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Functional Screening of TLRs in Human Amniotic Epithelial Cells

Claire Gillaux,* Céline Méhats,* Daniel Vaiman,* Dominique Cabrol,† and Michelle Breuiller-Fouche*†

Intrauterine infection is a major cause of spontaneous preterm birth. Amniotic epithelial cells represent the first line of defense against intra-amniotic bacteria. We hypothesize that this epithelial cell barrier is able to recognize and respond to pathogens through the function of TLRs, which are crucial regulators of the innate immune system. In this study, we describe the expression of transcripts for TLR1–TLR10 in human amniotic epithelial cells. We show that amniotic epithelial cells express functional TLR5, TLR6/2, and TLR4. Activation by TLR5 and TLR6/2 agonists produces IL-6 and IL-8, concomitantly with the activation of NF-κB signaling pathway, matrix metalloproteinase-9 induction, and PTGS2 expression. In contrast, TLR4 activation reduced amniotic epithelial cell viability and induced cell apoptosis evidenced by an elevated Bax/Bcl-2 ratio and cleavage of caspase-3. These data suggest specific TLR-mediated functions in human amniotic epithelial cells for initiating different immune responses, which ultimately may lead to preterm birth. The Journal of Immunology, 2011, 187: 2766–2774.

Recognition of pathogens by innate immune cells is mediated by pattern recognition receptors that recognize conserved pathogen-associated molecular patterns (PAMPs) (for reviews, see Refs. 10–12). To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR1–9 being conserved in both species. Each TLR senses different PAMPs. TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface, whereas TLR3, 7, 8, and 9 are expressed in cytoplasmic organelles, mainly in the endosomes. Briefly, TLR2 is able to recognize a wide set of bacterial products including components of Gram-positive bacterial cell walls, peptidoglycans, lipoproteins, and lipoteichoic acid, in addition to yeast cell-wall particles (zymosan). TLR2 complexes as a heterodimer with TLR6 or TLR1, interacting with microbial diacylated or triacylated lipopeptides, respectively. TLR4 is activated by LPS, a constituent of the cell wall of Gram-negative bacteria, TLR5 by bacterial flagellin, and TLR9 by unmethylated CpG motifs commonly present in bacterial and viral genomes. TLR7 and 8 sense viral constituents such as ssRNA, whereas dsRNA is recognized by TLR3. No specific activator of TLR10 has been identified to date.

Engagement of TLRs by PAMPs triggers intracellular signaling cascades through a set of Toll/IL-1 receptor-domain containing adapter proteins (13). All TLRs except TLR3 initiate MyD88-dependent signaling pathway to activate NF-κB and AP-1 and induce the expression of cytokines through various kinases. As expected, much attention has been paid to TLR expression and function in the female genital tract (14–17). Some studies have demonstrated that human placenta expresses TLRs and responds to corresponding PAMPs by producing proinflammatory cytokines. Others have reported divergent responses of trophoblast cells to invading pathogens: stimulation of TLR4 leading to cytokine production, whereas TLR2 activation promotes trophoblast cell death (18–20). In addition, spontaneous term labor and preterm delivery are associated with an increase expression of TLR2 and TLR4 in the chorioamniotic membranes, suggesting a role for the fetal membranes in the immune system (21, 22). Traditionally, the fetal membranes, which consist of the fetal-facing amnion and maternal-facing chorion, are of considerable importance for the successful maintenance and termination of pregnancy. The amnion is derived from the embryo and is formed on the 8th day after

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Abbreviations used in this article: MMP, matrix metalloproteinase; PAMP, pathogen-associated molecular pattern; poly(I:C), poly inosinic-polycytidylic acid; PPROM, preterm premature rupture of the fetal membranes; PTB, preterm birth; PTL, preterm labor.

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fertilization. The innermost layer, the amniotic epithelium, is in direct contact with the amniotic fluid and serves to protect the developing fetus (23). Thus, this amniotic epithelial cell monolayer provides a physical barrier that serves as the first line of defense encountered by invading pathogens. Understanding the functional role of TLR in amnion may therefore be informative for elucidating the complexity of the innate immune system to the site of an intrauterine infection.

