

# Toll-like receptor 4 activation on human amniotic epithelial cells is a risk factor for pregnancy loss

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**Background:** Maternal–fetal tolerance plays a fundamental role in the maintenance of pregnancy. However, this immunological tolerance can be influenced by intrauterine infections. Human amniotic epithelial cells (hAECs) have immunomodulatory effects and respond to invading pathogens through expressing various toll-like receptors (TLRs). We hypothesize that bacteria or bacterial products affect the immunosuppressive effects of hAECs through TLR stimulation. Here, we investigated how a successful pregnancy can be threatened by TLR4 activation on hAECs on lipopolysaccharide (LPS) engagement. **Materials and Methods:** hAECs were isolated from the amniotic membrane received from six healthy pregnant women. The immunophenotyping of hAECs was studied by flow cytometry. The isolated hAECs ( $4 \times 10^5$  cells/ml) were cultured in 24-well plates in the presence or absence of LPS ( $5 \mu\text{g/ml}$ ). After 24, 48, and 72 h of incubation, the culture supernatants of hAECs were collected, and the levels of interleukin-5 (IL-5), IL-6, IL-1 $\beta$ , tumor necrosis factor-alpha (TNF- $\alpha$ ), transforming growth factor-beta 1 (TGF- $\beta$ 1), and prostaglandin E2 (PGE2) were measured by enzyme-linked immunosorbent assay. **Results:** TLR4 activation showed a stimulatory effect on TGF- $\beta$ 1 production of hAECs ( $P < 0.001-0.05$ ). PGE2 production of LPS-stimulated hAECs was significantly increased ( $P < 0.01-0.05$ ). Moreover, TLR4 could induce TNF- $\alpha$  and IL-1 $\beta$  production of hAECs ( $P < 0.0001-0.01$ ), while this effect was not observed on IL-6 production of hAECs. The IL-5 was produced at a very low level in two culture supernatants of hAECs, in which its production was independent of LPS effect. **Conclusion:** TLR4 activation by bacterial components on hAECs may be a potential risk factor for pregnancy complications.

**Key words:** Human amniotic epithelial cell, immunosuppressive effect, lipopolysaccharide, pro-inflammatory mediators, toll-like receptor

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## INTRODUCTION

Immunological tolerance at the feto–maternal interface has an indispensable role in the implantation and maintenance of pregnancy.<sup>[1]</sup> On fertilization, maternal immune responses normally shift toward those which are critical to the successful pregnancy.<sup>[2,3]</sup> It has been reported that T helper 2 and T regulatory cells secrete immunosuppressive mediators such as interleukin (IL)-4, IL-5, transforming growth factor-beta (TGF- $\beta$ ), and IL-10 that inhibit the maternal immune system and enhance maternal–fetal tolerance.<sup>[2,4]</sup>

During pregnancy, pregnant women can be threatened by pathogenic agents.<sup>[5]</sup> In this notion, intrauterine infection is considered to be an important risk factor for pregnancy failures including premature rupture of the fetal membranes (PROM), preterm labor, and recurrent miscarriage.<sup>[6]</sup> Numerous studies have indicated that intrauterine pathogens stimulate the cells from both the innate and adoptive immune system, leading to the weakening and the rupture of the fetal membranes through producing pro-inflammatory cytokines at the feto–maternal interface. Several lines of evidence indicate that pro-inflammatory cytokines upregulate the production of prostaglandins (PGs) and matrix

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metalloproteinase (MMP) and thereby enhance uterine contractions and programmed cell death (apoptosis) which cause PROM and preterm birth (PTB).<sup>[6,7]</sup>

Anatomical situation of the vaginal vault and cervix provides a suitable place for colonizing many pathogenic agents such as *Ureaplasma urealyticum*, *Streptococcus agalactiae*, and other Gram-negative and Gram-positive bacteria, which have an identified role in pregnancy failures.<sup>[6,8]</sup> Recognition of microorganisms colonized in the female genital tract is mediated by various receptors of the immune system such as pattern recognition receptors (PRRs) of the innate immune system. Toll-like receptors (TLRs), as a known PRR expressed on different cells of the immune system, detect conserved pathogen-associated molecular patterns (PAMPs).<sup>[9]</sup> Until now, 10 and 12 functional TLRs have been identified in human and mice, respectively, in which recognize different PAMPs and have a distinct specificity for detecting a wide set of bacterial products.<sup>[10]</sup> In this regard, TLR4 senses lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls, and in turn triggers intracellular signaling cascades which lead to activated nuclear factor-kappa B and activator protein-1, which are considered as major transcription factors responsible for the expression of many genes involved in initiation and enhancement of inflammatory responses.<sup>[6,11,12]</sup>

