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Expression of toll-like receptors in the human decidua

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Summary. Background: Successful trophoblast invasion and transformation of the maternal spiral arteries requires that the pregnant endometrium (i.e., decidua) act in an immunologically paradoxical fashion, accepting the semi-allogenic placenta, while maintaining host defenses against an array of microbial pathogens. In contrast to the growing evidence that the immune surveillance molecules known as Toll-like receptors (TLRs) are expressed by trophoblasts and fetal membranes, to date, no studies have been conducted on the decidua. Methods: Decidual tissues and cells were obtained from women undergoing first trimester elective terminations or repeat Cesarean sections and analyzed at both the protein and mRNA level. Results: We now demonstrate for the first time that human decidua differentially express TLRs and their downstream signaling molecules as well as TLR stimulated induction of cytokine production in the first and third trimester of pregnancy. Conclusions: These findings suggest that the decidua is a critical component of the innate immune response in pregnancy. Moreover, the results have implications for the success or failure of compromised pregnancies in early or late gestation.

Key words: Decidua, endometrium, TLRs, cytokines

Introduction

The placenta has a unique structural organization that allows fetal cells expressing paternal alloantigens to establish a peaceful cohabitation with the maternal immune system (Bulla et al., 2005). The allogenic placenta is exposed to the maternal immune system present in the intervillous space from decidual vessels (Bulla et al., 2005). Specific populations of decidual leukocytes account for at least 15% of all cells in the decidualized uterine wall (Red-Horse et al., 2001). However, as in the case of uterine natural killer cells, their immunogenic responses (ie, cytolytic activity) may be suppressed in comparison to that of peripheral leukocytes in order to prevent fetal rejection (Red-Horse et al., 2001). Therefore, we hypothesized that the decidualized stromal cells as well as endometrial endothelial cells, are part of an innate immune response which supports trophoblast invasion and transformation but at the same time prevents large scale invasion of pathogens. This host protection may occur, in part, through the expression of Toll-like receptors (TLRs). TLRs are a family of transmembrane proteins, necessary to control invading pathogens and strengthen the innate immune system (Janeway and Medzhitov, 1999). Toll-like receptors play a primary role in the innate immune system's pathogen recognition (Dushay and Eldon, 1998; Kopp and Medzhitov, 1999; Means et al., 2000). These receptors bind to several microbial components and in turn trigger an array of signaling pathways that ultimately activate molecules such as nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3) (Means et al., 2000; Oshiumi et al., 2003; Mansell et al., 2001; Abrahams et al., 2004).

The specificity of downstream signaling is achieved through the coupling of TLRs with intracellular adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88)-dependent NF-κB, and TIR domain-containing adapter inducing IFN-b (TRIF)-dependent activation of the transcription factor (IRF-3) (Soboll et al., 2006). Moreover, recent information, points to signaling mechanisms unique to each TLR subtype (Takeuchi, 2001; O'Neill, 2002).

Ten members of the TLR family have been described in humans (Abrahams et al., 2004; Kirschning and Bauer, 2001) and are widely expressed throughout the immune system. Toll-like receptors can also be expressed by non-immune cells, particularly if these cells participate in an inflammatory response (Abrahams and Mor, 2005). In addition to classic bacterial, fungal and viral by-products, TLRs can also recognize extracellular matrix components including fibronectin, oligosaccharides of hyaluronic acid, and polysacharide

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fragments of heparin sulfate that are degraded by proteases produced during the course of inflammation. Fibrinogen stimulates chemokine production in a TLR4-dependent manner (Smiley et al., 2001; Vabulas et al., 2002).

Based on this data as well as the present findings, we suggest that decidual TLRs may be involved in responding to inflammatory processes in the presence or absence of infection. We hypothesize that abnormalities in decidual TLRs expression or function may be linked to abnormal placentation, inflammation, and adverse pregnancy outcomes.

Materials and methods

Reagents

LPS was purchased from Sigma-Aldrich (St. Louis, MO). Poly (I:C) and peptidoglycan (PG) were from InvivoGen (Montreal, Quebec).

