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Toll-like Receptor-Mediated Responses by Placental Hofbauer Cells (HBCs): A Potential Pro-Inflammatory Role for Fetal M2 Macrophages*

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Abstract

Problem—Microbial-driven responses in placenta are linked with adverse pregnancy outcomes. The role of Toll-like receptor (TLR) function in Hofbauer cells (HBCs), fetal macrophages of the placental villous core, remains understudied.

Method of Study—Flow cytometry and immunohistochemistry (IHC) were used to establish the phenotype of HBCs. Regulation of cytokine secretion in response to treatment with TLR agonists, and expression levels of TLRs and co-activators, were compared in HBCs, placental fibroblasts (FIBs), and human umbilical vein endothelial cells (HUVECs) using ELISA and qPCR.

Results—Although flow cytometry and IHC revealed HBCs to be M2 (anti-inflammatory) macrophages, LPS and Poly (I:C) treatments markedly enhanced IL-6 secretion by HBCs, and expression of TLR-2, TLR-3, TLR-4, CD14, and MD-2 were highest in HBCs.

Conclusion—These results indicate that although HBCs are M2 macrophages, inflammatory responses are induced through TLR-3 and TLR-4 in this cell type, suggesting a role in microbial-driven placental/fetal inflammation.

Keywords

Placenta; Hofbauer cells; macrophages; TLRs

INTRODUCTION

Cerebral palsy and related neurological disorders occur in approximately 2 to 3/1000 live births, and are a major cause of neonatal morbidity and long-term disability [1, 2]. Cerebral palsy is associated with preterm delivery, low birth weight, and chorioamnionitis (CAM) when accompanied by fetal inflammatory response syndrome [(FIRS, a multisystemic

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microbial invasion of the fetus with the hallmark presentation of funisitis, or umbilical cord inflammation [3-5]. CAM is most often caused by ascending genital tract 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 [4]. Specific placental inflammatory lesions occurring in association with cerebral palsy include thrombotic vasculopathy, chronic villitis with obliterative vasculopathy, and CAM with intense chronic vasculitis and meconium-associated vascular necrosis [3, 6]. Given the pro-inflammatory nature of FIRS, it is not surprising that cerebral palsy is associated with enhanced expression of inflammatory cytokines in amniotic fluid and cord blood [7, 8].

Placental inflammation and infection are suggested to be mediated through pattern recognition receptors (PRRs), mediators of innate immune response, which recognize pathogen-associated molecular patterns found in microbes but not in "self tissues"[9-11]. Human TLRs are the most completely studied PRRs, and 10 different human TLRs have been identified [9-11]. Major TLRs include: TLR-2 which recognizes Gram-positive bacterial peptidoglycan (PG), TLR-3, the receptor for viral double-stranded RNA, Poly (I:C) serves as an *in vitro* analogue, and TLR-4, the receptor for Gram-negative bacterial lipopolysaccharide (LPS) [10]. In cells expressing TLR-4 and the co-receptor CD14, binding of LPS to lipopolysaccharide binding protein (LBP) initiates the signaling cascade [12]. While CD14 is not required for TLR-4 mediated signaling, its presence may enhance TLR-4-mediated responses in some cell types [12, 13]. The binding of LPS to LBP facilitates the formation of a ternary complex with CD14, enabling LPS to be transferred to the LPS receptor complex composed of TLR-4 and MD-2, a membrane-associated as well as secreted glycoprotein [12].

To date, studies of placental TLR expression and function have focused on the role of cytotrophoblasts, a cell-type which may differentiate along non-invasive or invasive pathways giving rise to syncytiotrophoblast (SCT) as well as to extravillous trophoblasts, which invade matenal decidua and myometrium [14]. Immunohistochemstry revealed TLR-4 and TLR-2 protein were expressed in both preterm and term placental cytotrophoblasts [15, 16]. Quantitative PCR showed placental expression of mRNAs encoding TLR-1-10 of term placenta [17], suggesting that the placenta expresses a complete repertoire of TLR proteins. Functional studies have shown that, in first trimester trophoblasts, TLR-2-dependent signaling promotes apoptosis, whereas TLR-4 signaling induces secretion of inflammatory cytokines [18]. Moreover, TLR-3 and TLR-4 activation triggers trophoblast-mediated immune cell migration through the production of chemokines [19]. Collectively, these studies clearly indicate that the placenta senses microbial and viral products and generates an innate immune response. At term, the human placental villus largely consists of SCT, the outer trophoblast layer lining the intervillous space in direct contact with maternal blood, and underlying stromal cells which are adjacent to fetal capillaries and are comprised of fibroblasts (FIBs) and Hofbauer cells (HBCs, fetal macrophages)[20, 21]. HBCs, like other tissue macrophages, can be classified as M1 (proinflammatory), which characteristically express high levels of IL-1, TNF-α, CD11b, and CD40, or M2 (anti-inflammatory/pro-angiogenic), characterized by elevated levels of IL-10, TGF-β, CD163, and folate receptor (FR)-β [22, 23]. Immunohistochemical studies suggest that in normal placenta, HBCs are M2

macrophages [24] which supports a putative role in early placental angiogenesis and development [25, 26].

In light of the clear role of TLRs in infection-associated preterm delivery [27-29], and the specific association of inflammatory and pro-thrombotic processes in placenta and umbilical cord in FIRS [4, 5, 30], the goal of the current study was to examine TLR-mediated regulation of IL-6, IL-8, and tissue factor (TF, the primary initiator of hemostasis [31]) production in HBCs, a largely understudied major cell type of the placental villous core. Fibroblasts, a second major cell type of the villous core, and human umbilical vein endothelial cells (HUVECs) served as models for responses in the placental mesenchyme and fetal endothelium, respectively. Our studies revealed that although HBCs express an M2 phenotype *in vivo and vitro*, they respond to the TLR-4 agonist, LPS, and the TLR-3 agonist, Poly (I:C), with elevated levels of factors shown to be associated with FIRS and preterm delivery.