In line with the expression and function of TLRs in the female genital tract, it has been demonstrated that the human placenta contributes to maternal immune responses against various pathogenic agents through expressing different TLRs and producing pro-inflammatory mediators.<sup>[6,13]</sup> Conventionally, the placenta comprised the amnion, chorion, and decidua. The amnion, which is the closest layer to the fetus and derived from the embryo, consists of both cubical and columnar epithelial cells.<sup>[14]</sup> Human amniotic epithelial cells (hAECs) have stem cell-like and immunomodulatory features which play an important role in the maintenance and outcome of pregnancy. Previous reports indicated that hAECs can produce the immunosuppressive compounds such as TGF- $\beta$ , prostaglandin E2 (PGE2), and IL-4.<sup>[15,16]</sup> On the other hand, others have shown that the expression of functional TLRs on hAECs makes these cells as a physical barrier to protect the developing fetus against intra-amniotic infections through upregulating the production of antimicrobial  $\beta$ -defensins and key pro-inflammatory cytokines involved in PROM and PTB.<sup>[6,17,18]</sup> Therefore, thought that hAECs may exert various functions under different conditions.

Although TLRs' expression in the urogenital tract and their role in pregnancy loss are well known, there are a few reports that show the function of TLRs and molecular mechanisms involved in the possible effects of these receptors of the

innate immune on hAECs in pregnancy complications. In this study, we investigated whether activation of TLR4, as a known TLR of hAECs, on LPS engagement in hAECs can be a potential threat to the successful pregnancy through influencing the production of IL-1 beta (IL-1 $\beta$ ), IL-6, IL-5, tumor necrosis factor-alpha (TNF- $\alpha$ ), PGE2, and TGF- $\beta$ 1 mediators of hAECs.

## MATERIALS AND METHODS

### Study design and subjects

This is a cohort study performed on six healthy pregnant women. The nonrandom sampling was used to select the participants. The study was conducted from September 2016 to March 2017 in Isfahan University of Medical Sciences, Isfahan, Iran. All protocols and experiments used in this study were approved by the Ethics Committee of Isfahan University of Medical Sciences (protocol number: 394205) that was in accordance with the Declaration of Helsinki for medical research involving human subjects. The sample size was determined by a statistician using statistical tests according to the similar studies in the literature.<sup>[19]</sup>

### Procedures and variables assessment

#### Isolation of human amniotic epithelial cells

The amnion ( $n = 6$ ) was retrieved from the full-term placentas delivered by normal pregnant women during uncomplicated elective cesarean deliveries before the onset of labor. The hAECs were isolated according to the method previously described by Motedayyen *et al.*<sup>[14]</sup> Briefly, the amniotic membrane was mechanically peeled off from the chorion and cut into small pieces. The amnion layer was then washed several times with phosphate-buffered saline (PBS, pH 7.2) and incubated at 37°C for 10 min with predigestion buffer (calcium and magnesium-free Hank's balanced salt solution (HBSS) containing 0.53 mM ethylenediaminetetraacetic acid [EDTA]-4Na) to remove blood clots, torn pieces, and cellular debris. The digestion of the amnion was initiated at 37°C for 10 min with 0.05% EDTA/trypsin (Gibco, USA). Afterward, the amnion was digested twice at 37°C for 30 min with 0.05% EDTA/trypsin. The cells isolated from the amnion at the second and third digests were pooled and washed with ice-cold HBSS (Gibco, USA). Cell counts were performed using a Burker Neubauer chamber (hemocytometer, 40443001, Hecht Assistent, Germany). The viability of the cells was determined by trypan blue dye exclusion. The purity of the cells was measured after the fixation and permeabilization of the cells using an Intracellular Fixation and Permeabilization Buffer Set (eBiosciences, USA). To determine the cell purity, cytokeratin as an intracellular epithelial marker was stained with Alexa Fluor® 488 anti-cytokeratin or matched isotype control antibodies for 30 min at 4°C [Table 1]. The matched

isotype control antibody was considered as negative control. The cells were then washed several times with cell staining buffer (BioLegend, USA) and centrifuged at  $200 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The data were acquired using a FACSCalibur system (Becton Dickinson, San Jose, CA, USA) and then analyzed using FlowJo software (v 10.1, FlowJo, Ashland, OR, USA). The cells with the purity more than 96% were used as hAECs.