In this study, we postulated that primary human epithelial amniotic cells express various TLR family members and respond to multiple TLR ligands. We show that human epithelial amniotic cells express functional TLR6/2, 4, and 5. Upon stimulation with TLR6/2 and 5 agonists, human epithelial amniotic cells produce proinflammatory cytokines, concomitantly with the activation of NF-κB signaling pathway, MMP-9 induction, and PTGS2 expression. We also demonstrate that LPS activates apoptotic signaling pathways through TLR4.

Materials and Methods

Human fetal collection and amniotic epithelial cell cultures

Placentas with attached fetal membranes from term normal uncomplicated singleton pregnancies were collected after elective cesarean section prior to the onset of labor (range 38.5–40 wk of pregnancy) because of a diagnosed cephalo-pelvic disproportion in normal pregnancies at the Port-Royal Cohin Hospital (Paris, France). All patients gave informed consent. Fetal membranes were dissected free of the placenta under sterile conditions and washed 3 times with PBS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin (Invitrogen, Cergy-Pontoise, France) to eliminate blood clots. The isolation of amniotic epithelial cells from amnion tissue was based on modifications of procedure originally described by Casey and MacDonald (24). Briefly, the amnion manually separated from the choriondeciidae was cut into 1-cm squares and then digested two times for 60 min with DMEM–F12 containing 0.5% trypsin (Sigma-Aldrich). The cell suspension digest was vigorously shaken and filtered through a 100-µm nylon gauze. Undigested tissues were discarded. Epithelial amniotic cells were pelleted by centrifugation at 1500 × g for 10 min at 4˚C, suspended in complete medium (DMEM–F12 supplemented with 10% FCS, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin (Invitrogen), 1% insulin, transferrin, selenium, and 0.02 µg/ml epidermal growth factor (Sigma-Aldrich)) before being seeded at a density 2 × 10^5 cells/cm². Cell culture medium was changed after 3 d of culture. Upon reaching confluence (6–7 d), adherent cells were trypsinized and resuspended at a density of 2 × 10^6 cells per well in 24-well plates for protein extraction or 2 × 10^5 cells/ml, cultured in the second Alexa Fluor R 488 donkey anti-rabbit IgG-labeled solution (dilution 1:500) (Interchim, Montluçon, France) for 45 min at room temperature and protected from light. Nuclei were labeled with Hoechst 33342 (dilution 1:500) (Interchim, Montluçon, France) for 45 min at room temperature and protected from light. Nuclei were labeled with Hoechst 33342 (dilution 1:500) (Interchim, Montluçon, France) for 45 min at room temperature and protected from light. Nuclei were labeled with Hoechst 33342 (dilution 1:500) (Interchim, Montluçon, France) for 45 min at room temperature and protected from light.

Immunofluorescence analysis

Subconfluent amniotic epithelial cells were cultured for 2 d in 24-well dishes on coverslips and then transferred to serum-deprived media for 24 h before conducting experiments. To induce nuclear translocation of NF-κB p65, cells were incubated with or without TLR ligands at the indicated time at 37˚C. After fixation for 15 min with 4% paraformaldehyde in PBS, cells were rendered permeable by incubation in 0.1% Triton X-100 in 10% FCS–PBS for 15 min. They were incubated with the Ab directed against NF-κB p65 (dilution 1:200) (Santa Cruz Biotechnology) and Alexa Fluor 488 donkey anti-rabbit IgG-labeled solution (dilution 1:500) (Interchim, Montluçon, France) for 45 min at room temperature and protected from light. Nuclei were labeled with Hoechst 33342 (2 µg/ml). Coverslips were mounted on slides using fluorescent mounting medium from Dako France (Trappes, France). An Nikon E-600 microscope was used for conventional fluorescence microscopy, and photographs were taken using CoolSnap digital (RS Photometrics, Evry, France). For quantitative analysis of the NF-κB p65 nuclear translocation, total and unlabeled nuclei were counted in five distinct random fields per coverslip. The results are expressed in percentage of labeled nuclei. In each condition, at least 40 cells were quantified.

RT-PCR analysis

Total RNA was extracted from amniotic epithelial cells using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA using 1 µg total RNA, superscript III reverse transcriptase, and oligonucleotide primers (Invitrogen). Amplification was then performed as follows. Similar amounts of the resulting first-strand cDNA were used as template for PCR reactions: denaturation at 94˚C for 45 s, annealing at 60˚C for 45 s, and extension at 72˚C for 45 s for 35 cycles with a final extension step at 72˚C for 10 min. The primer sequences of TLRs 1–10 are detailed in Table I. The RT-PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide under UV light. To check the size of PCR products, a DNA molecular mass standard ladder (123-bp DNA ladder; Invitrogen) was run in parallel. Before performing RT-PCR, the samples were checked for any DNA contamination by performing PCR reactions done without reverse transcription of the RNA extracts. A cDNA from human lung (Bioschain, Hayward, CA) was used as a positive control.

