviving even within harsh acidic phagosomes [40]. Moreover, trophoblast cells exhibited an inherent defect in phagosome biogenesis even after uptake of inert beads. Thus, trophoblast cells appear to conveniently provide a safe early endosomal niche for the phagosomally adapted *Salmonella* wherein profound pathogen proliferation can occur under conditions of minimum stress.

The human immunodeficiency virus (HIV) is internalized by trophoblasts by a clathrin/caveolae/dynamin-independent pathway [41] followed by transition of the virus from Rab5-expressing early endosomes to Rab7 endosomes, and final accumulation of virions in CD63-positive organelles [26]. Thus, similar to *Salmonella*, HIV resides within non-maturing endosomal vesicles in trophoblasts. Characterization of *C. burnetii* containing endocytic compartment in BeWo cells indicated that initially the organisms co-localized with LAMP-1 but not cathepsin D whereas after 6 days post-infection the organisms co-localized with cathepsin D indicating their presence within phagolysosomes [25]. Although, presence of *C. burnetii* in phagolysosomes suggests that trophoblasts may have the ability to fuse with lysosomes at later time points after infection, virulent pathogens such as *Salmonella* cause rapid trophoblast death [9].

The production of IL-10 at the feto-maternal interface *in vivo* has pleiotropic effects for fetal survival. In a murine model, FoxP3⁺ T regulatory cells at the feto-maternal interface increased maternal susceptibility to *Salmonella* and *Listeria* infections due to IL-10 production [42;43]. Human cytotrophoblast cells produce IL-10, although production by choriocarcinoma cells has been controversial [13]. Our data indicate a low level constitutive IL-10 mRNA expression by JEG-3 cells which is upregulated upon ST infection. However, constitutive production of IL-10 protein was not detected in JEG-3 supernatants by ELISA (data not shown). Thus, IL-10 production by JEG-3 cells may be triggered only by certain evasive pathogens such as ST. Indeed, it has been previously shown that over-expression of SOCS3 gene triggers IL-10 production by JEG-3 cells [44]. Neutralizing IL-10 activity strikingly resulted in increased phagosome maturation and curtailment of *Salmonella* replication by JEG-3 cells. The production of IL-10 by macrophages correlated to increased replication of *Mycobacterium tuberculosis* [45]. Moreover, IL-10 blocked phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages [46]. Recently, murine recombinant IL-10 treatment of RAW 264.7 macrophages was shown to increase internalization of ST and bacterial survival [47]. The *in vivo* effect of neutralizing IL-10 is complex as the increased inflammation may provide protective immunity yet IL-10-deficient mice are also more sensitive to the ill-effects of TLR-ligand-induced inflammation during pregnancy [48]. Thus, we studied the effect of IL-10 depletion on bacterial burden within 24 h post-infection to eliminate any bystander effects. The specific decrease in placental bacterial numbers in IL-10 deficient mice reiterates our *in-vitro* observation that IL-10 production by trophoblast may support bacterial growth. Thus, our studies suggest a direct role for IL-10 produced by trophoblasts in modulating susceptibility to *Salmonella* infections and may have implications for other placental-tropic pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ST	<i>Salmonella enterica</i> Typhimurium
SCV	<i>Salmonella</i> containing vacuole
LAMP-1	lysosomal activating membrane protein-1
TTSS	Type III secretion system
SPI	<i>Salmonella</i> pathogenicity Island
CFU	colony forming unit