#### Immunophenotyping of human amniotic epithelial cells

To analyze the phenotypic characteristics of freshly isolated hAECs, the cells were stained by different primary or relevant isotype control antibodies. The hAECs ( $8\text{--}10 \times 10^5$ ) were suspended in  $100 \mu\text{l}$  PBS/1% bovine serum albumin and incubated with different antibodies or matched to isotype control immunoglobulin G for 30 min at  $4^{\circ}\text{C}$  [Table 1]. The cells were then washed three times with cell staining buffer, and the percentage of the stained cells was determined by a FACSCalibur flow cytometer and analyzed using FlowJo software.

#### Human amniotic epithelial cells' culture

The isolated hAECs ( $9\text{--}10 \times 10^7$ ) were suspended in 50 ml Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA), and then cultured in  $75\text{-cm}^2$  tissue culture flasks at a density of  $3 \times 10^5$  cells/ $\text{cm}^2$ . After 24-h incubation at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ , adhered hAECs were dissociated by trypsin and washed twice with PBS. The cells were counted by a hemocytometer and cell viability was determined using trypan blue dye exclusion.

#### Human amniotic epithelial cells' stimulation and cytokine assay

To determine the effect of TLR4 stimulation by LPS on production of some mediators of hAECs, the detached cells ( $4 \times 10^5$  cells/ml) were seeded in 24-well plates (Biofil, Canada) and incubated in the presence or absence of LPS ( $5 \mu\text{g/ml}$ , Sigma-Aldrich, USA) at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . The hAECs stimulated with LPS served as test group, while

the cells cultured in the absence of LPS were considered as control group. All cultures were performed in duplicate. To measure the possible effect of LPS-stimulated TLR4 on production of pro- and anti-inflammatory mediators of hAECs, the culture supernatants of hAECs in test and control groups were collected after 24, 48, and 72 h. The levels of IL- $1\beta$ , IL-6, TNF- $\alpha$ , IL-5, TGF- $\beta$ 1, and PGE2 were measured by an enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (Mabtech, Sweden).

#### Statistical analysis

The results are expressed as mean  $\pm$  standard error of the mean. Statistical analyses were done using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). In the present study, comparison was performed between the test group and control group. Unpaired *t*-test was used to compare the two groups with normal distribution, while Mann-Whitney test was performed in the case of nonnormal distribution.  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Human amniotic epithelial cells' characterization

Given that hAECs are not a cell line and do not have a defined immunological profile, we examined the expression of some known markers of these cells isolated from five distinct amnion donors. Flow cytometry results revealed that hAECs were strongly positive for the expression of cytokeratin (an epithelial marker) and stage-specific embryonic antigen-4 (an embryonic stem cell marker) [Figure 1]. hAECs failed to express CD90 and CD105 (mesenchymal stem cell markers) and some of hematopoietic stem cell markers including CD34, CD133, and CD45 [Figure 1]. Furthermore, our data indicated that hAECs were negative for human leukocyte antigen-DR (HLA-DR), in which its expression on the cell surface increases the risk of immune responses against the cells [Figure 1]. Nearly all hAECs expressed high level of TLR4, a well-known receptor for recognizing LPS [Figure 1].

