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Cell Type-Specific Expression and Function of Toll-Like Receptors 2 and 4 in Human Placenta: Implications in Fetal Infection

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Abstract

Placental infection is associated with adverse fetal outcomes. Toll-like receptors (TLRs) are critical regulators of the innate immune response based on their ability to recognize and respond to pathogen-associated molecular patterns expressed by microbes. To date, cell-type specific expression and regulation of TLR function in human term placenta remains largely unelucidated. The goal of the current study was to examine the *in vivo* and *in vitro* patterns of TLR expression and function in major cell types of term placenta.

Immunohistochemical analysis of terminal and stem villi localized TLR-2, which recognizes peptidoglycan (PG) from Gram-positive bacteria, to endothelial cells and macrophages, and to a lesser extent to syncytiotrophoblast (SCTs) and fibroblasts (FIBs). Staining for TLR-4, the receptor for Gram-negative bacterial lipopolysaccharide (LPS), was most prominent in SCTs and endothelial cells. Results from Western blotting, conventional, and quantitative PCR (qRTPCR) analyses using protein and mRNA isolated from cultures of SCTs and myofibroblasts (mFIBs) revealed that SCTs expressed TLR-2 and TLR-4, whereas mFIBs expressed only TLR-4. In addition, qRTPCR showed that LPS treatment increased TLR-2 expression in SCTs, indicating that infection with Gram-negative bacteria may enhance innate immune responses in placenta toward a broad range of microorganisms. In addition, treatment with LPS increased IL-8 levels in both SCTs and mFIBs, whereas PG treatment only stimulated IL-8 levels in SCTs. Our results indicate that there exist cell type-specific patterns of TLR function in placenta which likely regulate innate immune response at the maternal-fetal interface.

Keywords

Placenta; TLRs; syncytiotrophoblast; fetal infection

INTRODUCTION

Human TLRs are critical components in the innate immune response based on their ability to recognize pathogen-associated molecular patterns, which are found in microbes, but not in “self tissues” [1–3]. To date, 10 different TLRs have been identified in humans [1,3]. The major ligands and receptors relevant to this study are TLR-2 which recognizes peptidoglycan (PG)

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and bacterial lipoprotein from Gram-positive bacteria, and TLR-4, the receptor for lipopolysaccharide (LPS) from Gram-negative bacteria [2,3]. Recently, TLRs have been identified in both first trimester and term placenta [4–8]. Messenger RNAs encoding TLR-1–10 and proteins for TLR-2 and TLR-4 were expressed in villous and intermediate trophoblast of term placenta [4,6]. It was also reported that TLR-2-dependent signaling promoted apoptosis of first trimester cytotrophoblasts, whereas TLR-4 signaling induced synthesis of cytokines [7]. This suggested that intrauterine infections may result in either trophoblast apoptosis or in trophoblast production of cytokines that signal immune cells and promote trophoblast survival. A further study indicated that ligation of TLR-3 and TLR-4 in first trimester trophoblasts promotes immune cell migration [8]. Collectively, these studies clearly indicate that the placenta recognizes microbes through TLR receptors and promotes an innate immune response.

Microorganisms may gain access to the placenta and fetal membranes by two major routes: ascending from the genital tract, and hematogenous dissemination through the placenta [9]. Chorioamnionitis (CA), infection of placental tissues, is most often caused by ascending bacteria, mycoplasma or fungi, which result in a marked infiltration of neutrophils in maternal decidua and fetal membranes, with or without a neutrophilic response in the placenta and fetus [10]. CA is associated with preterm delivery, a major cause of neonatal mortality and morbidity [11]. Also of note, cerebral palsy (CP) and related neurological disorders are associated with chorioamnionitis (CA) when it is accompanied by fetal inflammatory response syndrome (FIRS, a multisystemic microbial invasion of the fetus with the hallmark presentation of umbilical cord inflammation (i.e. funisitis) [9–12]. Recent studies implicate placental infection and inflammation at this site with severe neurological sequelae in the newborn [13,14].

