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Toll-like Receptor 4 Expression in Decidual cells and Interstitial Trophoblasts across Human Pregnancy

Frederic Schatz, Ph.D.^{1,2}, Umit A. Kayisli, Ph.D.¹, Emre Vatandaslar, B.S.¹, Nehir Ocak, M.S.¹, Seth Guller, Ph.D.¹, Vikki M. Abrahams, Ph.D.¹, Graciela Krikun, Ph.D.¹, and Charles J. Lockwood, M.D., M.H.C.M.²

¹Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT, 06520, USA

²Department of Obstetrics and Gynecology, The Ohio State University College of Medicine, Columbus, OH, 43210, USA

Abstract

Problem—Toll receptor-4 (TLR-4) protects against gram-negative bacteria expressed lipopolysaccharide and “danger signals” from injured or dying cells. Although decidual cells (DCs) and interstitial trophoblasts (ITs) are in close contact, TLR-4 has been studied extensively only in ITs.

Method of Study—Formalin-fixed, paraffin-embedded serial sections of endometrium in follicular and luteal phases and deciduas from first and second trimester elective terminations and third trimester normal deliveries were immunostained for TLR-4, trophoblast-specific cytokeratin and DC-specific vimentin. HSCORE assessed TLR-4 immunostaining in DCs vs. ITs.

Results—TLR-4 HSCORES were significantly higher in: 1) first trimester DCs than luteal phase pre-decidual stromal cells; 2) first and third vs. second trimester DCs, but similar between third trimester deciduas parietalis and basalis; 3) first vs. second trimester ITs; 4) DCs vs. ITs across gestation.

Conclusion—Higher TLR-4 in DCs than ITs suggests DCs as primary targets for gram negative bacteria and/or inflammation-related danger signals.

Keywords

basalis; decidua; endometrium; HSCORE; immunohistochemistry; parietalis

Introduction

Human extravillous trophoblasts (EVTs) invade an underlying decidua basalis comprised primarily of resident decidual cells (DCs) (50%) and a diverse immune cell population (40%) dominated by innate immunity-mediating decidual natural killer cells (dNK cells) (70–80%), macrophages (20–25%) and dendritic cells (2%).^{1, 2} As they cross the decidua and enter the upper one third of the myometrium, EVT's breach and convert spiral arteries into low resistance high capacity vessels that increase utero-placental blood flow to the developing maternal-fetal unit.^{3, 4} The basalis contacts invading interstitial trophoblasts (ITs) and is closely associated with fetal extra-embryonic tissue at the start of implantation. The parietalis lines the remainder of the uterine cavity, first contacting the fetal extra-

*Corresponding author: Frederick Schatz, Ph.D., Department of Obstetrics and Gynecology, Ohio State University, Wexner Medical Center, 460 W. 12th Ave., Columbus, OH, 43210, USA. Frederick.Schatz@osumc.edu.

embryonic tissue towards the end of the first trimester when fetal growth obliterates the uterine cavity.⁵

Toll-like receptors (TLRs) belong to a major family of pattern recognition receptors that mediate innate immunity by recognizing conserved pattern recognition sequences on infectious microorganisms to generate an inflammatory response against the invading pathogen.⁶ Immune cells are generally the primary expressers of TLRs. However, at the maternal-fetal interface across human pregnancy, various TLRs are also expressed by trophoblasts and DCs with the overwhelming majority of studies directed at the former.⁷ One of these, TLR-4, is the primary mediator of host responses against gram negative bacterial LPS⁸ and “danger signals” emanating from injured or dying cells in response to inflammation despite the absence of infection⁹ as exemplified by patients with preeclampsia.¹⁰ TLR-4 has been studied extensively in trophoblasts across pregnancy. By comparison, relatively few studies have assessed TLR-4 expression by DCs.⁷

Extensive investigations have produced a detailed characterization of the immune cell population in first trimester human decidua,^{1, 2, 11} whereas there are far fewer assessments of decidual immune cells in later pregnancy. The majority of the latter were directed at term basalis, with the corresponding parietalis studied only relatively recently.^{12, 13} The resulting reports yielded contradictory information as to the identity and nature of leukocytes in the decidua at term and whether these differ between the basalis and parietalis. This uncertainty concerning the immune cell population taken together with the threat posed by gram negative bacteria and “danger signals” encouraged the current study to compare TLR-4 expression in DCs with neighboring ITs at the maternal-fetal interface across pregnancy. Serial sections of first and second trimester basalis and gestationally age-matched third trimester basalis and parietalis were subjected to immunohistochemical staining for TLR-4, as well as vimentin, a DC marker, and cytokeratin, a trophoblast marker. Semi quantitative HSCORE assessment of TLR-4 immunohistochemical staining revealed the dominance of TLR-4 expression in DCs versus ITs across gestation as well as in DCs versus pre-decidual stromal cells in first trimester and luteal phase endometrium.