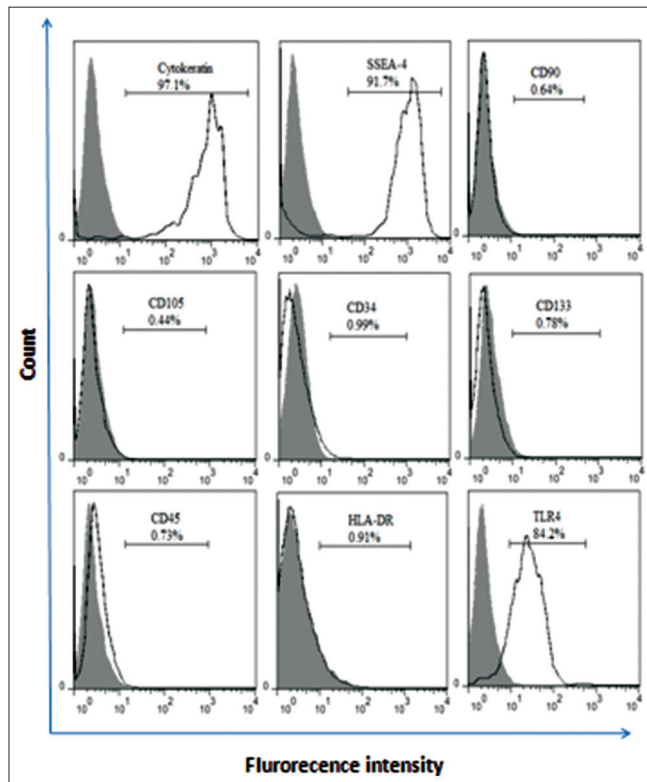
**Table 1: Primary and matched isotype control antibodies used to determine the purity and immunophenotyping of human amniotic epithelial cells by flow cytometry**

Fluorochrome/antibody	Isotype control	Working volume	Source of primary antibodies
Alexa Fluor 488 anti-cytokeratin (pan reactive)	Mouse IgG 1, $\kappa$	5 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
FITC anti-human CD105	Mouse IgG 1, $\kappa$	5 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
FITC anti-human CD90	Mouse IgG 1, $\kappa$	5 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
FITC anti-human CD45	Mouse IgG 1, $\kappa$	5 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
PE anti-human SSEA-4	Mouse IgG3, $\kappa$	20 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
PE anti-human CD133	Mouse IgG 1, $\kappa$	20 $\mu\text{l}$ /test	BioLegend, San Diego, CA, USA
FITC anti-human HLA-DR	Mouse IgG2a, $\kappa$	20 $\mu\text{l}$ /test	BD Biosciences, San Jose, CA, USA
FITC anti-human CD34	Mouse IgG 1, $\kappa$	20 $\mu\text{l}$ /test	BD Biosciences, San Jose, CA, USA
PE anti-human TLR4	Mouse IgG 1, $\kappa$	5 $\mu\text{l}$ /test	BD Biosciences, San Jose, CA, USA

TLR=Toll-like receptor; SSEA=Stage-specific embryonic antigen-4

**The effect of lipopolysaccharide-stimulated toll-like receptor 4 on production of anti-inflammatory mediators of human amniotic epithelial cells**

Regarding the fact that anti-inflammatory mediators have an indispensable role in the maintenance and outcome of pregnancy, the effects of LPS-stimulated TLR4 on TGF-β1, PGE2, and IL-5 production of hAECs were assessed. Our data demonstrated that TLR4 activation by LPS affected the production of TGF-β1 of hAECs. After 48 and 72 h of incubation, TGF-β1 production of LPS-treated hAECs was significantly higher than TGF-β1 produced by untreated hAECs ( $P < 0.001-0.05$ ) [Figure 2a]. The mean production of TGF-β1 of stimulated and nonstimulated hAECs after 24, 48, and 72 h is shown in Table 2. Moreover, we observed that TLR4 activation significantly increased PGE2 production of hAECs after 24 and 48 h of culture ( $P < 0.01-0.05$ ) [Figure 2b and Table 2]. In spite of measurement and assessment of the effects of TLR4 activation on TGF-β1 and PGE2 production of hAECs, we could not determine the effects of TLR4 stimulation by LPS on the IL-5 production of hAECs. Our results indicated that the IL-5 level in culture supernatants of hAECs was below the detection limit, and the IL-5 production did not influence by LPS stimulation. The IL-5 level was only detectable in hAEC samples No. 3 and 5 (data not shown).