Tissues

After obtaining informed consent, specimens for cell culture were obtained from repeat cesarean sections (n=8) derived from patients without complications or with elective first term terminations (n=7) as previously described (Cakmak et al., 2005).

Isolation of first trimester and term decidual cells

First trimester decidual cell cultures were prepared as described previously (Cakmak et al., 2005) and seeded on polystyrene tissue culture dishes. Decidua obtained from patients undergoing cesarean deliveries at term was scraped from the maternal surface of the chorion. Third trimester decidual cell cultures were then prepared as described previously (Cakmak et al., 2005) and cultured on polystyrene cell culture dishes. Cells were harvested using trypsin/EDTA and purity analyzed by flow cytometric analysis using an anti-CD45 mAb (BD Pharmingen, San Jose, CA) to monitor the presence of leukocytes after each passage. After three or four passages, cell cultures were leukocyte free (<1%). In order to simulate the environment of pregnancy, decidual cells were maintained in estradiol (E2) at 10^{-8} M plus medroxyprogesterone acetate (MPA) at 10^{-7} M for 7 days before additional treatment of cells was conducted.

Endometrial endothelial cell line

Endometrial endothelial cells were isolated and immortalized as previously described (Krikun et al., 2005) with telomerase (hTERT), using a retroviral system consisting of the pA317 hTERT-expressing cell line.

Immunohistochemistry (IHC)

Peroxidase staining was performed on 5 µm sections of paraffin-embedded tissues as described previously (Krikun et al., 2004). Staining for TLR-2 and -4 was performed using antibodies obtained from R&D (Minneapolis, MN) and Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA), respectively. Antibodies for TLR-2 were diluted 1-1000 and for TLR-4 1-500. Antibodies for cytokeratin-7 and CD-68 were from Dako (Carpinteria, CA) (both antibodies were diluted 1-200). Negative control slides were prepared by incubating with pre-immune serum for 2 hr. at room temperature. Treatment with the appropriate peroxidase conjugate and color development with DAB was carried out using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Samples were counterstained with hematoxylin and visualization and photography conducted with an inverted contrast microscope (Olympus, Mellville, NY).

Immunocytochemistry

Cultured, non confluent endometrial cells were stained for NFκB as described above with an antibody from R&D. Antibody was diluted at 1-200.

Western blotting

Analysis for TLR-2 (diluted 1-1000), TLR-4 (diluted 1-1000), MyD88 (diluted 1-500), TRIF (diluted 1-500) and ß-actin (diluted 1-10,000) were conducted as previously described (Krikun et al., 2005). The antibodies for TLR-2 and TLR-4 were the same as those used for IHC (see above). Antibodies for MyD88, TRIF and ß-actin were from R&D, Abcam (Cambridge, UK) and Sigma-Aldrich respectively. ECL was used for detection (Amersham, Piscataway, NJ) with secondary antibodies from Biosource International. Densitometric analyses were carried out with the Image J NIH analysis program.

Real time-quantitative RT-PCR

After assessing correct product formation by semi-quantitative RT-PCR, quantitative real time RT-PCR was conducted with the primers described below which were either designed by our laboratory or previously published (Bulut et al., 2001; Mempel et al., 2003; Nishimura and Naito, 2005). The identity of the amplified products was confirmed by DNA sequencing (Table 1).

Statistics

The Sigma Stat 3.0 program (Jandell Scientific) was utilized to conduct the appropriate statistical analyses.

Results

Expression of TLRs in first and third trimester decidual cells

Decidual cells were isolated and cultured as described in Methods and incubated for 7 days in basal media containing estradiol (10^{-8}M) plus

medroxyprogesterone acetate (10^{-7}M) to mimic the hormonal milieu of pregnancy. Cultures were then harvested and assessed by quantitative real time RT-PCR for the expression of TLRs 1-10. TLR expression was first normalized to that of ß-actin to correct for total cDNA content. Due to differences in primer pair efficiencies, TLR expression in term decidual cells was expressed as a percent of the levels in first trimester decidual cells, which was randomly assigned a value of 1. Figure 1 summarizes the results in first and third trimester decidual cells. While no statistical significant changes were observed in TLR-1-10 mRNA expression levels among first and term trimester decidual cells, it is noteworthy that with the exception of TLR-2, -7 and -9, all other TLRs were expressed at lower levels in term than in first trimester decidual cells.