Material and Methods

Patients and tissue

Paraffin sections of follicular phase (n=6) and luteal phase (n=6) endometrial tissues were obtained from Department of Pathology, Yale School of Medicine. Biopsies of decidua were obtained from first (n=5) and second trimester (n=4) elective terminations and normal deliveries in the third trimester (n=10) under approval of the Yale University School of Medicine Human Investigation Committee (HIC). Third trimester decidua were derived from placentas following idiopathic preterm labor without clinical or histological evidence of chorioamnionitis or chronic villitis, and following either cesarean or vaginal delivery at term.

Immunohistochemistry

Immunostaining for TLR-4, vimentin and cytokeratin 7 was performed as described previously¹⁴. Briefly, five- μ m formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated through a descending ethanol series. Antigen retrieval was performed by boiling the slides in citrate buffer (10 mM; pH 6.0) for 15 min, followed by endogenous peroxidase quenching with 3% hydrogen peroxide (in 50% methanol/50% distilled water) for 15 min. The slides were incubated with 5% goat or donkey blocking serums (Vector Labs, Burlingame, CA) in Tris-buffered saline (TBS) for 30 min at room temperature in a humidified chamber. After removal of excess serum, serial

sections were incubated with either rabbit polyclonal TLR-4 antibody (Abcam Inc, Cambridge, MA), or monoclonal chicken anti-vimentin antibody (Abcam) or rabbit monoclonal anti-cytokeratin 7 antibody (Abcam) overnight in a humidified chamber at +4°C. Normal rabbit IgG antibody were used at the same concentrations as the primary antibodies for negative controls. After washing, the slides were incubated with biotinylated goat anti-rabbit or donkey anti-chicken secondary antibodies (Vector Labs) for 30 min at room temperature. After washing again in TBS, the antigen-antibody complex was detected with a streptavidin-biotin peroxidase or alkaline phosphatase kit (Vector Labs). DAB (3, 3'-diaminobenzidine tetrahydrochloride dehydrate) and Fast Red (Vector Labs) were used as chromogens to detect TLR-4, vimentin and cytokeratin immunoreactivity. The slides were then counterstained with hematoxylin and mounted.

Immunostaining for TLR-4 was evaluated semi-quantitatively using HSCORE analysis as described previously.¹⁴ Immunostaining intensity was categorized by the following scores: 0 (no staining), 1 (weak, but detectable, staining), 2 (moderate staining), and 3+ (intense staining). An HSCORE value was derived for each specimen by calculating the sum of the percentage of cells that stained at each intensity category multiplied by its respective score, using the formula $HSCORE = \sum i \cdot P_i$, where i represents the intensity category score, and P_i is the corresponding percentage of cells. For each slide, five different fields were evaluated microscopically at 200X magnification. HSCORE evaluation was performed independently by two investigators blinded to the source of the samples as well as to each other's results; the average score of both was then used.

Statistical Analysis

Immunohistochemistry scores (HSCORE) were found to be normally distributed according to the Kolmogorow-Smirnov test. Thus, analysis of variance (ANOVA) and post hoc Tukey test for pair-wise multiple comparisons were used for statistical analysis. $P < 0.05$ was considered to be significant. Statistical calculations were performed using Sigmaxstat for Windows, version 2.0 (Jandel Scientific Corporation, San Rafael, CA).