Zymography

The proteolytic activity of pro–MMP-9 secreted into the culture medium was assessed by gelatin zymography on 10% SDS gel incorporating 1 mg/ml gelatin as previously described (25). Briefly, 3 µg protein was loaded and subjected to electrophoresis under nonreducing conditions. The gels

Western blot analysis

Amniotic epithelial cells were lysed on ice and scraped into a lysis buffer [50 mM NaCl, 25 mM HEPES, pH 7.6, 2.5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 50 mM NaF, 30 mM Na2PO4, 5 mM Na3VO4, and the protease inhibitor mixture, P-2714 (Sigma-Aldrich, Saint Quentin Fallavier, France)]. Lysates were then sonicated briefly and frozen at −80˚C until use. The protein concentrations were determined by the method of Bradford using BSA as standard. Equal amounts of proteins (20 µg) were resolved by SDS-PAGE on 8 or 10% gels and transferred onto a Hybond-P membrane. Non-specific binding sites were blocked by incubating the membranes with 5% low-fat dried milk in Tris buffer saline Tween (10 mM Tris HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20). The membranes were then incubated overnight at 4˚C in Tris buffer saline Tween/1% milk with the indicated primary Abs at the appropriate concentrations, Bax (1:1000), Bcl-2 (1:500), cleaved caspase-3 (1:500), and IcAM (1:1000) (Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France), and PTGS2 (1:1000) (Cayman Chemical, Spibio, Montigny le Bretonneux, France) followed by incubation with HRP-conjugated secondary Abs (dilution 1:7000). The blots were developed with ECL reagents (Amer sham Biosciences ECL reagent, GE Healthcare, Orsay, France) and visualized on Kodak X-ray films (GE Healthcare Biosciences). Molecular weight markers were run in parallel.

For standardization, the membranes were stripped with a buffer containing 62.5 mM Tris (pH 6.2), 2% NaDodSO4, and 100 mM 2-mercaptoethanol at 50 C for 30 min and reprobed with a polyclonal Ab raised against GAPDH (Santa Cruz Biotechnology). Densitometric analysis was performed using the National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/) developed by W.S. Rasband (National Institutes of Health, Bethesda, MD), which allowed us to control that the intensity of the signal for GAPDH exhibited no statistical difference in all the samples studied.

Determination of cytokine production by ELISA

Amniotic epithelial cells were plated at 5 × 10⁵ cells/ml, cultured in the aforementioned media for 6 d, and then stimulated with TLR ligands in serum-free medium for 6 h and 24 h. After stimulation, culture media were collected, and IL-6, IL-8, and TNF-α were analyzed using commercially available kits (e-Bioscience, San Diego, CA) according to the manufacturer’s instructions.

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were washed twice in 2.5% Triton X-100 for 30 min and incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.6), 5 mM CaCl₂, 200 mM NaCl, and 0.02% sodium azide. Thereafter, gels were stained with Coomassie brilliant blue R-250 in 30% ethanol and 10% acetic acid for 1 h and then destained in 25% ethanol and 10% acetic acid. Clear bands on a blue background indicate the presence of gelatin-degrading proteinases. The gelatinolytic activity of pro–MMP-9 (92 kDa) was estimated by scanning the wet gels.

**Cell viability**

The amniotic epithelial cell viability was checked by exclusion of trypan blue. At the indicated times after treatment, cells were detached by trypsin, pelleted, and resuspended in DMEM supplemented with 10% FCS. After staining with trypan blue, viable cells were counted with a hemocytometer. Three replicate wells were used for each test condition. All experiments were repeated at least six times on cells from six different patients. Cell viability data were validated by the PrestoBlue Cell Viability Reagent (Invitrogen) protocol. Briefly, cells plated in 96-well plates were treated at the indicated concentration of TLR agonists and allowed to grow for 72 h. Cell viability was expressed as the 570-nm absorbance ratio of drug-treated cells compared with cells incubated in the absence of drug (medium). The result was expressed as percentage of the control (defined as 100%).