Reference List

1. Valdez Y, Ferreira RB, Finlay BB. Molecular mechanisms of *Salmonella* virulence and host resistance. *Curr Top Microbiol Immunol*. 2009; 337:93–127. [PubMed: 19812981]
2. Gordon MA. Invasive nontyphoidal *Salmonella* disease: epidemiology, pathogenesis and diagnosis. *Curr Opin Infect Dis*. 2011 Oct; 24(5):484–9. [PubMed: 21844803]
3. Schloesser RL, Schaefer V, Groll AH. Fatal transplacental infection with non-typhoidal *Salmonella*. *Scand J Infect Dis*. 2004; 36(10):773–4. [PubMed: 15513410]
4. Scialli AR, Rarick TL. *Salmonella* sepsis and second-trimester pregnancy loss. *Obstet Gynecol*. 1992 May; 79(5 (Pt 2)):820–1. [PubMed: 1565377]
5. Gyang A, Saunders M. *Salmonella* Mississippi: a rare cause of second trimester miscarriage. *Arch Gynecol Obstet*. 2008 May; 277(5):437–8. [PubMed: 18004579]
6. Cagiola M, Severi G, Forti K, Menichelli M, Papa P, De Giuseppe A, et al. 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*. 2007 Apr 15; 121(3–4):330–7. [PubMed: 17257785]
7. Linde K, Bondarenko V, Sviridenko V. Prophylaxis of *Salmonella abortus ovis*-induced abortion of sheep by a *Salmonella typhimurium* live vaccine. *Vaccine*. 1992; 10(5):337–40. [PubMed: 1574918]
8. Holden DW. Trafficking of the *Salmonella* vacuole in macrophages. *Traffic*. 2002 Mar; 3(3):161–9. [PubMed: 11886586]
9. Chattopadhyay A, Robinson N, Sandhu JK, Finlay BB, Sad S, Krishnan L. *Salmonella enterica* serovar Typhimurium-induced placental inflammation and not bacterial burden correlates with pathology and fatal maternal disease. *Infect Immun*. 2010 May; 78(5):2292–301. [PubMed: 20194592]
10. Robbins JR, Bakardjiev AI. Pathogens and the placental fortress. *Curr Opin Microbiol*. 2012 Feb; 15(1):36–43. [PubMed: 22169833]
11. Koi H, Zhang J, Makrigiannakis A, Getsios S, MacCalman CD, Strauss JF III, et al. Syncytiotrophoblast is a barrier to maternal-fetal transmission of herpes simplex virus. *Biol Reprod*. 2002 Nov; 67(5):1572–9. [PubMed: 12390890]
12. Robbins JR, Skrzypczynska KM, Zeldovich VB, Kapidzic M, Bakardjiev AI. Placental syncytiotrophoblast constitutes a major barrier to vertical transmission of *Listeria monocytogenes*. *PLoS Pathog*. 2010; 6(1):e1000732. [PubMed: 20107601]
13. Fisher S, Genbacev O, Maidji E, Pereira L. Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis. *J Virol*. 2000 Aug; 74(15):6808–20. [PubMed: 10888620]
14. Le Monnier A, Autret N, Join-Lambert OF, Jaubert F, Charbit A, Berche P, et al. ActA is required for crossing of the fetoplacental barrier by *Listeria monocytogenes*. *Infect Immun*. 2007 Feb; 75(2):950–7. [PubMed: 17118980]
15. Fried M, Domingo GJ, Gowda CD, Mutabingwa TK, Duffy PE. Plasmodium falciparum: chondroitin sulfate A is the major receptor for adhesion of parasitized erythrocytes in the placenta. *Exp Parasitol*. 2006 May; 113(1):36–42. [PubMed: 16430888]