Results

Figure 1 displays immunohistochemical staining for TLR-4 in menstrual cycle-derived endometrial sections obtained during: (A) the follicular phase, readily identified by the presence of circular glands (GI) and fibroblast like endometrial stromal cells (ESC); and (B) the mid-luteal phase containing enlarged glands and pre-decidual stromal cells characterized by round, slightly enlarged cytoplasm (arrows). The first trimester decidual section displayed in (C) contains areas of both pre-decidual stromal cells (arrows) and cuboidal, epithelioid DCs (arrowheads). Double label immunostaining in the serial section shown in (D) verifies that both pre decidual stromal cells (arrows) and DCs (arrowheads) are vimentin positive. The HSCOREs shown in (E) reveal significantly higher immunoreactive TLR-4 in first trimester DCs ($p < 0.03$) compared with ESC in the follicular phase or pre-decidual stromal cells in the luteal phase endometrium.

Figure 2 displays immunohistochemical staining for TLR-4 in DCs and ITs in first trimester decidua. The presence of immunoreactive TLR-4 in both DCs (arrowheads) and ITs (arrows) is indicated in 2A. The identity of both cell types is confirmed in the serial section displayed in 2B in which the arrowheads denote vimentin-positive DCs (brown stain) and the red stain indicated by the arrowheads point to cytokeratin-positive ITs. The additional section in 2C indicates localization of immunoreactive TLR-4 in both the cell membrane (arrowheads) and cytoplasm (arrows) of DCs. A similar subcellular distribution of TLR-4 immunostaining is also evident in ITs (not shown). Although glandular epithelial cells (GI)

and spiral artery endothelial cells (*) in 2A and 2E, respectively, display TLR-4 immunoreactivity, evaluating this expression is beyond the scope of the current study.

Figures 3A, B and 3C, D display serial sections for second trimester and third trimester decidua basalis, respectively. The arrowheads in B and D specify vimentin-positive DCs (red stain) and the arrows point to cytokeratin-positive ITs (brown stain). Inspection of the corresponding serial sections denoting TLR-4 brown immunostaining in ITs (arrows) and DCs (arrowheads) indicates that third trimester DCs display more prominent TLR-4 staining than second trimester DCs. However, similar TLR-4 immunostaining is seen in second and third trimester ITs (arrows).

Figure 3E compares HSCORES for TLR-4 immunostaining in DCs versus ITs in first, second and third trimester sections of decidua basalis. At each stage of pregnancy, TLR-4 HSCOREs are higher in DCs than corresponding ITs, with statistical significance attained in the third trimester ($p < 0.001$). TLR-4 immunostaining is also significantly higher in first trimester than second trimester ITs ($p < 0.01$). Moreover, TLR-4 HSCORES are significantly higher in first and third versus than second trimester DCs ($p < 0.01$).

Inspection of TLR-4 immunostaining in the representative sections depicted for paired decidua basalis (A) and decidua parietalis (B) in Fig. 4 indicates that immunoreactivity is more prominent in DCs (arrowheads) than in ITs (arrows). The HSCOREs displayed in the bar graphs in Fig. 4C indicates statistical equivalence between DCs in the basalis and parietalis. However, while DCs displayed significantly greater TLR-4 immunostaining than corresponding ITs in 3rd trimester decidua basalis (Fig. 3A), irregular spatial distribution of ITs among sections prevents compiling accurate HSCORES in the parietalis.

Discussion

The maternal-fetal interface is a site of immune privilege that promotes tolerance of the semi-allogeneic fetal transplant while protecting it against the presence of pathogens.¹⁵ The former prevents fetal rejection, whereas the importance of the latter is evident in the strong positive correlation that exists between intrauterine bacterial and viral infections and such disorders of pregnancy as spontaneous abortion, preterm labor and delivery, intrauterine growth restriction and preeclampsia.^{16, 17} By distinguishing “self,” i.e. embryo, placenta, mother, from “nonself, i.e. bacteria, viruses, fungus”, the innate immune system provides the first line of defense against these pathogens.¹⁵ Activation of specific TLRs expressed by immune cells by specific pathogen-associated molecular pattern molecules (PAMPs) generates a protective inflammatory response against these bacteria and viruses.⁶ However, a number of studies also demonstrate that non-immune cells of the maternal-fetal interface also have this capacity.¹⁸