**Statistical analysis**

Results are expressed as the mean ± SEM. Statistical significance was determined using one-way ANOVA, followed by post hoc tests with StatView software (SAS Institute, Cary, NC). The p values <0.05 were considered statistically significant.

**Results**

**Human amniotic epithelial cells express mRNAs for TLRs 1–10**

The expression profile of TLRs 1–10 (primers are listed in Table I) was assessed by end-point RT-PCR in human amniotic epithelial cells. Transcripts for TLR1–TLR10 were expressed in all of the amniotic epithelial cells studied (n = 5). An example for cell preparation obtained from one patient is shown in Fig. 1. All amplified products were of the predicted size. As a positive control, human lung was shown to express all mRNAs of TLRs 1–10.

**TLR5 and TLR6/2 stimulation in human amniotic epithelial cells triggers the secretion of proinflammatory cytokines**

To characterize the functional relevance of TLRs in human amniotic epithelial cells, the production of IL-6, IL-8, and TNF-α were determined at 6 h and 24 h from the five patients studied.

**Amniotic epithelial cells were stimulated with TLR ligands at the indicated doses: the TLR1/2 ligand, Pam3Cys-Ser-(Lys)₄, 100 ng/ml; the TLR3 ligand, poly(I:C), 100 ng/ml; the TLR4 ligand, LPS, 5 µg/ml; the TLR5 ligand, flagellin, 100 ng/ml; the TLR6/2 ligand, MALP-2, 100 ng/ml; the TLR7 ligand, imiquimod, 10 µg/ml; and CpG, 2.5 µg/ml, which binds TLR9. Subsequently, the concentration level of IL-6, IL-8, and TNF-α was determined in the cell media by ELISA (Fig. 2). Among all TLR ligands examined, only flagellin and MALP-2 significantly induce the production of IL-6 and IL-8 within 6 h and then remain stable during the 24-h test. As shown in Supplemental Fig. 1, flagellin and MALP-2 induced the production of IL-6 and IL-8 in a concentration-dependent manner, peaking at 100 ng/ml after 6 h of treatment. Flagellin appeared to be more potent than MALP-2 to induce secretion of IL-6, but MALP-2 preferentially induced IL-8 secretion. We also found that TNF-α concentration in the media was below the limits of detection of the ELISA kit used in our experimental conditions and was not affected by the presence of the TLR agonists used (data not shown).

**TLR5 and TLR6/2 stimulation in human amniotic epithelial cells induces nuclear translocation of the NF-κB subunit p65**

The effects of TLR ligands on NF-κB activation were evaluated by its ability to induce the translocation of the NF-κB p65 subunit into the nucleus of amniotic epithelial cells. As shown in Fig. 3A, p65 immunoreactivity was entirely absent from the nucleus of untreated cells, and it was evenly distributed throughout the cytoplasm. Incubation with flagellin (100 ng/ml) resulted in p65 accumulation in nuclei of amniotic epithelial cells after 40 min of treatment. The nuclear p65 staining reached a maximum at ~60 min. In parallel, MALP-2 (100 ng/ml) was also able to translocate NF-κB p65, but only a partial translocation was observed at a time as long as 90 min. To evaluate the magnitude of TLR agonist-induced translocation of NF-κB p65, the percentage of cells showing nuclear immunostaining was determined (Fig. 3B). Cells treated with flagellin or MALP-2 showed a significant difference from control conditions at 40, 60, or 90 min. By contrast, the TLR1/2, TLR4, TLR7, or TLR9 ligands failed to translocate NF-κB p65 (data not shown).

A critical regulatory control point on the pathway to NF-κB activation is the phosphorylation, ubiquination, and subsequent degradation of IκBα. We therefore estimated the level of IκBα in the cytosol using Western blot. As shown in Fig. 3C, stimulation of amniotic epithelial cells with TLR5 and TLR6/2 ligands results in the degradation of IκBα. The effects of flagellin are more rapid than those of MALP-2.