16. Mor G. Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction. *Ann N Y Acad Sci.* 2008 Apr;1127:121–8. [PubMed: 18443339]
17. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. *Placenta.* 2005 Aug; 26(7):540–7. [PubMed: 15993703]
18. Cossart P, Sansonetti PJ. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science.* 2004 Apr 9; 304(5668):242–8. [PubMed: 15073367]
19. Bevilacqua E, Hoshida MS, Amarante-Paffaro A, Albieri-Borges A, Zago GS. Trophoblast phagocytic program: roles in different placental systems. *Int J Dev Biol.* 2010; 54(2–3):495–505. [PubMed: 19757392]
20. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA.* 2000 Jun 6; 97(12):6640–5. [PubMed: 10829079]
21. Robinson N, Kolter T, Wolke M, Rybníček J, Hartmann P, Plum G. Mycobacterial phenolic glycolipid inhibits phagosome maturation and subverts the pro-inflammatory cytokine response. *Traffic.* 2008 Nov; 9(11):1936–47. [PubMed: 18764820]
22. Orendi K, Kivity V, Sammar M, Grimpel Y, Gonen R, Meiri H, et al. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. *Placenta.* 2011 Feb; 32(Suppl):S49–S54. [PubMed: 21257083]
23. Barbosa BF, Silva DA, Costa IN, Mineo JR, Ferro EA. BeWo trophoblast cell susceptibility to *Toxoplasma gondii* is increased by interferon-gamma, interleukin-10 and transforming growth factor-beta1. *Clin Exp Immunol.* 2008 Mar; 151(3):536–45. [PubMed: 18234060]
24. Carvalho JV, Alves CM, Cardoso MR, Mota CM, Barbosa BF, Ferro EA, et al. Differential susceptibility of human trophoblastic (BeWo) and uterine cervical (HeLa) cells to *Neospora caninum* infection. *Int J Parasitol.* 2010 Dec; 40(14):1629–37. [PubMed: 20708622]
25. Ben AA, Ghigo E, Le PY, Lepolard C, Salcedo SP, Lemichez E, et al. *Coxiella burnetii*, the agent of Q fever, replicates within trophoblasts and induces a unique transcriptional response. *PLoS One.* 2010; 5(12):e15315. [PubMed: 21179488]
26. Vidricaire G, Tremblay MJ. Rab5 and Rab7, but not ARF6, govern the early events of HIV-1 infection in polarized human placental cells. *J Immunol.* 2005 Nov 15; 175(10):6517–30. [PubMed: 16272306]
27. Zeldovich VB, Robbins JR, Kapidzic M, Lauer P, Bakardjiev AI. Invasive extracellular trophoblasts restrict intracellular growth and spread of *Listeria monocytogenes*. *PLoS Pathog.* 2011 Mar;7(3):e1002005. [PubMed: 21408203]
28. Equils O, Lu D, Gatter M, Witkin SS, Bertolotto C, Arditi M, et al. Chlamydia heat shock protein 60 induces trophoblast apoptosis through TLR4. *J Immunol.* 2006 Jul 15; 177(2):1257–63. [PubMed: 16818785]
29. Koi H, Zhang J, Parry S. The mechanisms of placental viral infection. *Ann N Y Acad Sci.* 2001 Sep;943:148–56. [PubMed: 11594535]
30. Rosselin M, Abed N, Virlogeux-Payant I, Bottreau E, Sizaret PY, Velge P, et al. Heterogeneity of type III secretion system (T3SS)-1-independent entry mechanisms used by *Salmonella Enteritidis* to invade different cell types. *Microbiology.* 2011 Mar; 157(Pt 3):839–47. [PubMed: 21109565]
31. Amarante-Paffaro AM, Hoshida MS, Yokota S, Goncalves CR, Joazeiro PP, Bevilacqua E, et al. Localization of cathepsins D and B at the maternal-fetal interface and the invasiveness of the trophoblast during the postimplantation period in the mouse. *Cells Tissues Organs.* 2011; 193(6): 417–25. [PubMed: 21116106]
32. Amarante-Paffaro A, Queiroz GS, Correa ST, Spira B, Bevilacqua E. Phagocytosis as a potential mechanism for microbial defense of mouse placental trophoblast cells. *Reproduction.* 2004 Aug; 128(2):207–18. [PubMed: 15280560]
33. Baardman ME, Kerstjens-Frederikse WS, Berger RM, Bakker MK, Hofstra RM, Plosch T. The role of maternal-fetal cholesterol transport in early fetal life: current insights. *Biol Reprod.* 2013 Jan;88(1):24. [PubMed: 23153566]
34. Linehan SA, Martinez-Pomares L, Gordon S. Mannose receptor and scavenger receptor: two macrophage pattern recognition receptors with diverse functions in tissue homeostasis and host defense. *Adv Exp Med Biol.* 2000; 479:1–14. [PubMed: 10897405]