The villous network of placenta include terminal villi which are the final villous branches (~50 µm in diameter) and the main site of nutrient exchange between mother and fetus, and stem villi which form the main trunk of the villous tree (~300 µm in diameter) [15]. Major cell types in placenta include syncytiotrophoblast (SCT), the cell layer that lines the intervillous space and is in direct contact with maternal blood, and several populations of underlying fibroblasts (FIBs) that manifest different phenotypes including perivascular myofibroblasts (mFIBs) [16]. To simplify terminology, both the single layer of SCT observed *in vivo* and the units of SCT generated *in vitro* will be referred to as SCTs. Also, mFIBs refer to myofibroblasts *in vivo*, as well as cell cultures with a myofibroblast phenotype. Based on the association between placental infection/inflammation and devastating fetal outcomes, the goal of the current study was to examine TLR expression and function in SCTs and mFIBs, cell types of placental villi that are uniquely positioned to affect release of cytokines to mother and fetus, respectively.

MATERIALS AND METHODS

Tissue procurement

Placental tissue used for immunohistochemical and cell culture studies was obtained from uncomplicated normal term pregnancies (36–41 weeks of gestation) with appropriately grown, singleton fetuses delivered by elective Cesarean section (n=13). Protocols were approved by the Human Investigation Committee at Yale University School of Medicine.

Immunohistochemistry

Immunohistochemical localization of proteins in human term placentas was carried out essentially as we have previously described [17]. Formalin-fixed samples were embedded in paraffin and cut into 5 µm sections. After deparaffinization in xylene and rehydration in a graded series of ethanol, slides were boiled in citrate buffer (10 mM, pH 6.0) for 15 min for antigen retrieval. Sections were immersed in 3% hydrogen peroxide (in 50% methanol/50%

distilled water) for 15 min to block endogenous peroxidase activity. Slides were then incubated in a humidified chamber with 5% horse serum in Tris-buffered saline (TBS; Lab-Vision, Fremont, CA) for 30 min at room temperature. The sections were then incubated overnight with primary antibodies in 1% horse serum in TBS at 4°C in a humidified chamber. Mouse monoclonal antibodies to cytokeratin 7 (clone TL 12/30, M7018, DAKO, Carpinteria, CA, 1:100 dilution), vimentin (clone V9, N1421, DAKO, 1:100 dilution) and α -smooth muscle actin (α SMA, clone 1A4, Sigma-Aldrich, St. Louis, MO, 1:15,000 dilution) were used to identify trophoblasts, fibroblasts, and myofibroblasts/muscle cells, respectively in placental villi. Immunolocalization of TLRs was carried out using goat anti-human antibodies to TLR-2 (AF2616, R&D Systems, Minneapolis, MN, 1:500 dilution) and TLR-4 (AF1478, R&D Systems, 1:1000). For negative controls, nonspecific mouse and goat IgG (Sigma, St. Louis, MO) were used at dilutions of 1:100 and 1:500, respectively. The sections were washed three times for 5 min each with TBS, then biotinylated horse anti-mouse or anti-goat antibody (Vector Laboratories, Inc., Burlingame, CA) was added at a 1:400 dilution for 30 min at room temperature. The antigen-antibody complex was detected with an avidin-biotin-peroxidase kit (LabVision). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride) was used as the chromogen, and sections were counterstained with hematoxylin and mounted with Permount (Fisher Chemicals, Springfield, NJ) on glass slides. Visualization and photography was conducted with an IX71 inverted microscope (Olympus, Mellville, NY). Six different placentas were used for immunohistochemical studies. Micrographs from one representative placenta are shown in Figures 1 and 2.