MATERIALS AND METHODS

Collection of Placentas

Placentas (n=16) from normal uncomplicated term pregnancies were brought to the laboratory within 30 min following elective cesarean without labor at Yale New Haven Hospital. Infection was excluded on the basis of standard clinical criteria (absence of fever, uterine tenderness, maternal/fetal tachycardia, foul vaginal discharge). Tissues were then processed immediately for isolation of placental cell cultures. Each placenta was processed separately (i.e. tissues were not pooled). Approval for this study was granted by the Yale University School of Medicine Human Investigation Committee.

Immunohistochemistry

Five μm placental villous tissue sections were deparaffinized at 58°C overnight, washed 3 times with xylene, and progressively rehydrated to 70% ethanol. Antigen retrieval was achieved by microwaving for 20 min in 10 mM sodium citrate buffer, pH 6.0. Sections were blocked with 10% donkey serum, and incubations were carried out overnight at 4°C with a 1:100 dilution of mouse anti-CD163 (Cat# MCA1853, ABDSerotec, Raleigh, NC), sheep anti-FR-β (Cat# AF5697, R&D, Minneapolis, MN), rabbit anti-CD40 (Cat# PAB11229, Abnova, Jhongli, Taiwan), and a 1:25 dilution of rabbit CD11b (Cat# HPA02274, Sigma, St. Louis, MO) primary antibodies. Mouse IgG (Cat# X0931, Dako, Carpinteria, CA) and Sheep IgG (Cat# 013000003, Jackson ImmunoResearch, West Grove, PA) served as negative controls. The following day, endogenous peroxidase was quenched with 3% hydrogen peroxide in 50% methanol for 12 min, and after washing in PBS, biotinylated donkey anti-mouse (Cat# 715065151, Jackson ImmunoResearch), anti-sheep (Cat# 713065003, Jackson ImmunoResearch), and anti-rabbit (Cat# 711-065-152, Jackson ImmunoResearch) secondary antibodies were added for 30 min at a dilution of 1:1000. Staining was developed using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) and diaminobenzidine substrate. Slides were counterstained with hematoxylin, dehydrated to 100% ethanol and then xylene, and were mounted with CytoSeal 60. Visualization and photography was conducted with an IX71 inverted microscope (Olympus, Melville, NY).

Isolation of Hofbauer cells (HBCs) and fibroblasts (FIBs)

Isolation of HBCs and FIBs was carried out using protocols previously developed in our laboratory [32]. Villous tissue was dissected free of membranes, minced, and rinsed with calcium- and magnesium-free phosphate-buffered saline (PBS). Membrane-free villous tissue fragments were then subjected to 3 sequential enzymatic digestions in a solution containing 0.25% trypsin, 0.2% DNase I, 25 mM HEPES, 2 mM $CaCl_2$, and 0.8 mM $MgSO_4$ in Hanks' Balanced Salt Solution (HBSS) at 37°C; the first was carried out for 15 min in 150 ml of digestion solution, and the second and third for 30 min each in 150 ml and 200 ml of digestion solution, respectively. After each step, undigested tissue was removed and saved following passage through gauze and a 100 μm sieve. Trypsin-digested tissue fragments were then washed using PBS and further digested with collagenase A (1mg/ml)/ DNase I (0.2 mg/ml) in RPMI-1640 containing 25 mM HEPES, 5% FBS, and 1% antibiotic-antimycotic (RPMI medium) for 1 h at 37°C. Cells were pelleted and resuspended in RPMI medium and were loaded onto a discontinuous Percoll gradient (40%/20%) and centrifuged without break at room temperature for 20 min at 1000 × g using a Beckman TJ-25 centrifuge and a TS-5.1-500 swinging bucket rotor (Beckman Instruments, Fullerton, CA, USA). Cells from the 40%/20% Percoll interface were pelleted and resuspended in RPMI medium. The cells were then loaded onto a second discontinuous Percoll gradient (35%/30%/25%/20%) and centrifuged for 30 min. Cells from 20%/25% to 30%/35% interfaces were combined and were immunopurified by negative selection using sequential treatment with anti-EGFR (clone #528, Santa Cruz Biotechnology, Santa Cruz, CA) and then anti-CD10 (clone #MEM-78, Biolegend, San Diego, CA) antibodies conjugated to magnetic beads [32]. Cells from the final supernatant were counted using a hemocytometer, then plated in RPMI medium, and floating and weakly attached cells were washed off after 1 h. The remaining adherent cells were treated as described below.

Cultures of FIBs were obtained from cells attached to magnetic beads containing anti-CD10 antibody obtained during HBC isolation. The bead-cell mixtures were washed and cultured in F12/DMEM supplemented with 10% FBS (FBSM medium) and fresh medium was added every 2-3 days until confluency was reached after approximately 2 to 3 weeks. Following trypsinization of first passage cells, magnetic beads with attached cells, comprising approximately 10% of the cell population, were removed with a magnet. Passage 3 to 7 FIBs were used for experiments.

Flow cytometry

HBCs were detached from the substratum 1 h following plating using 5 mM EDTA and 4 mg/ml lidocaine-HCl in HBSS, and were fixed with 4% paraformaldehyde and stored in PBS at 4°C. Two$\times 10^5$ fixed cells were washed once using staining buffer (0.5%BSA/PBS). Blocking of Fcγ receptor was then carried out by incubating fixed cells with human IgG (100 μg/ml) in staining buffer for 15 min at room temperature. Unconjugated sheep anti-human FR-β (Cat#AF5697, R&D Systems, Minneapolis, MN), APC-conjugated mouse anti-human CD163 (Cat#333609, BioLegend, San Diego, CA), PE-conjugated mouse anti-human CD11b/ITGAM (Cat#301305, BioLegend) and FITC-conjugated mouse anti-human CD40 (Cat#334305, BioLegend) primary antibodies and isotype-matched controls were then added, and incubated for 45 min at 4°C. Cells were then washed twice with staining buffer.