**Figure 1:** Phenotypic characteristics of human amniotic epithelial cells. Each histogram is representative of four independent experiments. Black line and gray-shaded histogram represent test- and isotype-matched control antibodies, respectively

**The effect of lipopolysaccharide-stimulated toll-like receptor 4 on pro-inflammatory cytokines production of human amniotic epithelial cells**

Having considered that inflammatory responses are harmful to the successful pregnancy, we examined the effects of TLR4 stimulation on the production of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α of hAECs. As shown in Figure 3a, there was a significant increase in the production of TNF-α of LPS-stimulated hAECs after 24, 48, and 72 h of incubation ( $P < 0.0001-0.01$ ) [Figure 3a and Table 3]. The same trend was also observed in the IL-1β production of LPS-treated hAECs [Figure 3b]. TLR4 activation had a potent effect on IL-1β secretion of hAECs ( $P < 0.01$ ) [Figure 3b]. After 72 h of culture, the mean production of IL-1β of stimulated and nonstimulated hAECs was  $21.19 \pm 2.08$  and  $12.88 \pm 2.69$ , respectively [Table 3]. Our results indicated that TLR4 activation could induce IL-6 production of hAECs, although this induction was not statistically significant [Figure 3c and Table 3].

**DISCUSSION**

Maternal immune responses to the fetus is a major challenge for the implantation and maintenance of

**Table 2: The effect of toll-like receptor 4 activation on the mean production of anti-inflammatory mediators of human amniotic epithelial cells**

	Incubation time (h)	LPS-treated hAECs	Untreated hAECs	P
TGF-β1	24	151.6±48.02	100.7±15.73	0.270
	48	192.9±22.07	132.3±37.08	0.478
	72	138.0±18.62	34.0±5.86	0.0003*
PGE2	24	31.78±3.8	14.18±5.59	0.03
	48	16.69±2.31	7.81±3.11	0.07
	72	13±1.5	7.19±2.94	0.1

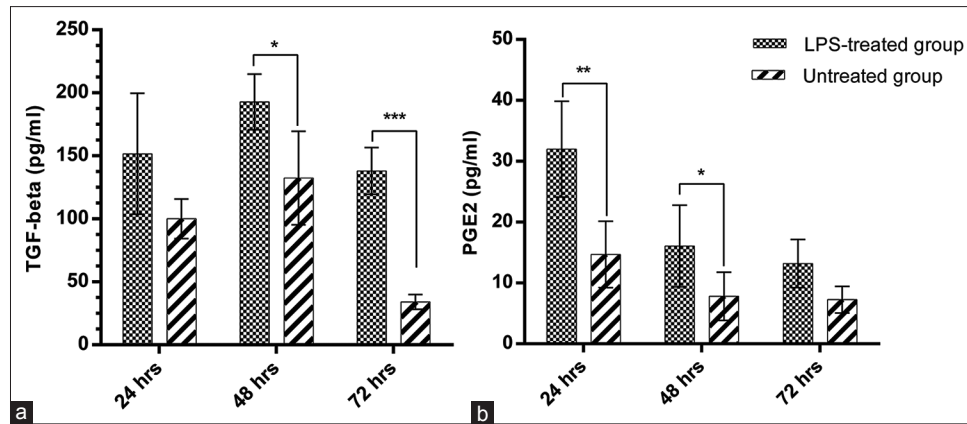
Data are shown as mean±SEM. \*P value is statistically significant. SEM=Standard error of the mean; TGF-β1=Transforming growth factor-beta 1; PGE2=Prostaglandin E2; LPS=Lipopolysaccharide; hAECs=Human amniotic epithelial cells

**Table 3: The effect of toll-like receptor 4 activation on the mean production of pro-inflammatory cytokines of human amniotic epithelial cells**