Cell culture

Cytotrophoblasts (CTs) and myofibroblasts (mFIBs) were isolated from the same placenta to allow for a direct comparison of TLR expression and TLR-dependent cytokine production in these cell types. Cytotrophoblasts were initially isolated from approximately 45 g of human villous tissue at term following trypsin digestion and centrifugation on Percoll gradients. We have previously employed this procedure [18] which is a modification of those developed by Kliman and colleagues [19] and Douglas and King [20]. Briefly, fresh placental tissue fragments were finely chopped, washed with saline, and treated with trypsin and DNAase. The digestate was poured through cheesecloth and two wire-mesh sieves with 0.0038 and 0.0021 inch openings. The effluent was collected and centrifuged at $500 \times g$ for 5 min. The cell pellets were resuspended for centrifugation on a continuous Percoll gradient. CTs sedimenting as a ring of cells at a density of approximately 1.05 g/ml, were washed and resuspended in basal medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 50 μ g/ml penicillin, and 50 μ g/ml streptomycin (Cellgro, Herndon, VA), i.e. FCS medium. Under these conditions we and others have found that we obtain a CT purity of approximately 90–95%, with the major contaminant being FIBs (~5%) and immune cells (1%) [18,19,21]. Immunopurification was carried out to remove these cell types using procedures as we have previously described [18]. One hundred microliters each of immunomagnetic microspheres conjugated to mouse anti-human CD-45 (immune cell marker) and CD-9 (fibroblast and extravillous trophoblast marker) obtained from Clemente Associates Inc. (Madison, CT) were added to approximately 3×10^8 cells. The mixture was then incubated on ice for 30 min with intermittent mixing. Magnetic microparticles were removed using a magnet, and the supernatant was subjected to a second round of immunopurification. Immunopurified CTs were washed and used to generate SCTs (see below). FCS medium was added to magnetic particles attached to CD9 and CD45 positive cells, and the mixture was plated in a T-75 culture flask.

SCTs were generated by culturing CTs for 72 h in FCS medium. Under these conditions CTs spontaneously differentiate into SCTs as previously described by Kliman et al. [19].

On reaching 80% confluency after approximately 2 weeks, cells in the flask containing the CD9+/CD45+ population were subcultured. These cells which express a myofibroblast phenotype (see Table 1) are denoted “mFIBs” and were used for experiments between passage 3–10. The process of isolation of SCTs and mFIBs from the same placenta was carried out in 5 different term placentas. Immunocytochemistry of methanol-fixed mFIBs was carried out in 6-well chamber slides using antibodies to cytokeratin-7, vimentin, and α SMA as described above for immunohistochemical procedures. Immunocytochemistry was carried out in SCTs and mFIBs isolated from 3 different placentas, representative results from one are shown in Table 1. For Western blot and PCR studies, cells were plated in FCS medium in 10 cm culture dishes. Cells were plated in 24-well dishes for studies that examined the effect of LPS and PG treatments on IL-8 expression.

Cultures were maintained at 37°C in FCS medium in a humidified atmosphere of 5% CO₂/95% air.

Western Blotting

For immunoblotting studies, cells were placed on ice; washed twice with phosphate-buffer saline (PBS); and lysed in 0.2 ml of buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol, and protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 mM α -glycerophosphate, 8 mM sodium pyrophosphate, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml aprotinin (Roche Molecular Biochemicals, Indianapolis, IN). The lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the soluble supernatants were stored at –80°C. Samples were normalized for total protein concentration using the Bio-Rad (Hercules, CA) protein assay. For Western blotting, 20 μ g of cell protein was boiled for 3 min in 2X SDS sample buffer containing 50 μ M of the reducing agent mercaptoethanol. Electrophoresis was then carried out using 4–15% Tris-HCl polyacrylamide gradient gels (Bio-Rad). Proteins were then transferred to Immobilon paper (Millipore Corp, Bedford, MA) at 100 volts for 80 min. Following transfer, blots were incubated with primary antibodies (the sources are described above in immunohistochemical studies) at the following dilutions: goat anti-human TLR-2 (1:1000); TLR-4 (1:500); mouse anti-human α SMA (1:15,000); and rabbit anti-human cyclophilin B (Abcam, Cambridge, MA, 1:2000). Overnight incubation with primary antibody at 4° C was followed by a 1 h incubation with rabbit anti-goat (1:2000), goat anti-mouse (1:15,000), or sheep anti-rabbit (1:5,000) horseradish secondary antibody conjugates (Bio-Rad, Hercules, CA). Proteins were detected using an enhanced chemiluminescence reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL) as we have previously described [18]. The Western blot in figure 3 depicts results obtained for mFIBs and SCTs isolated from 3 different placentas.