Since little is known about the expression and function of TLRs in non-immune cells in decidua basalis versus parietalis across pregnancy, this prompted the current study to focus on TLR expression in DCs with ITs. Both cell types are closely associated across gestation with each readily identified by the expression of specific immunohistochemical markers. Several studies have assessed the expression of TLRs, particularly that of TLR-4, in trophoblasts. In the first trimester, prominent TLR-4 expression was observed in villous and extravillous trophoblast, but found to be absent from syncytiotrophoblasts.¹⁹ In contrast, strong TLR-4 immunostaining was observed in syncytiotrophoblasts of term placenta.²⁰ While LPS expressed in the cell wall of gram negative bacteria is the classic TLR-4 ligand, TLR-4 also responds to heat shock proteins and other host-derived molecules released during tissue damage from dead or dying cells with or without infection (reviewed in ⁷). Chlamydia-derived Heat shock protein 60, but not LPS, induces apoptosis of first trimester

human trophoblasts.²¹ Differential responses to these ligands may reflect different downstream signaling and adapter molecule mediation.⁷

Ligation of TLR-4 expressed by HTR-8/SVneo, a first trimester EVT-derived cell line, directly inhibits their migration and enhances expression of monocyte recruiting and activating chemokines.^{7, 22} The preeclamptic decidua contains excess macrophages.²³ Consistent with inhibition of trophoblast invasion, tumor necrosis factor alpha derived from activated macrophages: 1) enhances plasminogen activator inhibitor-1 (PAI-1) expression in cytotrophoblasts, which inhibits urokinase-type PA at the leading edge of trophoblast invasion;²⁴ 2) induces apoptosis of trophoblasts.²⁵ Impaired human EVT migration is accompanied by incomplete transformation of the spiral vessels to low resistance high capacitance conduits that reduce uteroplacental blood flow. The resulting placental hypoxia is a significant risk factor for the later development of preeclampsia.³

In contrast with several studies of TLR-4 expression in trophoblasts during human pregnancy, evaluation of TLR-4 mRNA and protein in human DCs appears to be limited to the first trimester.^{26, 27} Seeking to fill this information gap, the current study initially assessed TLR-4 expression during decidualization (DZ) of human endometrial stromal cells. In humans, DZ involves marked changes in morphological and biochemical differentiation that are initiated during the luteal phase of the menstrual cycle as rising circulating progesterone levels act on estradiol-primed stromal cells. During DZ, the cytoplasmic component of the fibroblast-like precursor stromal cells enlarges. Under continued progesterone stimulation the DZ reaction spreads throughout the early pregnant endometrium, resulting in distinct areas containing round decidualized stromal cells or more mature cuboidal, epithelioid DCs.²⁸⁻³⁰

In human endometrial sections, the current study found no change in immunohistochemical levels of TLR-4 during the transition of endometrial stromal cells to predecidual stromal cells during the luteal phase or early pregnancy. By contrast, significantly enhanced TLR-4 expression was evident in the mature DCs in decidual sections during early pregnancy. Further use of immunostaining in the current study found that in sections of decidua basalis in the first, second and third trimester, as well as in third trimester decidua parietalis, TLR-4 immunoreactivity was greater in DCs than in neighboring ITs. Specifically, significantly higher TLR-4 HSCORE values were observed in DCs of first and third trimester than in second trimester basalis, whereas TLR-4 immunoreactive levels are similar in DCs of third trimester decidua basalis and parietalis. Moreover, TLR-4 HSCORES are significantly higher in first trimester DCs and ITs than in second trimester ITs. The relatively higher expression level of TLR-4 in first and third versus second trimester could reflect the enhanced inflammatory environment present at the maternal-fetal interface in the beginning and end of pregnancy compared to mid pregnancy.

The dominance of TLR-4 expression in DCs in decidual sections across pregnancy revealed in the current study, suggests that maternally derived cells may be major protectors against gram negative bacteria and “danger signals” emanating from severe inflammation with or without infection. The localization of TLR-4 in cell membranes is consistent with its known protective actions. The cytoplasmic localization of TLR-4 also demonstrated in this study may reflect active synthesis as well as possible TLR signaling which may require endocytosis.³¹ Human trophoblasts undergo syncytialization in culture, thereby mandating use of freshly isolated cells or bioengineered cell lines for *in vitro* studies. In contrast, human DCs isolated across pregnancy can be passaged and expanded several times and stored frozen without apparent loss in responsiveness to ovarian steroids or pro-inflammatory cytokines.