Consistent with the above findings, Fig. 2 displays immunohistochemistry studies performed on first trimester decidua. The results demonstrate strong staining for both TLR-2 and TLR-4, the major receptors for components of gram-positive and gram-negative bacteria respectively. While TLR-4 appears to be present throughout all decidual cell types, TLR-2 appears to be absent from the endothelial cells. These results were confirmed by RT-PCR studies in isolated endometrial endothelial cells (data not shown). To establish cell specificity of TLRs staining, non sequential sections of first trimester decidua were also stained for cytokeratin-7 or CD68. As can be seen in Fig. 3, cytokeratin-7 staining is only limited to the glandular epithelium whereas CD68 staining revealed only a minimal number of macrophages. No staining was observed in the absence of primary antibody or the presence of pre-immune serum in any sample (not shown).

The specificity of the antibodies used for TLR-2 immunohistochemistry was confirmed by Western blot. As shown is Fig. 4, first trimester decidual cells expressed a single band at 84 kDa corresponding to the TLR-2 protein and a band at 91 kDa corresponding to the TLR-4 protein. In addition a weaker second band is consistently observed with the use of this polyclonal antibody, but the presence of TLR-4 has been clearly confirmed by our quantitative RT-PCR results. As expected, the housekeeping protein ß-actin was detected at similar levels and at the anticipated molecular weight at 48 kDa.

Decidual cells express TLR signaling intermediates

As mentioned above, TLR signaling pathways are

Table 1. DNA Sequencing.

TLR	SENSE	ANTI-SENSE	SIZE (BP)
TLR1	TTTGAAAATTGTGGGCACCTTACTG	AAGCAACATTGAGTTCTTGCAAAGC	350
TLR2	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCT	400
TLR3	AGCCGCCAACTTCACAAG	AGCTCTTGGAGATTTTCCAGC	400
TLR4	TGGATACGTTTCCTTATAAG	GAAATGGAGGCACCCCTTC	449
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	355
TLR6	AGAACTCACCAGAGGTCCAACC	GAAGGCATATCCTTCGTCATGAG	500
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	551
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	581
TLR9	TTATGGACTTCCTGCTGGAGGTGC	CTGCGTTTTGTCGAAGACCA	310
TLR10	CAATCTAGAGAAGGAAGATGGTTC	GCCCTTATAAACTTGTGAAGGTGT	556
IL-6	ATGCAATAACCACCCCT	AGTGTCCTAACGCTCATAC	277
IL-8	GACAAGAGCCAGGAAGAAAC	CTACAACAGACCCACACAATAC	459