For CD163, CD11b/ITGAM and CD40 staining, cells were then resuspended in 300 μl of PBS for analysis. For FR-β staining, cells were resuspended in staining buffer and incubated with FITC conjugated donkey anti-sheep IgG (Cat# 713096147, Jackson ImmunoResearch) secondary antibody for 45 min at 4°C. Cells then were washed and resuspended in 300 μl of PBS for analysis [32]. The forward scatter threshold was set to exclude cell debris and then ten thousand events were collected using FACSCalibur and CellQuest software (BD Biosciences, Franklin Lakes, New Jersey). The results were analyzed using FlowJo software (Tree Star, Ashland, OR). The percentage of positive cells was based on comparison with the isotype-matched control antibody, for which gating was set at 1%.

Treatment of cell cultures with TLR agonists

Prior to experiments, HBCs and FIBs were maintained in FBSM, whereas HUVECs, obtained through the American Type Culture Collection (Manassas, VA), were maintained in EGM-2 as described [33]. For experiments, HBCs and FIBs cells were maintained for the indicated time in either FBSM or serum-free medium (SFM, DMEM/F12 with 50 μg/ml ascorbic acid and ITS+ Premix, a culture supplement which yields a final concentration of insulin of 6.25 mg/ml; transferrin, 6.25 mg/ml; selenous acid, 6.25 ng/ml; bovine serum albumin, 1.25 mg/ml; and linoleic acid, 5.35 μg/ml) with and without 1 pg/ml to1 μg/ml LPS (from *Escherichia coli* 055:B5; purified by phenol extraction; Cat# L2880, Sigma-Aldrich, St. Louis, MO), 50 ng/ml LBP (Cat# 870-LP, R&D Systems, Minneapolis, MN), 1 μg/ml PG (from *Staphylococcus aureus*, Cat# tlrl-pgnsa, Invivogen, San Diego, CA) and 1 μg/ml Poly (I:C) (synthetic analog of dsRNA, Cat# tlrl-pic, Invivogen). HUVECs were maintained in FBSM with and without TLR ligands due to the loss of viability which occurred in SFM as evidenced by their detachment from the substratum. Alternatively, HBCs and FIBs were washed twice using PBS and were incubated for 1 h with mouse anti-human CD14 antibody (R&D systems, Cat# MAB3832) or mouse IgG1 isotype control antibody (R&D systems, Cat# MAB002) each diluted in SFM to achieve a final concentration of 10 μg/ml. LPS and LBP were then added to a final concentration of 1 μg/ml and 50 ng/ml, respectively. Cells were then maintained for an additional 24 h and levels of IL-6 and IL-8 in conditioned media were measured by ELISA.

ELISA

Levels of IL-6 and IL-8 in conditioned media from the 3 cell types were quantitated using R&D Systems DuoSet ELISAs (Cat# DY206 and DY208, respectively). Levels of cytokines in culture media were normalized to total cellular protein, determined using a DC Protein Assay (BioRad Laboratories, Hercules, CA).

qPCR

Levels of mRNA were determined with an ABI 7500 RealTime PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays for IL-6 (Cat# Hs00174131), IL-8 (Cat# 99999034), TF (Cat# 00175225), TLR-2 (Cat# 01014511), TLR-3 (Cat# 01551078), TLR-4 (Cat# 00152939), CD14 (Cat# 00169122), MD-2 (Cat# 01026734), and 18S RNA (Cat# 99999901). PCR reactions were performed in duplicate in a 20 μl volume of TaqMan Universal PCR Master Mix containing 1 μl of reverse transcription

cDNA and 1 μl of assay primer-probe mix. Mean CT values were analyzed using the 7500 System SDS software 1.3.1. Gene expression was normalized to the housekeeping gene 18S using the formula $2^{-\Delta\Delta CT}$. Results are expressed as relative expression compared to an endogenous control in each experiment as we have previously described [34].

Statistics

It is of note that not all of the 16 placentas were used in the same cohort and the indicated number of independent experiments from separate placentas are provided in the figure legends. Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare results that were normally distributed and are presented as a mean + SE. Wilcoxan rank-sum and Kruskal-Wallis ANOVA were carried out for data that were not normally distributed and are presented as a median with quartiles. SigmaStat software was used for statistical analyses. A $P < 0.05$ was considered significant in all studies.

RESULTS

HBCs isolated from normal term placentas are M2 macrophages

Immunohistochemistry of normal term placental tissue revealed no staining for the M1 macrophage marker CD11b (Fig. 1A). Low levels of CD40, a second marker of M1 macrophages, were observed throughout the villus and no specific localization to HBCs was noted (Fig. 1B). Conversely, staining for CD163 (Fig. 1C) and FR-β (Fig.1D), markers of M2 macrophages, was revealed in numerous pleiomorphic cells beneath the syncytium and above fetal capillaries, the site of placental HBCs [20, 21]. Similarly, flow cytometry indicated that immuno-purified cultures of HBCs did not express CD11b, express variable levels of CD40 (5% to 50% positive cells), whereas 98-99% of HBCs were positive for CD163 and FR-β (Fig. 2). This indicates that isolated cultures of HBCs maintain the M2 phenotype observed *in vivo* [25, 26].

TLR regulation of IL-6 and IL-8 protein secretion by HBCs

To test the effects of TLR activation on cytokine production by HBCs, cells were incubated for 24 h with 1 μg/ml LPS, PG, and Poly (I:C) in medium with (FBSM) and without (SFM) serum, and levels of IL-6 and IL-8 secreted into the culture media were assessed by ELISA. In both FBSM (Fig. 3A) and SFM (Fig. 3B), the presence of LPS promoted a significant approximate 1,000-fold increase in IL-6 levels. Incubation with LPS promoted a significant 3- to 6-fold stimulation of IL-8 levels in HBCs in both the presence (Fig. 3D) and absence (Fig. 3E) of serum. LPS induced a time-dependent increase in IL-6 and IL-8 levels in SFM between 4 and 48 h (Fig. 3C and F), and a dose-dependent increase between 1 pg/ml and 1 μg/ml (Fig. 3G and H). Incubation with PG did not significantly affect levels of IL-6 or IL-8 in either media, whereas Poly (I:C) triggered a 2- to 3-fold stimulation of IL-8 secretion in both FBSM and SFM, and a significant 200-fold increase in IL-6 secretion in SFM (Fig. 3B,D and E).