PCR

Procedures for conventional and quantitative real-time PCR analysis are essentially as we have previously described [22]. Total cellular RNA was initially extracted from whole placental tissue and cultures of mFIBs and SCTs using Tri Reagent (Sigma, St. Louis, MO). Five μ g of RNA was reverse transcribed to cDNA with AMV reverse transcriptase (Invitrogen, San Diego, CA). A quantitative standard curve ranging from 500 pg to 250 ng of cDNA was created with the Roche Light Cycler (Roche, Indianapolis, IN) by monitoring the increasing fluorescence of PCR products during amplification. Once the standard curve was established, quantitation of TLR-2 and TLR-4 levels was determined and adjusted to the expression of 18S RNA. Melting curve analysis was conducted to determine the specificity of the amplified products and to ensure the absence of primer-dimer formation. All products obtained yielded the correct melting temperature. Electrophoresis of amplified products revealed a single product of the expected size for TLR-2 (347 bp), TLR-4 (548 bp), and 18S RNA (236 bp). DNA sequencing verified their identity. The following primers were synthesized and gel-purified at the Yale

DNA Synthesis Laboratory, Critical Technologies: TLR-2, Forward 5'-GCCAAAGTCTTGATTGATTGG-3', Reverse 5'-TTGAAGT-TCTCCAGCTCCTG-3'; TLR-4, Forward 5'-TGGATACGTTTCCTTATAAG-3', Reverse 5'-GAAATGGAGGCACCCCTTC -3'; 18S, Forward 5'-GATATGCTC-ATGTGGTGTG; Reverse, 5'-AATCTTCTTCAGTCGCTCCA-3'. Conventional and qRT-PCR studies shown in figures 4 and 5 depict results obtained for tissue or cultures isolated from 3 different placentas.

ELISA

The concentration of IL-8 in culture media was determined by immunoassay (Quantikine, R & D Systems). For experiments, media were added to 96-well dishes coated with IL-8 antibody and washing and treatment with secondary antibody was carried out as described by the manufacturer. Levels of IL-8 were derived from optical densities using a microtiter-plate reader and the Soft Max software program (Molecular Devices, Menlo Park, CA) and were normalized to total cell protein. The time course of IL-8 levels in cultures of SCTs and mFIBs shown in figure 6 depicts a representative experiment from one placenta representing 3 identically ones conducted from different placentas. Cumulative results for LPS and PG effects on IL-8 levels in cell cultures from 3 independent experiments are shown in figure 7.

Statistics

Results are expressed as a mean $\pm$ SE. Data were analyzed by ANOVA followed by pairwise multiple comparison by the Student-Newman-Keuls Method using SigmaStat software from Jandel Scientific (San Rafael, CA). A P-value <0.05 was considered significant.

RESULTS

In vivo expression of TLR-2 and TLR-4 in term placenta

Initially, we established the distribution of cell types within terminal villi (Fig. 1) and stem villi (Fig. 2) of human term placenta. Immunohistochemistry using antibodies against cytokeratin 7 was used to identify cells of trophoblast origin. We noted strong staining (i.e. the appearance of brown peroxidatic product) in syncytiotrophoblasts (SCTs), the continuous cell layer in direct contact with the intervillous space (arrows, Fig. 1D and 2D). Based on vimentin staining, fibroblasts (FIBs) were observed in the villous core in terminal villi (arrows, Fig. 1E), and individual FIBs were more easily visualized at extravascular sites in stem villi (arrows, Fig. 2E) at varying distances from fetal vessels (denoted by "V", Fig. 2E). Staining for α -SMA identified perivascular smooth muscle cells and myofibroblasts (mFIBs) in terminal and stem villi (arrows, Figs. 1F and 2F). This is consistent with previous findings showing that vimentin-positive FIBs are distributed throughout the stem villus, whereas mFIBs and smooth muscle cells are found at perivascular sites [15,16]. It is of note, that no staining was observed in the presence of isotype-matched non-specific antibody (Figs. 1C and 2C).