**TLR5 and TLR6/2 stimulation in human amniotic epithelial cells induces PTGS2 expression**

Because TLR activation mediates inflammation, we evaluated the level of PTGS2 in amniotic epithelial cells after induction by TLR
and MALP-2 resulted in a 3-fold increase in pro–MMP-9 activity. MMP-9 protein. As shown in Fig. 5B, gelatin zymography in the culture medium of amniotic epithelial cells induced pro–MMP-9 production TLR5 and TLR6/2 stimulation in human amniotic epithelial cells induces pro–MMP-9 production

TLR activation also results in modification of the extracellular matrix. As a marker of this activity, we analyzed MMP-9 activity by gelatin zymography in the culture medium of amniotic epithelial cells treated or not with 100 ng/ml flagellin, 100 ng/ml MALP-2, or 5 μg/ml LPS for 6 h. Fig. 5A shows a representative zymography gel with bands at 92-kDa corresponding in size with the pro–MMP-9 protein. As shown in Fig. 5B, incubation with flagellin and MALP-2 resulted in a 3-fold increase in pro–MMP-9 activity.

In contrast, the expression of MMP-9 was unaffected by LPS. None of the other ligands of TLR tested was capable of inducing the secretion of pro–MMP-9 (data not shown).

**TLR4 ligand, but not TLR5 or TLR6/2 ligands, reduces human amniotic epithelial cell viability**

Cell number and viability were examined by either a trypan blue dye exclusion assay or by PrestoBlue reagent, which is quickly reduced by metabolically active cells. As shown in the time-course study (Fig. 6), subconfluent amniotic epithelial cells were maintained for up to 3 d in serum-free medium in absence of stimuli without a significant change in cell viability. Treatment with flagellin or MALP-2 had no significant effect on the number of viable cells at 24, 48, or 72 h. When amniotic epithelial cells were treated with LPS (5 μg/ml), the number of viable cells did not change the first 24 h, but a significant decrease was observed at 48 and 72 h poststimulation with LPS. The concentration-dependent effect of LPS (0.05–5 μg/ml) was also determined (Fig. 6). A significant decrease in the number of viable cells was detected at 5 μg/ml at 48 h (data not shown) and 72 h. None of the other ligands of TLR tested has an effect on cell viability.

**TLR4 ligand, but not TLR5 or TLR6/2 ligands, affects Bcl-2 family proteins in human amniotic epithelial cells**

To determine whether the treatment of amniotic epithelial cells with TLR ligands affects the Bcl-2 family proteins, we performed Western blot analysis for the proapoptotic Bax protein and the antiapoptotic Bcl-2 protein. As shown in Fig. 7A, treatment of cells for 72 h with LPS (5 μg/ml) resulted in an increased expression of Bax protein (23 kDa), whereas the level of Bcl-2 (26 kDa) was decreased. In contrast, the levels of Bax/Bcl-2 proteins did not change after stimulation of amniotic epithelial cells with MALP-2 or flagellin. The relative intensities of the protein signals quantified by densitometric analyses and normalized to the corresponding GAPDH are illustrated in Fig. 7B.

**TLR4 ligand affects cleaved caspase-3 in human amniotic epithelial cells**

Treatment of amniotic epithelial cells with increasing concentrations of LPS (0.5–5 μg/ml) resulted in cleavage of caspase-3, as evidenced by appearance of the proteolytic 17-kDa fragment at 72 h, whereas little or no activated caspase-3 was detected at earlier time points (Fig. 8A). As depicted in Fig. 8B, the cell-permeable caspase inhibitor Z-VAD-FMK (25 μM) impairs caspase-3 activation in amniotic epithelial cells exposed to LPS for 72 h. These results strongly suggest that activation of the caspase cascade was essential for LPS-induced apoptosis in amniotic epithelial cells.

**Discussion**

Despite extensive literature on TLR expression in the female genital tract (14, 17, 18, 26, 27), little is known about the TLR repertoire and its response upon TLR ligand engagement in human amniotic epithelial cells, which represent the first line of host defense against intra-amniotic bacteria. It seems then important to determine if this final epithelial cell barrier has a specific mechanism to recognize and respond to invading pathogens. In this study, we evaluated the role of specific human TLRs, in particular those relevant to key proinflammatory mediators involved in PTB. We found that transcripts for TLR1–10 were detectable in amniotic epithelial cells, which was similar to the TLR gene expression profile previously reported in human placenta as well as in human choriocarcinoma cell line (19, 28). To date, only the expression of TLR2 and TLR4 by amniotic epithelial cells has been described. Upregulation of both receptors was reported for amniotic mem-

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brane from women who delivered after spontaneous PTL and women with chorioamnionitis (16, 21, 29, 30). Recently, the presence and functional role of a soluble truncated form of TLR2, which acts as a decoy receptor, was described in human amniotic fluid (31).