Initial studies in our laboratory revealed that progesterone enhances mRNA and protein levels of tissue factor (TF), the major mediator of hemostasis via thrombin generation,³² in primary cultures of stromal cells derived from pre-decidualized cycling human endometrium.³³ Furthermore, this progesterone response is retained by primary DCs derived from third trimester deciduas.³⁴ Complementing these *in vitro* observations, immunoreactive TF levels are significantly elevated in decidualized stromal cells in luteal phase endometrial sections compared with endometrial stromal cells in the follicular phase.³³ Moreover, among the cells in third trimester decidual sections, immunoreactive TF levels are highest in DCs. Thus, the DZ-associated elevation in TF that begins in the luteal phase and continues throughout pregnancy differs from the current observation that immunoreactive TLR-4 levels are first increased in DCs of early pregnant endometrium. This enhanced TLR-4 expression may have evolved as a pregnancy-related protective mechanism against specific microbes and other inflammation-related “danger signals.” Experiments with primary DCs alone and/or in co-culture with ITs may elucidate mechanisms underlying the DZ-related increase in TLR-4 expression.

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Figure 1.

TLR-4 immunoreactivity in cyclic endometrium and in first trimester decidua. (A) Weak staining for TLR-4 (brown) is exhibited by fibroblast-like endometrial stromal cells (ESC) in follicular phase endometrium, (B) in pre-decidual stromal cells (PDC; arrows) in luteal phase, and (C) in first trimester decidua. Also in first trimester decidua (C), prominent brown immunostaining is evident in decidual cells (DC; arrowheads) and glandular epithelium. (D) Double label immunohistochemistry for vimentin (brown) identifies DCs (arrowheads) and PDC [arrows], whereas cytokeratin 7 (red) identifies glandular epithelial cells (GI). Insert shows negative control with primary TLR-4 antibody replaced by normal rabbit IgG. (E) indicates HSCOREs and specimen numbers (n) evaluated. Bars represent Mean $\pm$ SEM. Follicular phase ESCs (FP ESC); Luteal phase PDCs (LP-PDC); First trimester DCs (FT DC).

Figure 2.

TLR-4 immunoreactivity in decidual cells (DCs) and interstitial trophoblasts (ITs) in first trimester decidua. **(A)** TLR-4 expression (brown) in DCs (arrowheads) and ITs (arrows). **(B)** Double labeling immunohistochemistry for vimentin (brown) to identify DCs (arrowheads), and cytokeratin 7 (red) to identify ITs (arrows). In addition to DCs and ITs, TLR-4 expression is also evident in cytokeratin 7 positive glandular epithelial cells (GI) and spiral artery endothelial cells (*). **(C)** TLR-4 expression in cell membrane (arrowheads) and cytoplasm (arrows) of DCs. **(D)** Serial section of **C**, shows double label immunohistochemistry for vimentin (brown), which identifies DCs, and cytokeratin 7 (red) which identifies glandular epithelial cells (GI).

Figure 3.

TLR-4 immunoreactivity in decidual cells (DCs) and interstitial trophoblasts (ITs) in second and third trimester decidua. In second trimester decidua basalis: (A) TLR-4 expression (brown) in DCs (arrowheads), ITs (arrows). Relatively weak TLR-4 immunoreactivity is localized in both DCs and ITs. (B) Double label immunohistochemistry for vimentin (red) to identify DCs (arrowheads), and cytokeratin 7 (brown) to identify ITs (arrows). In third trimester decidua basalis: (C) TLR-4 expression (brown) in DCs (arrowheads) and ITs (arrows). Note stronger TLR-4 immunoreactivity localized to DCs (arrowheads) versus ITs (arrows). (D) Double label immunohistochemistry for vimentin (red) to identify DCs (arrowheads), and cytokeratin 7 (brown) for ITs (arrows). (E) HSCORES compare TLR-4 immunostaining in DCs and ITs in first, second and third trimester decidua. Bars represent Mean $\pm$ SEM.

Figure 4.

TLR-4 immunoreactivity in decidual cells (DCs) and interstitial trophoblasts (ITs) in third trimester decidua basalis and parietalis. TLR-4 immunoreactivity in DCs (arrowheads) and ITs (arrows) in paired (A) decidua basalis, and (B) decidua parietalis. (C) TLR-4 HSCORES in DCs in decidua basalis versus decidua parietalis indicate similar immunostaining at the two sites. Bars represent Mean $\pm$ SEM.