Regulation of IL-6, IL-8, and TF mRNA expression in HBCs by TLR agonists

IL-6 and IL-8 mRNA levels were measured by qPCR in cultures of HBCs maintained in FBSM (+Serum) and SFM (-Serum) with and without 1 μg/ml LPS, PG, and Poly (I:C) for

24 h. Levels of IL-6 mRNA in HBCs were significantly stimulated approximately 6,000-fold by LPS treatment in both FBSM and SFM (Fig. 4A). LPS treatment significantly enhanced IL-8 mRNA levels in HBCs approximately 7-fold using both media (Fig. 4B), a much smaller response compared to its effect on IL-6 mRNA. PG treatment did not enhance IL-6 and IL-8 mRNA levels in HBCs (Fig. 4A and B). Poly (I:C) treatment of HBCs induced 40- and 4-fold increases in the levels of IL-6 and IL-8 mRNA (Fig. 4A and B), respectively. Since LPS-mediated changes in TF mRNA have previously been reported to be maximal at 2 to 6 h in endothelial cells [35, 36], in this current study HBCs were incubated with LPS, PG, and Poly (I:C) for 4 h in medium with and without serum, and levels of TF mRNA were determined by qPCR. The patterns of TLR-dependent regulation of TF-mRNA differed from that of IL-6 and IL-8 mRNA as LPS promoted a 60- to 100-fold increase in expression in HBCs (Fig. 4C).

Regulation of IL-6, IL-8, and TF in FIBs and HUVECs by TLR agonists

Interestingly, in response to LPS, levels of IL-6 and IL-8 secreted by FIBs were significantly enhanced 60- and 150-fold, respectively at 24 h in the presence of serum, whereas LPS had no significant effect in the absence of serum (Fig. 5A). Importantly, treatment of FIBs with LPS in the presence of LBP enhanced IL-6 and IL-8 levels approximately 100- and 70-fold, respectively in FIBs maintained in SFM (Fig. 5A). In contrast, the presence of LBP did not significantly alter LPS effects on cytokine levels in HBCs maintained with or without serum (Fig. 3G and H). This indicates that LBP is required for LPS-mediated enhancement of cytokine expression in FIBs but not HBCs. Treatment of FIBs with PG or Poly (I:C) had no effect on the secreted levels of IL-6 and IL-8 under either culture condition (not shown). In FBS medium, LPS treatment of FIBs significantly enhanced IL-6 and IL-8 mRNA levels approximately 20- and 1,000-fold, respectively (Fig. 5B). In SFM, only combined treatment of FIBs with LPS and LBP significantly enhanced IL-8 mRNA 600-fold, whereas the 30-fold effect on IL-6 levels did not reach statistical significance (Fig. 5B). Unlike HBCs, LPS treatment had no significant effect on TF mRNA levels in FIBs (Fig. 5B).

Treatment of HUVECs with LPS in FBSM for 24 h promoted approximately a 20- and 180-fold increase in IL-6 and IL-8 secretion, respectively (Fig. 6A). This indicated that LPS treatment more markedly stimulated IL-6 levels in HBCs compared to FIBs and HUVECs, whereas IL-8 levels were more profoundly enhanced by LPS treatment in these two cell types compared to HBCs. Similar to FIBs, levels of IL-6 and IL-8 in HUVECs were not significantly affected by PG and Poly (I:C) treatment (not shown). It is of note, that viability of HUVECs was markedly reduced by maintenance in SFM for >8 h as evidenced by their detachment from the substratum (not shown). This precluded analysis of cytokine regulation in the absence of serum in this cell type and, therefore, studies on the role of LBP in the HUVECs' response to LPS. The patterns of TLR-mediated regulation of cytokine mRNA expression in HUVECs was similar to that noted in FIBs, as LPS promoted approximately 2- and 180-fold increases in the expression of IL-6 and IL-8 mRNA, respectively (Fig. 6B). In contrast, the 20-fold increase in TF mRNA levels in response to LPS treatment of HUVECs (Fig. 6B) was similar to responses noted in HBCs.

Expression of TLRs and TLR-4 co-activators in HBCs, FIBs, and HUVECs

Based on the differential patterns of TLR agonist-driven cytokine regulation in the three cell types studied, and their differential dependency on LBP when stimulated with LPS, we next examined the relative expression levels of the relevant TLRs and co-activators. TLR-2 mRNA levels in HBCs were approximately 3,000-fold greater than that observed in FIBs and HUVECs (Fig. 7). HBC levels of TLR-3 mRNA were approximately 12- and 7-fold-fold greater than that noted in FIBs and HUVECs, respectively. Levels of TLR-4 mRNA in HBCs were 12- and 8-fold greater than that observed in FIBs and HUVECs, respectively. Levels of CD14 mRNA in HBCs was approximately 7,000-fold that noted in FIBs and HUVECs. Expression of MD-2 in HBCs was 38- and 6-fold that noted in FIBs and HUVECs, respectively. Thus, markedly higher levels of the TLRs 2,3,4 and the two TLR-4 co-activators, CD14 and MD-2, were found in HBCs compared to the two other cell types (Fig. 7).

Requirement of CD14 in LPS-mediated effects on cytokine expression in HBCs and FIBs

The role of CD14 in LPS-mediated changes in cytokine production was next examined. HBCs and FIBs, initially pretreated in SFM with and without a function-perturbing anti-CD14 antibody or control IgG1 antibody, were then maintained with antibodies for an additional 24 h in the presence and absence of 1 μg/ml LPS and 50 ng/ml LBP. Levels of IL-6 and IL-8 secreted into the conditioned media were then measured by ELISA. We observed that the CD14 antibody significantly reduced IL-6 secretion by the HBCs by 40%, whereas it had no effect on the secretion of IL-8 (Fig. 8). Of note, treatment with CD14 antibody significantly reduced both IL-6 and IL-8 secretion by 87% and 83%, respectively in FIBs. Control antibody had no effect on cytokine levels in both cell types. These results demonstrate that LPS effects on cytokine secretion in HBCs were mostly CD14-independent, whereas LPS effects in FIBs required CD14.