35. Thelen T, Hao Y, Medeiros AI, Curtis JL, Serezani CH, Kobzik L, et al. The class A scavenger receptor, macrophage receptor with collagenous structure, is the major phagocytic receptor for *Clostridium sordellii* expressed by human decidual macrophages. *J Immunol.* 2010 Oct 1; 185(7): 4328–35. [PubMed: 20810988]
36. Watanabe K, Shin EK, Hashino M, Tachibana M, Watarai M. Toll-like receptor 2 and class B scavenger receptor type I are required for bacterial uptake by trophoblast giant cells. *Mol Immunol.* 2010 Jul; 47(11–12):1989–96. [PubMed: 20471681]
37. Haas A. The phagosome: compartment with a license to kill. *Traffic.* 2007 Apr; 8(4):311–30. [PubMed: 17274798]
38. Albaghdadi H, Robinson N, Finlay B, Krishnan L, Sad S. Selectively reduced intracellular proliferation of *Salmonella enterica* serovar typhimurium within APCs limits antigen presentation and development of a rapid CD8 T cell response. *J Immunol.* 2009 Sep 15; 183(6):3778–87. [PubMed: 19692639]
39. Glomski IJ, Decatur AL, Portnoy DA. *Listeria monocytogenes* mutants that fail to compartmentalize listeriolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses. *Infect Immun.* 2003 Dec; 71(12):6754–65. [PubMed: 14638761]
40. Thompson JA, Liu M, Helaine S, Holden DW. Contribution of the PhoP/Q regulon to survival and replication of *Salmonella enterica* serovar Typhimurium in macrophages. *Microbiology.* 2011 Jul; 157(Pt 7):2084–93. [PubMed: 21511762]
41. Vidricaire G, Tremblay MJ. A clathrin, caveolae, and dynamin-independent endocytic pathway requiring free membrane cholesterol drives HIV-1 internalization and infection in polarized trophoblastic cells. *J Mol Biol.* 2007 May 18; 368(5):1267–83. [PubMed: 17395200]
42. Rowe JH, Ertelt JM, Aguilera MN, Farrar MA, Way SS. Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens. *Cell Host Microbe.* 2011 Jul 21; 10(1):54–64. [PubMed: 21767812]
43. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity.* 2008 Apr; 28(4):546–58. [PubMed: 18387831]
44. Dong Q, Fan R, Zhao S, Wang Y. Over-expression of SOCS-3 gene promotes IL-10 production by JEG-3 trophoblast cells. *Placenta.* 2009 Jan; 30(1):11–4. [PubMed: 19036437]
45. Flesch IE, Hess JH, Oswald IP, Kaufmann SH. Growth inhibition of *Mycobacterium bovis* by IFN- γ stimulated macrophages: regulation by endogenous tumor necrosis factor- α and by IL-10. *Int Immunol.* 1994 May; 6(5):693–700. [PubMed: 8080840]
46. O’Leary S, O’Sullivan MP, Keane J. IL-10 blocks phagosome maturation in mycobacterium tuberculosis-infected human macrophages. *Am J Respir Cell Mol Biol.* 2011 Jul; 45(1):172–80. [PubMed: 20889800]
47. Lee KS, Jeong ES, Heo SH, Seo JH, Jeong DG, Choi YK. IL-10 suppresses bactericidal response of macrophages against *Salmonella Typhimurium*. *J Microbiol.* 2011 Dec; 49(6):1050–3. [PubMed: 22203573]
48. Murphy SP, Fast LD, Hanna NN, Sharma S. Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. *J Immunol.* 2005 Sep 15; 175(6):4084–90. [PubMed: 16148158]

Figure 1. ST infection of cell lines

HeLa, THP-1, or THP-1 differentiated to macrophages with PMA (a), JEG-3, BeWo and HTR-8 (b) cells (5×10^5 /ml/24 well) were infected with wild-type ST (ST-WT), ST- *invA*, ST- *rck* or ST- *rck invA* at a MOI of 10. The intracellular bacterial burden at various times (a, c) was determined based on colony forming units (CFU) after cell lysis and doubling time was calculated (b, d). Data indicate mean $\pm$ standard deviation of triplicate infected cultures. **, Data in panel A for HeLa cells infected with ST-WT is significantly different from ST- *invA* and ST- *rck invA* by two-way ANOVA for both bacterial strain ($p < 0.01$) and time ($p < 0.0001$). The data are representative three independent experiments.

Figure 2. Mechanism of ST entry into trophoblasts

HeLa, THP-1 macrophages (activated with PMA) and JEG-3 cells were treated with inhibitors; cytochalasin B (a), cytochalasin D (b), LY294002 (c), and Wortmannin (d) fucoidan (e) or Mannan (f). The control non-inhibitor treated cultures were treated with DMSO at the same concentration utilized for solubilizing the inhibitors. The number of internalized bacteria was determined at 2 hours after exposure of infected cells to gentamycin (to remove extracellular bacteria). The number of bacteria internalized in control non-inhibitor treated cultures was calculated as 100 % and the relative percentage of internalized bacteria in inhibitor-treated cultures is shown. Data indicate mean $\pm$ standard deviation of triplicate infected cultures. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$ in comparison to entry of ST into HeLa cells in the presence of the same concentration of inhibitor as calculated by Student's t test ($n=3$). The data are representative three independent experiments conducted.