Having established the cell type distribution within the placental architecture, we examined the patterns of TLR-2 and TLR-4 expression by immunohistochemistry. We observed weak TLR-2 immunoreactivity in SCTs of terminal and stem villi, denoted by arrows in Figs. 1A and 2A, respectively. TLR-2 staining was most pronounced in endothelial cells lining fetal vessels of terminal and stem villi (denoted "V" in Figs 1A and 2A). Strong punctate staining of TLR-2 was observed in cells of the terminal villous stroma, most likely reflecting expression in Hofbauer cells or fetal macrophages (arrows, Fig. 1A inset), based on CD-68 immunoreactivity examined in parallel sections (not shown). In stem villi, TLR-2 staining was not detected in perivascular α SMA-positive mFIBs (small arrows, Fig. 2A), whereas high levels of TLR-2 staining was noted in groups of extravascular α SMA-negative FIBs (arrowheads, Fig. 2A). Strong TLR-4 staining was noted in SCTs of terminal and stem villi (arrows, Figs. 1B and 2B). Prominent endothelial staining for TLR-4 was also noted at these

sites, whereas staining in the placental villous stroma was less restrictive and more diffuse than that noted for TLR-2. These results indicate that there are cell-type specific patterns of TLR-2 and TLR-4 expression in the human placental villus.

Expression of TLRs in cultures of SCTs and mFIBs

We then cultured human placental FIBs, and SCTs, and analyzed TLR expression by immunoblotting and PCR methodologies. As shown in Table 1, immunocytochemistry initially revealed that SCTs were positive for the trophoblast/epithelial cell marker cytokeratin 7, but negative for vimentin (fibroblast marker) and α SMA (myofibroblast marker). Conversely, cultured FIBs manifested a myofibroblast phenotype based on staining for vimentin and α SMA, but not cytokeratin, and are therefore referred to as myofibroblasts (mFIBs). Consistent with these results, we noted that Western blotting of cell extracts detected α SMA expression at the anticipated molecular weight of 42 kD [23] in each of 3 independent cultures of mFIBs, but not in SCTs (3rd panel, Fig. 3). Western blotting showed that SCTs express both TLR-2 and TLR-4, whereas mFIBs express only TLR-4 (top two panels, Fig. 3). Both TLRs were detected at a molecular weight of approximately 90 kDa, which is consistent with a previous report [7]. Cyclophilin B, a 21 kDa protein, utilized as a housekeeping protein [24], was detected in both SCTs and mFIBs (bottom panel, Fig. 3).

Messenger RNA was then isolated from three different whole placental tissues and cultures of mFIBs and SCTs. Following generation of cDNA, levels of TLR-2 and TLR-4 expression were determined by conventional PCR. Consistent with Western blot results, we noted that TLR-2 and TLR-4 mRNA were expressed in SCTs and term placental tissue, whereas mFIBs expressed TLR-4 mRNA, but little if any TLR-2 mRNA (Fig. 4). Levels of the 18S RNA housekeeping gene were similar in the two cell types. We then determined whether treatment with peptidoglycan (PG, a TLR-2 ligand) and lipopolysaccharide (LPS, a TLR-4 ligand) altered expression of TLR-2 and TLR-4 in cultures of SCTs and mFIBs. Cells were maintained for 24 h with and without 1 μ g/ml PG or LPS and expression of TLRs was examined using qRT-PCR. Consistent with results obtained from conventional PCR analysis and Western blotting, we noted that TLR-2 and TLR-4 mRNA were detected in SCTs whereas only TLR-4 mRNA was detected in mFIBs (Fig. 5). Of note, the presence of LPS promoted a significant 6-fold increase ($p < 0.05$) in TLR-2 mRNA levels in SCTs (Fig. 5A). Treatment with PG did not markedly affect levels of TLR-2 in SCTs, and TLR-4 expression was not affected by either treatment in this cell type (Fig. 5B). Although LPS and PG treatments induced a 2 to 4-fold induction in TLR-4 expression in mFIBs (Fig. 5B), these results did not reach statistical significance.