DISCUSSION

Release of inflammatory cytokines and hemostatic factors by placental stromal cells, such as HBCs and FIBs, adjacent to fetal capillaries, as well as by fetal endothelial cells themselves, likely play important roles in the initiation and propogation of fetal inflammatory processes noted in pregnancies with and without intrauterine infection [3-5]. However, little is known about the mechanisms by which these three cell types generate inflammatory responses. Therefore, in the present study we used cultures of HBCs, FIBs, and HUVECs to establish cell type-specific patterns of TLR-driven regulation of cytokine mRNA expression and protein secretion at the maternal-fetal interface. Our laboratory has recently developed techniques for the concommittant isolation of HBCs, FIBs, and cytotrophoblasts from placenta [32]. Of note, the most marked cellular response observed in the current study was the up-regulation of IL-6 mRNA levels and protein secretion by HBCs treated with LPS. Although basal levels of IL-8 in HBCs were high, they were less responsive to LPS treatment. Conversely, in FIBs and HUVECs, IL-8 levels were more substantially up-regulated than IL-6 levels by LPS treatment. Also of interest is that LPS treatment enhanced TF gene expression in HBCs and HUVECs but not in FIBs. Although the effects of Poly (I:C) were less pronounced than those promoted by LPS in HBCs, it is likely that the Poly

(I:C)-mediated increases in IL-6 and IL-8 levels is biologically relevant and suggests that HBCs respond to viral infections through a TLR-3-mediated mechansim as has been noted in non-placental tissue macrophages [37]. In addition, Poly (I:C) treatment did not significantly affect cytokine secretion by FIBs and HUVECs, and PG treatment did not promote significant responses in any of the three cell types. It is possible that treatment with Poly (I:C) and PG at concentrations greater than the 1 μg/ml level used in the current study would enhance cytokine secretion in these cell types. Using flow cytometry, we demonstrated that cultures of HBCs maintain an M2 phenotype observed *in vivo* by immunohistochemistry. This suggests that HBCs, despite their M2 phenotype, release pro-inflammatory cytokines in response to microbial stimuli at the maternal-fetal interface. Our results are consistent with recent findings demonstarting that Hofbauer cell phenotype (i.e. relative expression of M1 vs M2 markers) is not altered in pregnancies with CAM [24]. Interestingly, the ability of HBCs to form multinucleated giant cells was reduced in pregnancies complicated by CAM[38]. Of note, increased expression of TLR-4 in HBCs has been previously documented in placentas from pregnancies with CAM [16], supporting a direct role of infection in the regulation of HBC immune function. Importantly, our previous study indicated that there are focal increases in HBCs in placental villi from pregnacies associated with histological CAM [39], suggesting an important role of this cell type in mediating local inflammatory changes associated with placental infection.

Several studies specifically implicate CAM, funisitis, and increased levels of IL-6 and IL-8 levels in amniotic fluid and fetal blood with the development of FIRS and cerebral palsy [7, 40, 41]. The differential patterns of cell type-specific cytokine regulation we observed in response to LPS treatment suggests that IL-6 production is liklely to be markedly enhanced in HBCs in pregnancies with Gram-negative intrauterine infections including *Ureaplasma urealyticum* and *Staphylococcus aureus* which have been implicated in CAM with funistis [30, 42]. IL-6 is known to regulate acute phase inflammatory responses as well as metabolic processes, including modulation of glucose metabolism and differential regulation of the hypothalamic-pituitary-adrenal axis [43]. Therefore, IL-6 generated by HBCs in the setting of CAM may also affect the proliferation of leukocytes and metabolic processes in the fetus. IL-8 is a major neutrophil chemoattractant [44]. Our findings showing LPS-mediated increases in IL-8 levels in placental FIBs and fetal endothelial cells support a role of these cell types, as well as HBCs, in the marked infiltration of neutrophils to the maternal-fetal interface associated with CAM [4]. Since LPS treatment stimulated TF expression in HBCs and HUVECs, but not in FIBs, HBCs and fetal endothelial cells may play a role in the thrombotic vasculopathy associated with CAM and cerebral palsy [3, 6].

Binding of LPS to LBP promotes the formation of a complex with CD14 which facilatates the subsequent transfer of LPS to the TLR-4/MD-2 receptor complex [12]. LBP is an acute phase protein synthesized by liver, more recently its presence has been demonstrated in airway and oral epithelia [45-47] which are exposed to commensal pathogens. Modeling studies based on structural analysis suggests that MD-2, not TLR-4, is an endotoxin-binding protein [48]. In the current study, we observed that, under serum-free conditions, LBP was required for the LPS action in FIBs, but not in HBCs. Our results also indicated that levels of CD14, TLR-4 and MD-2 were significantly higher in HBCs compared to FIBs,

suggesting that the LBP requirement in FIBs was due to relatively low levels of TLR-4 and its co-activators in this cell type. Moreover, our studies using function-perturbing anti-CD14 Ab indicated a more strict requirement for CD14 in TLR-4 signaling in FIBs compared to HBCs, which may also reflect the relative abundance of TLR-4 and co-activators in these cell types. These results suggest, that in the absence of an endogenous source of LBP, HBCs would be expected to be the primary mediators of innate immune reponse to Gram-negative bacteria present in the placental stroma.