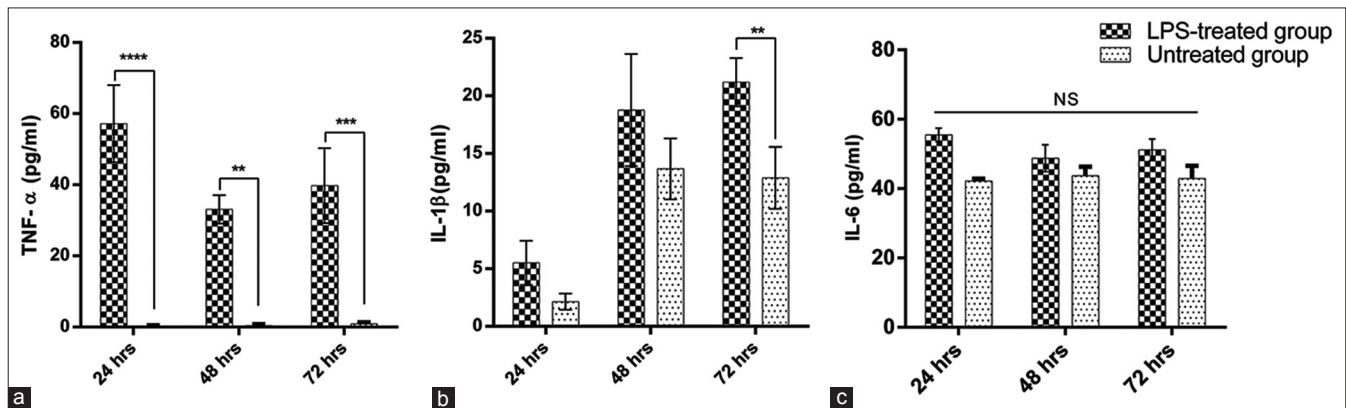
	Incubation time (h)	LPS-treated hAECs	Untreated hAECs	P
TNF-α	24	57.85±18.62	0.28±0.18	0.0001****
	48	33.9±2.07	0.9±0.08	0.007**
	72	39±1.07	0.8±0.07	0.001***
IL-1β	24	5.5±1.91	2.14±0.7	0.13
	48	18.77±4.8	13.67±2.64	0.7
	72	21.19±2.08	12.88±2.69	0.02*
IL-6	24	55.503±1.919	42.14±0.703	0.08
	48	48.77±3.865	43.67±2.64	0.3
	72	51.19±3.086	42.88±3.69	0.2

Data are shown as mean±SEM. \*\*\*\*,\*\*\*,\*\*\*P value is statistically significant. SEM=Standard error of the mean; LPS=Lipopolysaccharide; hAECs=Human amniotic epithelial cells; TNF-α=Tumor necrosis factor-alpha; IL-1β=Interleukin-1β; IL-6=Interleukin-6





**Figure 2:** Toll-like receptor 4 stimulation influences the production of anti-inflammatory mediators of human amniotic epithelial cells. The isolated human amniotic epithelial cells were cultured in 24-well plates and stimulated with lipopolysaccharide. After 24, 48, and 72 h, the levels of transforming growth factor- $\beta$ 1 and prostaglandin E2 produced by human amniotic epithelial cells in culture supernatants were measured by enzyme-linked immunosorbent assay (a and b). The results are representative of six independent experiments. Each bar in A and B shows mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



**Figure 3:** Toll-like receptor 4 activation affects pro-inflammatory cytokines production of human amniotic epithelial cells. The human amniotic epithelial cells were cultured in the presence or absence of lipopolysaccharide. The culture supernatants of the cells were collected after 24, 48 and 72 h of incubation and the effect of lipopolysaccharide-stimulated toll-like receptor 4 on the levels of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6 of human amniotic epithelial cells were assessed using enzyme-linked immunosorbent assay (a-c). The depicted results are representative of six independent experiments. Data show mean  $\pm$  standard error of the mean. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

pregnancy.<sup>[3]</sup> In normal pregnancy, maternal–fetal tolerance plays a fundamental role in suppressing abnormal immune responses and inhibiting immunological rejection of the fetus.<sup>[20]</sup> However, intrauterine infection is a well-known risk factor for disrupting feto–maternal immunologic tolerance, leading to PROM, PTB, and recurrent abortion.<sup>[5,21]</sup> Therefore, we investigated whether TLR4 activation by LPS on hAECs may contribute to abnormalities in maternal immune responses at the feto–maternal interface through affecting the production of anti- and pro-inflammatory mediators of hAECs.

Although numerous reports have revealed TLR expression and its function in the female genital tract, a few studies have been carried out on the TLR repertoire and its role in hAEC responses to intra-amniotic pathogens.<sup>[5,6,22]</sup> Some studies conducted on TLR expression and its response on TLR ligand engagement in hAECs have demonstrated that TLR activation has different results on functions of hAECs,