TLR-mediated cytokine expression in cultures of SCTs and mFIBs

We then determined whether TLR-mediated responses in SCTs and mFIBs reflected the patterns of TLR expression in these cell types. For these experiments, cells were maintained for 8, 24, and 48 h with and without 1 μ g/ml LPS or PG and levels of IL-8 in culture media were measured by ELISA and normalized to cell protein. We noted that treatment of SCTs with PG or LPS induced a time-dependent, 6- and 12-fold increase in IL-8 levels, respectively (Fig. 6A). Conversely, whereas LPS treatment induced a time-dependent 50-fold increase in IL-8 levels in mFIBs, treatment with PG treatment had no effect on IL-8 levels in these cultures (Fig. 6B). We further noted that treatment with 10 μ g/ml PG also had no effect on IL-8 levels in mFIBs (not shown). Cumulative results from 3 independent experiments indicated that a 48 h treatment of SCTs with PG and LPS promoted a significant 6- and 10-fold increase in IL-8 levels, respectively (Fig. 7A). Conversely, although LPS treatment promoted a significant 50-fold increase in IL-8 levels in mFIBs, PG treatment was without effect in this cell type (Fig. 7B). Similar patterns of regulation of IL-6 expression in SCTs and mFIBs by LPS and PG treatments were noted (not shown).

Our *in vivo* and *in vitro* studies indicate that SCTs and mFIBs of human term placenta manifest differential patterns of TLR-2 and TLR-4 expression and function.

DISCUSSION

Recent studies indicate that placental infection is associated with adverse neonatal outcomes [13,14]. *In situ* RT-PCR revealed an infectious agent was present in 46 out of the 60 placentas from newborns with respiratory distress, severe neurological sequelae and cerebral palsy, or death of unknown etiology [13]. The infectious agents (bacteria, Coxsackie virus, parovirus, cytomegalovirus, and herpes simplex virus) were localized primarily to Hofbauer cells (fetal macrophages) and SCTs [13]. No infectious agent was revealed in the 17 control placentas. Similar results were reported in another study in which placentas were examined from 33 cases with significant neurodevelopmental delay [14]. It is of note, that in both studies, when autopsy material was available, the same infectious agent was found in the placenta and fetal tissue. These results indicate that placental infection and inflammation may pose serious health risks for the fetus and neonate. Thus, the goal of the current study was to elucidate the patterns of expression, regulation, and function of TLR-2 and TLR-4 in SCTs, mFIBs, and other major cell types found in human term placenta.

Initially, using immunohistochemistry, we observed that SCTs expressed TLR-4, and prominent staining was also noted in endothelial cells. TLR-4 expression in FIBs in the villous stroma was noted, but was fairly diffuse in nature. Western blotting and PCR analysis of cell extracts from cultures of SCTs and mFIBs supported these *in vivo* observations. For TLR-2, immunohistochemistry revealed pronounced staining in endothelial cells and Hofbauer cells, and low levels in SCTs. The patterns of TLR-2 localization in FIBs were more complex; strong expression was noted in groups of extravascular FIBs, but not in perivascular mFIBs. Western blot and PCR procedures detected TLR-2 in cultures of SCTs but not mFIBs, which does not conflict with the relative patterns of expression noted in these cell types by immunohistochemistry. However, based on observed *in vivo* patterns of expression, we expect that cultures of SCTs would express lower levels of TLR-2 expression compared to cultures of endothelial cells and macrophages. Cells designated mFIBs in current study were confirmed to have a myofibroblast phenotype based on immunocytochemistry which indicated expression of vimentin, α SMA, but not the trophoblast marker cytokeratin 7. Expression of α SMA in these cells and not in SCTs was revealed by Western blotting. Protocols using collagenase digestion without immunopurification have been used by our group and others to previously isolate vimentin-positive and α SMA-positive FIBs from term placenta [17,25]. The current protocols allowed for the simultaneous separation of SCTs and mFIBs from the same placenta. Others have generated CTs and FIBs from the same placenta [26].