A specific role for TLR-4 signaling in complications of pregnancy is suggested by the demonstration of increased TLR-4 expression in HBCs in pregnancies with CAM [16] and in interstitial trophoblasts in pregnancies with preeclampsia [49]. Studies using *in situ* 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 [50]. The infectious agents (bacteria, Coxsackie virus, parovirus, cytomegalovirus, and herpes simplex virus) were localized primarily to HBCs and SCT; no infectious agent was revealed in the 17 control placentas [50]. Similar results were reported in another study in which placentas were examined from 33 cases with significant neurodevelopmental delay [51]. Of note, in both studies, the same infectious agent was found in the placenta and fetal tissue of autopsy materials. Importantly, using mouse models TLR-4 was shown to play a critical role in preterm delivery promoted by intrauterine infusion of LPS or heat-killed *E. Coli* [28, 29]. A recent study demor

In conclusion, our results showed that, despite their M2 phenotype, HBCs expressed high levels of TLRs and co-activators, and are able to respond to infectious TLR agonists by producing inflammatory markers of FIRS in a cell-specific manner. These results suggest an important role of HBCs as mediators of placental/fetal inflammation.

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Abbreviations

CAM	chorioamnionitis
FIBs	fibroblasts
FIRS	fetal inflammatory response syndrome
HBC	Hofbauer cell
HUVEC	human umbilical vein endothelial cell
LBP	lipopolysaccharide (LPS)-binding protein
PG	peptidoglycan
poly (I:C)	polyinosinic:polycytidylic acid
SCT	syncytiotrophoblast
TF	tissue factor

TLR Toll-like receptor

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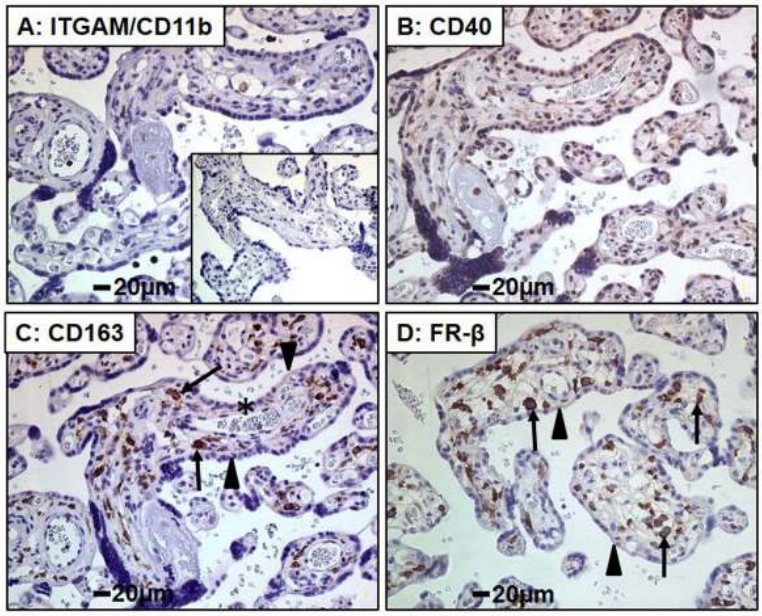

Figure 1. Immunohistochemical staining for macrophage markers in term placental tissue

Immunohistochemical staining for M1 macrophage markers, CD11b (ITGAM) (A) and CD40 (B) and M2 macrophage markers CD163 (C) and FR-β (D), was carried out using formalin-fixed paraffin embedded sections. Staining with negative control antibody is shown in the Panel A insert. Results are presented from one placenta representing three different placentas. Arrows indicate stained HBCs, arrowheads denote the syncytiotrophoblast, and the asterisk denotes a fetal capillary. Bar; 20 μm.

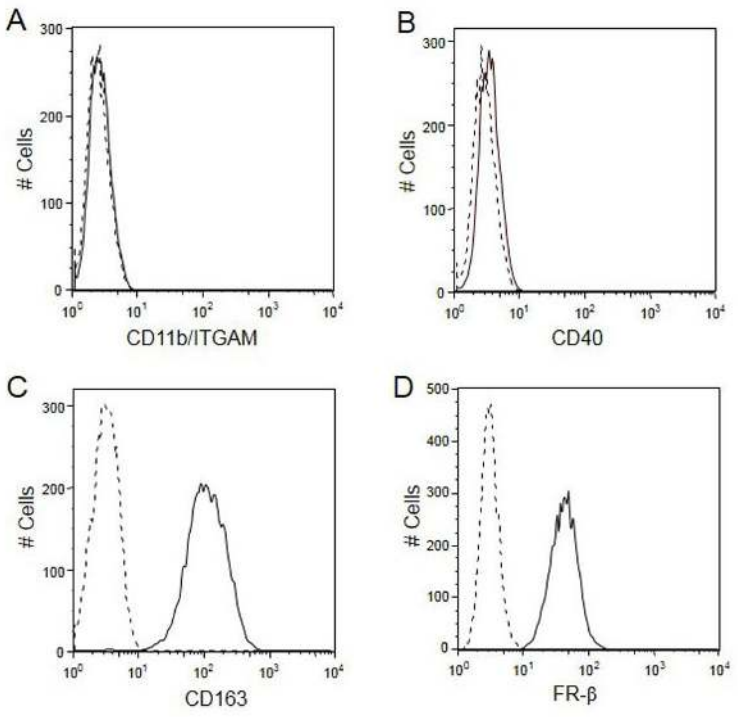

Figure 2. Flow cytometric analysis of M1 and M2 macrophage markers in cultures of HBCs

HBCs were stained for CD11b (A), CD40 (B), CD163 (C), and FR-β (D) and analyzed by flow cytometry indicated by the solid line in each panel. The dashed line in each panel represents the signal obtained using an isotype-matched control antibody. The results are representative of three independent experiments.