Figure 3. Characterization of Salmonella containing vacuoles by confocal microscopy
 Monolayers of THP-1 macrophages activated with PMA (a) and JEG-3 (b) were infected with ST-WT (10 MOI) and the immunofluorescence for expression of endosomal proteins and ST-lipopolysaccharide (ST) was carried out by confocal microscopy. Representative confocal image at 60 minutes post-ST-infection of THP-1 (a) and JEG-3 cells (b) indicating the staining of the endosomal markers Rab5, Rab7, and LAMP-1 (green), with ST (red), and nuclei (blue). White arrows within panels indicate the co-localization of endosomal marker expression with ST to give a merged yellow color. The magnification bar within each panel correlates to 10 μ M. Similar images were analyzed for 5 minutes as well. Enumeration of 100 intracellular ST with the specific endosomal marker at 5 and 60 minute post-infection for overlapping yellow staining was used to determine % co-localization for both cell types. (c), Composite data (Mean $\pm$ SD) quantified from images produced in 3 independent experiments. ***, $p < 0.0001$ ($n = 3$) for both cell type and time for expression on LAMP-1; *, $p < 0.01$ ($n = 3$) for expression of Rab 5 at 5 minutes in JEG-3 compared to THP-1 cells by two-way ANOVA.

Figure 4. Western blot analysis of isolated phagosomes after internalization of ST or beads
Cells (5×10^5) were infected with 10 MOI of ST-WT, or allowed to internalize beads and phagosomes were isolated at 5 and 60 minutes post infection. JEG-3 and THP-1 cells were used with or without activation with PMA. An equal amount of phagosomal protein was loaded into each well. The cell lysate was used as a loading control for actin. The expression of Rab 5, Rab 7, and cathepsin D in JEG-3 and THP-1 cells with or without PMA activation is indicated (a, c). MW indicates the molecular weight markers prior to western blotting. Western blots were quantified based on intensity using ImageJ, normalized to actin and data from 3 independent experiments were pooled and graphed (b, d). **, $p < 0.01$ ($n=3$) by

student t test for JEG-3 cells in comparison to THP-1+PMA cells at the specific time indicated.

Figure 5. Flow cytometric analysis of Phagolysosome fusion

THP-1 macrophages differentiated with PMA and JEG-3 cells (5×10^5) were incubated with FDG-beads or infected with FDG-ST (10 MOI). Cells were fixed after 5, 10, 30, or 60 minutes after incubation with beads or ST and acquired by flow cytometry to measure the release of green fluorescence which correlated to cleavage of FDG and phagosome-lysosome formation. Data were analysed by FlowJo® software. Representative histogram plot showing fluorescence intensity of cells prior to and at various times after internalization of beads or ST is indicated (a, b). Mean $\pm$ SD of Mean fluorescence intensity (MFI) from three independent experiments is plotted for each cell type after internalization of beads (c) or ST (d). ***, $p < 0.01$ by two-way ANOVA for JEG-3 cells overtime in comparison to THP-1 cells.

Figure 6. Role of IL-10 produced by trophoblast in modulating ST infection

Supernatants were collected from non-infected or ST infected THP-1 and JEG-3 cells. IL-6 cytokine production was assayed by ELISA (a). ***, $p < 0.001$ by student t test for cytokine production by infected THP-1 macrophages relative to JEG-3 cells ($n=3$). IL-10 expression was determined by q-RT-PCR and fold-change in mRNA expression relative to β -actin levels is indicated (b). **, $p < 0.01$ by student t test for expression of IL-10 by JEG-3 relative to THP-1 cells ($n=3$). JEG-3 cells were treated with an isotype or anti-IL-10 antibody (50 $\mu\text{g/ml}$) before infection with FDG-ST. Phagosome maturation kinetics was determined based on FDG cleavage. Representative histogram demonstrating the release of FDG, 30 minutes after infection is shown (c). The increase in MFI over time indicative of release of

FDG is shown (d). Intracellular bacterial burden (Mean $\pm$ SEM) of triplicate infected JEG-3 cells after treatment with anti-IL10 antibody is shown (e). Treatment ($p=0.0073$) and time ($p<0.0001$) are significantly different by two-way ANOVA. The doubling time was then calculated (f). Mean $\pm$ standard deviation of triplicate infected cultures is indicated (f). *, $p<0.05$ by Student's *t* test for doubling time in the presence of anti-IL-10 antibody in comparison to ST-infected control JEG-3 cells. All in-vitro data are representative of 3 independent experiments conducted. Pregnant untreated C57BL/6J, anti-IL-10 antibody treated C57BL/6J and IL-10 $^{-/-}$ mice ($n=3-4$ /group) were infected with ST-WT (i.v., 1000 CFU), and bacterial burden in placenta assessed at 24 h post-infection. *, $p<0.05$ data are significantly different from untreated C57BL/6J mice by one-tailed Mann Whitney U-test.