Although previous studies clearly indicated that mRNA encoding all 10 TLRs is expressed by whole placental tissue [6], the *in vivo* pattern of cell-type specific expression of TLR-2 and TLR-4 protein in the human term placenta remained controversial. Immunohistochemical studies have demonstrated syncytial expression of TLR-2 and TLR-4 in term placenta [4,27], yet another report localized TLR-4 to extravillous trophoblasts and Hofbauer cells, but not SCTs [5]. These disparate results were likely due to fact that different antibodies were used to detect TLR-4. In the first two reports, as in the current study, the antibody utilized was directed against TLR-4 alone. In the later report an antibody directed against TLR-4 complexed with MD-2, an accessory protein, was used. This may indicate that the villous syncytium expresses TLR-4 but not MD-2, or that a TLR-4/MD-2 complex is not present in human placenta. Previous immunohistochemical analysis did not previously note staining of TLR-2 or TLR-4 in the villous stroma or fetal vessels [4,5], but these reports did not examine stem villi in detail where different classes of mesenchymal cells and vessels are more easily discerned by conventional light microscopy and immunohistochemistry. In addition, ours is the first report

to verify immunohistochemical results with Western blot studies in cell cultures using the same antibody for both methodologies.

Of note, we also observed that treatment with LPS enhanced expression of TLR-2 mRNA in SCTs, which is consistent with findings in hepatocytes and adipocytes [29,30]. This suggests that infection with Gram-negative bacteria may enhance innate immune responses in SCTs toward a broad range of microorganisms. Our final experiments showed that treatment with LPS induced IL-8 expression in both SCTs and mFIBs, whereas PG treatment stimulated IL-8 levels in SCTs, but not in mFIBs. This finding is consistent with our Western blot and PCR results showing TLR-4 expression in SCTs and mFIBs, and absence of TLR-2 expression in mFIBs.

Previous publications by our group demonstrated the roles of TLR-2, TLR-3, and TLR-4 in regulating cytokine responses, immune cell migration, and antimicrobial factors in first trimester trophoblasts [7,8,30]. In addition, we showed that TLR-4 expression is induced in extravillous interstitial trophoblasts in the placental bed of women with preeclampsia [31]. Another group demonstrated increased TLR-4 expression in Hofbauer cells in pregnancies with chorioamnionitis [5], supporting a role for TLRs in this complication of pregnancy. We have also reported that LPS treatment enhances expression of pro-inflammatory cytokines by primary cultures of SCTs [32]. The current findings of differential patterns of TLR expression and function in SCTs, mFIBs, and endothelial cells (i.e. major components of the term placental villus) have not been previously reported by our group or others. These results have potential significance in terms of placental infections and directional cytokine release by the placenta. Based on our results showing relatively high basal levels of cytokine expression by SCTs, this suggests that the syncytium may act as a microbial sensor in pregnancy and release cytokines to maternal blood in response to low levels of commensal bacteria. In contrast, the finding that mFIBs express extremely low levels of cytokines under basal conditions, which are dramatically induced by LPS treatment, indicates that mFIBs may only release cytokines in response to Gram-negative bacteria that have breached the syncytium. Furthermore, based on their location this may be associated with inflammatory processes in the fetal endothelium and directional release of cytokines to the umbilical/fetal circulation leading to FIRS. The absence of TLR-2 in populations of FIBs suggests that these particular cell types may not play a major role in TLR-mediated cytokine responses to Gram-positive bacteria.

In conclusion, our results demonstrate that SCTs, mFIBs, and endothelial cells differentially express TLR-2 and TLR-4, and TLR-dependent cytokine production. These cell type-specific patterns of TLR function in placenta likely impact innate immune response and directionality of cytokine release in uncomplicated pregnancies and those with FIRS.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Table 1
Immunocytochemical analysis of cultures of myofibroblasts (mFIBs) and syncytiotrophoblasts (SCTs).

Cell type	Protein	% Positive Cells
mFIBs	Cytokeratin 7	<1
	Vimentin	>95
	α SMA	>95
SCTs	Cytokeratin 7	>95
	Vimentin	2
	α SMA	<1

Cell cultures from 3 different placentas were plated in 6-well chamber slides and immunocytochemistry of methanol-fixed cells were carried out using antibodies to cytokeratin-7 (trophoblast/epithelial marker), vimentin (fibroblast marker), and α SMA (myofibroblast cell marker).