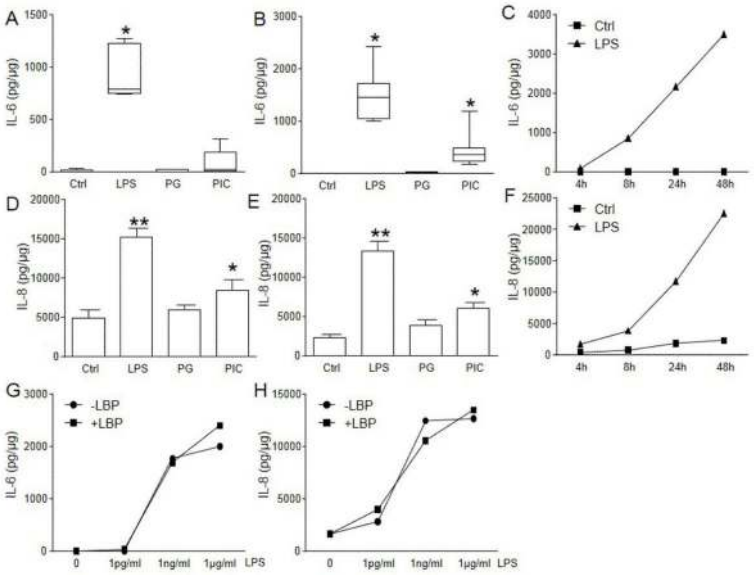

Figure 3. Regulation of IL-6 and IL-8 secretion by HBCs following treatment with LPS, PG and Poly (I:C)

HBCs were maintained without (control, Ctrl) or with 1μg/ml of LPS, PG, or Poly (I:C) (PIC) for 24 h in medium with (Panels A and D), or without (Panels B and E) serum. Levels of IL-6 and IL-8 secreted into conditioned media from three independent experiments were determined by ELISA and normalized to total cell protein. Time course of LPS effects (C and F) and response of HBCs to LPS and LBP (G and H) in medium without serum are shown from a single experiment representing 3 identically conducted ones. Results which are not normally distributed are presented as medians and percentiles; the lines inside the box indicate the median, the ends of the box describe the lower and upper quartiles, and the whiskers define the smallest and largest values (A and B). Normally distributed results are presented as a mean + SE (D and E).

*P<0.05 *vs* Ctrl; **P<0.001 *vs* Ctrl

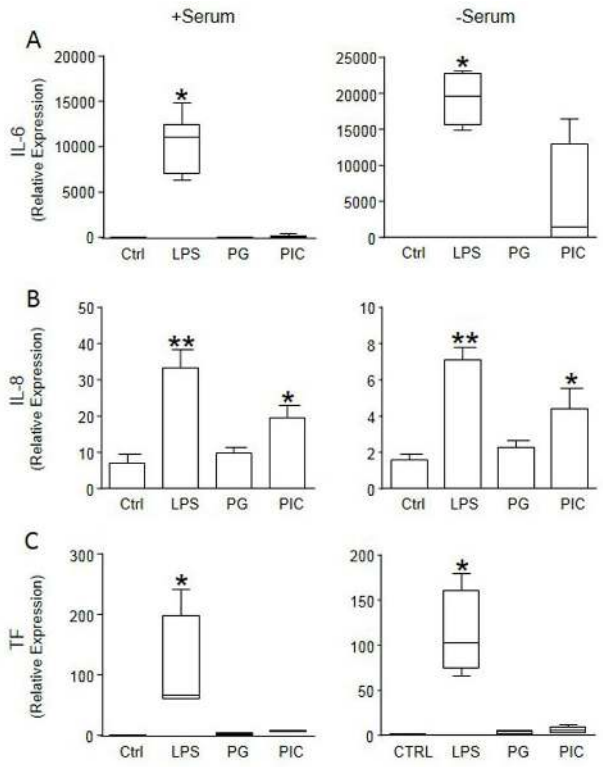

Figure 4. Effect of LPS, PG, and Poly (I:C) treatment on IL-6, IL-8, and TF mRNA levels in HBCs

HBCs were incubated in in FBSM (+Serum) or SFM (-Serum) for 24 h (IL-6 and IL-8 mRNA) or 4 h (TF mRNA) without (control, Ctrl) or with 1 μg/ml LPS, LPS and 50 ng/ml LBP, PG, or Poly (I:C) (PIC). Levels of IL-6 (A), IL-8 (B), and TF (C) mRNA expression were analyzed by qPCR and normalized to 18S RNA levels. Results are expressed as relative expression and are presented as a median and percentiles from five independent *P<0.05 *vs* Ctrl; **P<0.001 *vs* Ctrl

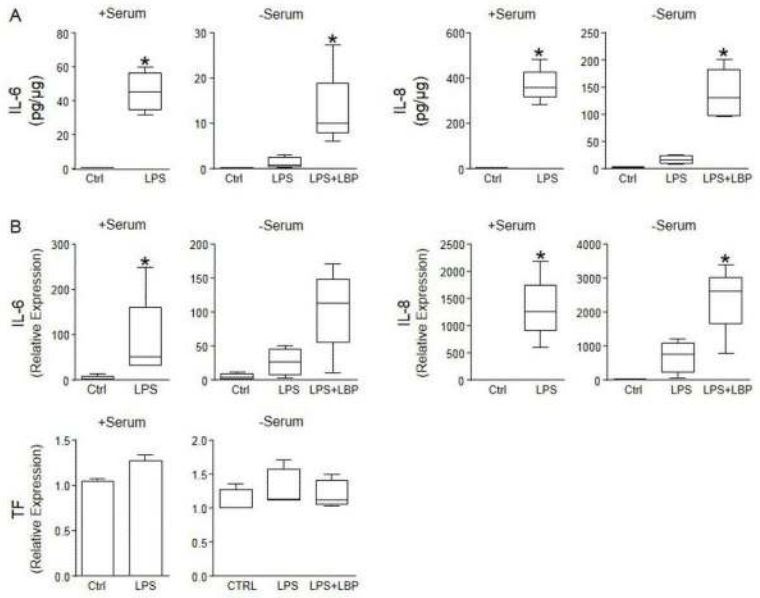

Figure 5. Effect of LPS and LBP treatments on levels on IL-6, IL-8, and TF expression by FIBs

FIBs were maintained in FBSM (+Serum) or SFM (-Serum) for 24 h (IL-6 and IL-8 secretion and mRNA expression) or 4h (TF mRNA expression) without (control, Ctrl) or with 1 µg/ml LPS, or LPS and 50 ng/ml LBP. Levels of IL-6 and IL-8 protein secretion were analyzed by ELISA and normalized to total protein level (A). Levels of mRNA were analyzed by qPCR and normalized to 18S RNA levels (B). Results are expressed as a mean + SE or as a median and percentiles from three or four independent experiments.
*P<0.05 *vs* Ctrl