Only activation by TLR5 and TLR6/2 by their cognate ligands (flagellin and MALP-2, respectively) induced proinflammatory responses in amniotic epithelial cells. Release of IL-6 and IL-8 was dose-dependently increased after stimulation with flagellin or MALP-2. MALP-2 was a less potent stimulus than flagellin to induce IL-6 production but could strongly induce IL-8 production. The other TLR activators cannot significantly induce IL-6 or IL-8 production. Amniotic epithelial cells were found to produce TNF-α, but the TNF-α levels were below the detection limit in our experimental conditions, and the levels were unchanged after stimulation with TLR agonists.

NF-κB is critically involved in TLR-mediated signaling pathways and is known to be implicated in the regulation of many key proteins participating in the parturition process including PTGS2, which promoter contains two NF-κB sites (32–35). In amniotic epithelial cells, activation of TLR5 and TLR6/2 by flagellin and MALP-2, respectively, appears to trigger a classical response characterized by a nuclear translocation of the NF-κB p65 subunit, as evaluated by indirect immunofluorescence. The nuclear translocation of NF-κB p65 was complete at 60 min together with a concomitant degradation of IκBα in amniotic epithelial cells after activation by TLR5 and TLR6/2 agonists. A question arising from these observations was whether activated NF-κB increases expression of target genes. One of these is PTGS2, which was shown to be selectively induced by flagellin and MALP-2 in amniotic epithelial cells. These data expand previous reports indicating that bacterial products affect PG synthesis by fetal membranes (36–40). We then hypothesized that the PGs consequently produced have the ability to induce the production of MMP. Thus, compelling clinical and ex vivo data point to PGs and MMPs as key effectors in PPROM. Increased MMP-9 secretion has been associated with pathological and pathophysiological processes, such as PTL, normal delivery, and PPROM. Additionally, microbial invasion of the amniotic cavity increased MMP-9 levels in amniotic fluid (5). Therefore, we investigated the role of TLR stimulation in the activation of MMP-9 in amniotic epithelial cells. By gelatin zymography, we have observed that amniotic epithelial cells exclusively produced pro–MMP-9. Moreover, an increase in pro–MMP-9 production was significantly

**FIGURE 3.** Activation of NF-κB signaling pathway in amniotic epithelial cells treated with TLR5 and TLR6/2 ligands. A, To detect the nuclear translocation of NF-κB p65, amniotic epithelial cells were cultured for 40, 60, and 90 min in the presence of flagellin (100 ng/ml) or MALP-2 (100 ng/ml), as described in Materials and Methods. Amniotic epithelial cells were then fixed, permeabilized, and immunostained with Ab against NF-κB p65. This is the result of five independent experiments performed with five patients. Original magnification ×200. B, Quantification of NF-κB p65 nuclear translocation. Results are expressed as the percentage of cells that express NF-κB p65 in nucleus and are the means ± SEM of at least five independent experiments performed in duplicate. *p < 0.05 (significant difference compared with control). C, Cell lysates from amniotic epithelial cells treated with flagellin (100 ng/ml) or MALP-2 (100 ng/ml) were characterized by immunoblot using Ab against IκBα. A representative Western blot is shown.
found after treatment of amniotic epithelial cells by flagellin and MALP-2.