depending on which TLR is activated.<sup>[6]</sup> Furthermore, immunosuppressive impacts of hAECs are mainly mediated through the secretion of immunomodulatory components.<sup>[23]</sup> Therefore, an important question proposed in this study was how TLR4 activation by bacterial components can be affected immunosuppressive impacts of hAECs. At the first step, the effects of TLR4 activation on TGF- $\beta$ 1 production of hAECs were studied. We observed that TLR4 stimulation had a potent effect on TGF- $\beta$ 1 production of hAECs after 48 and 72 h of culture. In consistent with this result, our previous study also showed that the TGF- $\beta$ 1 expression in hAECs could be significantly induced by TLR4 activation.<sup>[11]</sup> To discover the effects of LPS-stimulated TLR4 on PGE2 production of hAECs, our findings showed that the PGE2 levels in culture supernatants of hAECs cultured in the presence of LPS were significantly higher than those of hAECs cultured alone. Given that TGF- $\beta$ 1 and PGE2 are two anti-inflammatory agents, production of these mediators of hAECs suggests that they may participate in suppressing

immune responses against intra-amniotic infections and enhancing maternal immunological tolerance to the fetus; however, several lines of evidence indicate that TGF- $\beta$ 1 has a pivotal role in cell-mediated immunity through increasing activation, proliferation, and differentiation of T cells.<sup>[24-26]</sup> Moreover, previous reports have provided convincing evidence that PGs enhance MMP activation, programmed cell death (apoptosis), and stimulate uterine contractions.<sup>[6]</sup> Therefore, it is likely that TLR4 stimulation on LPS engagement on hAECs induces T cell-mediated cellular immune responses against intra-amniotic bacteria and participates in the weakening and the rupture of the fetal membranes through TGF- $\beta$ 1 and PGE2 production. However, the critical question that must be addressed in future studies is how the molecular mechanisms involved in the effects of TLR4 stimulation on the TGF- $\beta$ 1 and PGE2 secretion of hAECs.

Regarding the fact that intrauterine pathogens cause deleterious responses at the fetomaternal interface and thereby lead to the breakdown of maternal immunological tolerance to the fetus, the possible role of hAECs in enhancing harmful responses to the successful pregnancy was evaluated. We focused on determining how TLR4 activation by LPS disrupts pregnancy-friendly immunity at the fetomaternal interface. In the current study, we observed that in the presence of LPS, the TNF- $\alpha$  production of hAECs was significantly increased, which is consistent with the results of a study conducted by Li *et al.* that showed hAECs express TNF- $\alpha$ .<sup>[27]</sup> Regarding the fact that TNF- $\alpha$  acts as a trigger in the induction of apoptosis, production of this pro-inflammatory agent of hAECs may be a potential risk factor for the reduced survival of hAECs and the rupture of the fetal membranes. In the next step, to determine the role of TLR4 activation on stimulating production of other cytokines related to pregnancy complications, the effects of LPS on IL-1 $\beta$  and IL-6 secretion of hAECs were assessed. The results of this study demonstrated that hAECs significantly produced IL-1 $\beta$  when the cells were cultured in the presence of LPS, while IL-6 production did not influence by LPS. In contrast with our results, Gilliaux *et al.* indicated that TLR5 and TLR6/2 stimulation on hAECs induced pro-inflammatory cytokines such as IL-6 and IL-8; however, hAECs stimulation by LPS could not influence the production of these cytokines of hAECs.<sup>[6]</sup> The authors reported that TLR4 stimulation by LPS only led to the decreased viability of cells and increased rate of cell apoptosis.<sup>[6]</sup> Moreover, it has been shown that intra-amniotic bacteria such as *Ureaplasma parvum* and *U. urealyticum* serovars increased IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  production of hAECs through the stimulation of TLR2, TLR6, and TLR9.<sup>[18]</sup> Therefore, the results of this study provided evidence that shows TLR4 stimulation along with other TLRs may contribute to production of pro-inflammatory

cytokines of hAECs. In line with this notion, it is thought that the activation of various TLRs on hAECs along with other infiltrating immune cells and endogenous cells in the female genital tract plays a fundamental role in the pathogenesis of spontaneous abortion, PROM, and PTB.

Taken together, our data provide evidence to indicate that TLR4 stimulation by LPS on hAECs participates in maternal immunological abnormalities at the fetomaternal interface through inducing TNF- $\alpha$ , IL-1 $\beta$ , PGE2, and TGF- $\beta$ 1 production of hAECs. These findings suggest that TLR4 activation may be a potential risk factor for pregnancy failures. However, more robust studies and more information are required to clarify the causative role of TLRs of hAECs in pregnancy loss.

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### Conflicts of interest

There are no conflicts of interest.

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