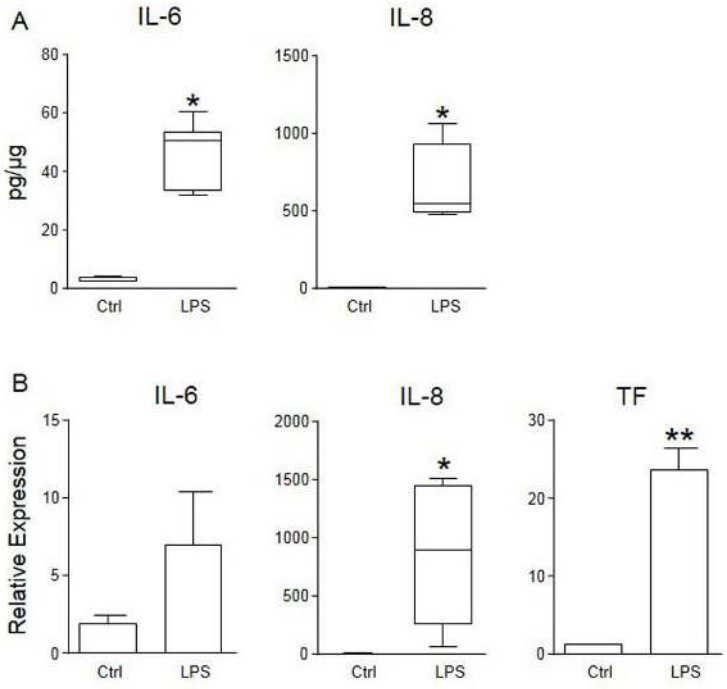

Figure 6. Effect of LPS treatment on IL-6, IL-8, and TF expression by HUVECs

HUVECs were maintained in FBSM for 24 h (IL-6 and IL-8 secretion and mRNA expression) or 4h (TF mRNA expression) without (control, Ctrl) or with 1 µg/ml LPS. Levels of IL-6 and IL-8 protein secretion were analyzed by ELISA and normalized to total protein level (A). Levels of mRNA were analyzed by qPCR and normalized to 18S RNA levels (B). Results are expressed as a median and percentiles or as a mean + SE from three to five independent experiments.

*P<0.05 *vs* Ctrl; **P<0.001 *vs* Ctrl

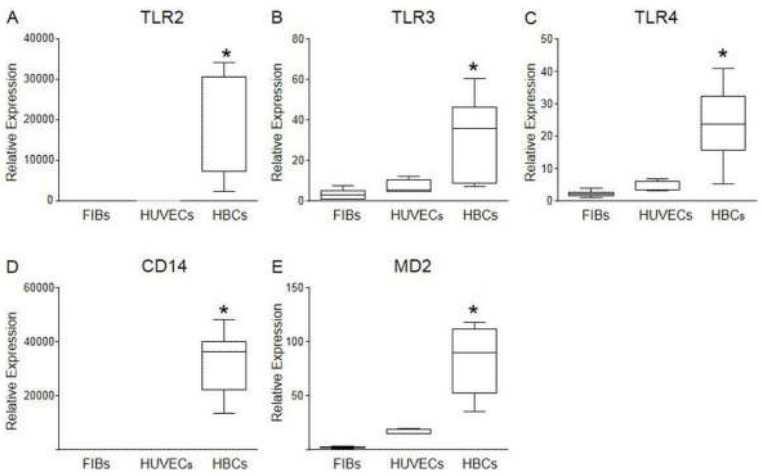

Figure 7. Comparison of TLR-2, TLR-3, TLR-4, CD14 and MD-2 mRNA levels in HBCs, FIBs, and HUVECs

Levels of TLR-2, TLR-3, TLR-4, CD14, and MD-2 mRNA in HBCs, FIBs, and HUVECs were analyzed by qPCR in three to six independent experiments, are expressed as relative expression, and presented as median and percentiles.

*P<0.05 vs FIBs

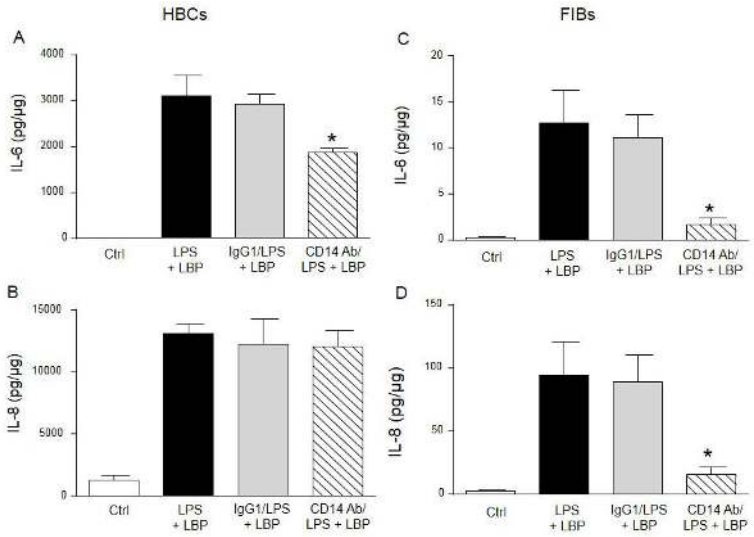

Figure 8. Role of CD14 in LPS-mediated effects on cytokine levels secreted by HBCs and FIBs

HBCs (A and B) and FIBs (C and D) were maintained for 24 h in SFM without (Ctrl) or with 1 μg/ml LPS and 50 ng/ml LBP and a function-perturbing anti-CD14 antibody or control IgG1 antibody. Levels of IL-6 and IL-8 secretion into conditioned media were quantitated by ELISA and normalized to cell protein. Results are presented as a mean + SE from 3 independent experiments for each cell type.

*P<0.05 vs LPS+LBP