Our data indicate that TLR5 and TLR6/2 were expressed and functionally active in terms of NF-κB activation, IL-6 and IL-8 production, and PTGS2 and MMP-9 activation in amniotic epithelial cells. These results are in line with the proposed role of NF-κB in the regulation of inflammatory processes in immune responses through the local production of proinflammatory factors responsible for modulating the synthesis of MMP-9. Moreover, the TLR5 and TLR6/2 responses that we observed in amniotic epithelial cells were somewhat predictable. Genital Mycoplasma (and in particular Ureaplasma urealyticum), Streptococcus agalactiae, as well as other Gram-positive and Gram-negative aerobic and anaerobic organisms are known to be associated with chorioamnionitis and PTB (9, 41, 42). Both TLR2 and TLR6 are necessary for responding to Mycoplasma-associated protein (43), and flagellin, the TLR5 ligand, is a highly immunogenic molecule expressed in flagellated Gram-positive and Gram-negative bacteria. Notably, the ligation of TLR1/2, TLR3, TLR4, TLR7, and TLR9 expressed by amniotic epithelial cells had a different effect as all cytokine levels were unchanged and we did not observe NF-κB activation, PTGS2 induction, or MMP-9 activation. The absence of these TLR responses may be indicative of a nonfunctional state of TLR1/2, TLR3, TLR4, TLR7, and TLR9, but alternatively the response of TLR ligands may involve different pathways to exert other specific cellular functions or may induce the production of certain cytokines and/or chemokines not detected in our experiments. Several studies have documented the role of fetal membrane apoptosis in the pathogenesis of PPROM (5, 44, 45). It is thought that amniotic epithelial cells are vulnerable to apoptosis by physiological agents (46). We thus hypothesized that TLR stimulation may induce apoptosis of amniotic epithelial cells.
Prolonged exposure of amniotic epithelial cells to LPS, the classical TLR4 ligand, decreased the number of viable cells. By contrast, activation of other TLRs did not alter the amniotic epithelial cell viability. Additional studies were performed with classical markers of apoptosis, which include the balance between the Bcl-2 family proteins and caspase-3 protein. It is well established that alteration of the Bax/Bcl-2 ratio is a key factor involved in a cell that undergoes apoptosis (47). In the current study, we observed that treatment of amniotic epithelial cells with LPS reduced the expression of Bcl-2 together with an increase in the level of Bax protein and induced the cleavage of caspase-3. In addition, treatment of cells with Z-VAD-FMK successfully blocked the activation of caspase-3 induced by LPS. Trophoblastic apoptosis in response to stimulation through TLR2 has been reported, in contrast to TLR4 ligation, which results in cytokine production (48). To date, functional TLR4 has been implicated in PTL triggered by administration of heat-killed Escherichia coli to mice (49, 50). In addition, an association between polymorphisms (Asp299Gly) in TLR4 and PTB has been reported (51, 52). Human TLR4 mutations are associated with an increased risk of Gram-negative infections (53). Among the bacterial strains associated with PTB outcomes, Mycoplasma hominis and Chlamydia trachomatis are Gram-negative and thus have the capacity to signal via TLR4 (54). TLR4 was immunodetected at the apical surface of amniotic epithelium after 25 wk of pregnancy and at the basal membrane at term pregnancy. This dynamic redistribution of TLR4 with gestational age suggests that amniotic epithelium presents distinctive defensive features. Also, chorioamnionitis was associated with a sequential redistribution of TLR4 from the apical to the basal membrane of amnion epithelium, which could be mimicked ex vivo by LPS stimulation (55). This activation of TLR4 allows a decreased LPS signaling early in an infection but allows the amnion epithelium to remain competent to invasive pathogens. Of greater interest, structural modifications of LPS have been shown to impact inflammatory responses within amniochorion in a non-human primate model (56). Adding to the complexity of LPS signaling in mammals, TLR4 is able to recognize not only LPS but also different endogenous ligands from damaged/stressed tissues (10).

Altogether, our data indicated that TLR5 and TLR6/2 were expressed and functionally active in terms of inflammatory responses in amniotic epithelial cells; by contrast, TLR4 stimulation did not affect inflammatory responses but can induce apoptosis, which might theoretically lead to PPROM or PTL. Each TLR shared specific functions and responded to a limited number of ligands. More than one TLR can respond to a variety of pathogens, and one TLR may interact with another with regard to recognition and signaling events. This is especially relevant, given the recently reported interaction of TLR2 and TLR3 in macrophages in a mouse model of pregnancy (57). These authors have demonstrated that activation of these two TLRs has a synergistic effect on inflammatory responses and PTB.

This study provides novel functional evidence for a unique repertoire of functional TLR expression and a response profile of...
amnion that stresses the importance of amniotic epithelial cells as sentinels for a wide range of pathogens. Activation of TLR6/2, TLR4, and TLR5 appears to generate distinct amniotic epithelial cell responses. Therefore, we can suppose that definite intrauterine infections may have either an inflammatory effect or an effect on amniotic epithelial cell survival during pregnancy, depending upon which TLR is activated. Further investigations into TLR signaling at the maternal–fetal interface could provide new insights into the role played by the immune system in maintaining pregnancy and combating preterm delivery.

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Disclosures

The authors have no financial conflicts of interest.

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