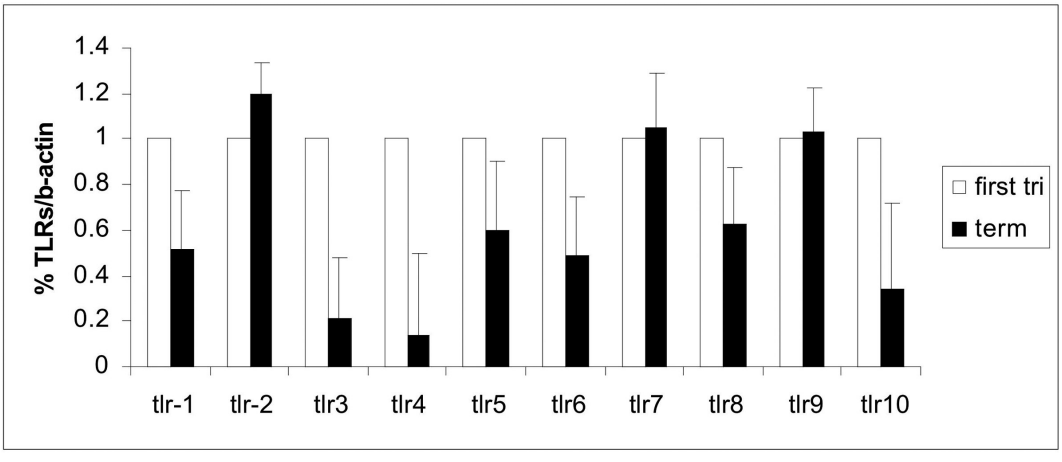

Fig. 1. Quantitative real time RT-PCR of TLR expression by decidual cells. The figure depicts the percent TLR expression by cultured term decidual cells compared to first trimester decidual cell expression which have been arbitrarily assigned a value of 1. All values were first normalized to ß-beta actin expression (n=4 for 1st trimester and n=4 for 3rd trimester) and analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks suggested for studies of small sample numbers.

finely regulated by TIR domain-containing adaptors, such as MyD88, TIRAP/Mal, TRIF and TRAM. Differential utilization of these TIR domain-containing adaptors provides specificity of individual TLR-mediated signaling pathways (Takeda and Akira, 2005). In order to determine whether the TLRs expressed in

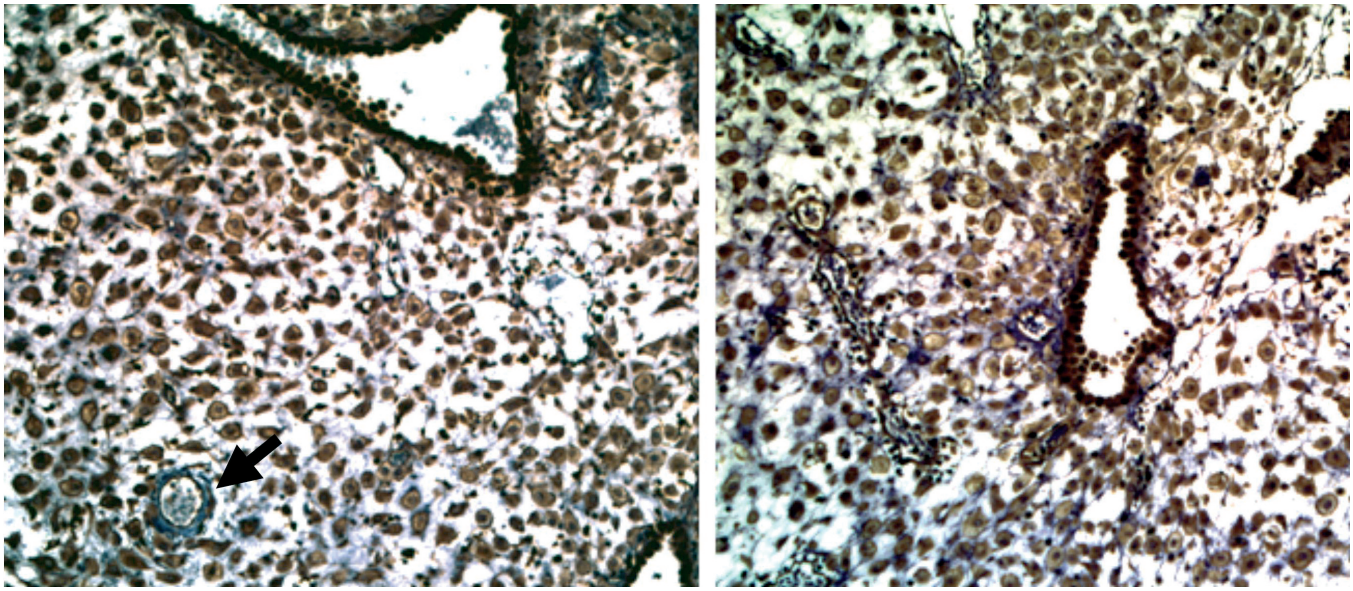

Fig. 2. Immunohistochemical staining for TLR-2 and TLR-4 in first trimester decidua. Non sequential formalin fixed paraffin embedded sections of first trimester decidua were stained for TLR-2 and TLR-4 as described in Methods. Brown color depicts positive staining. Black arrow points to endothelium. This is a representative photograph of one experiment, 8 other term and 7 other first trimester deciduas yielded similar results. x 40

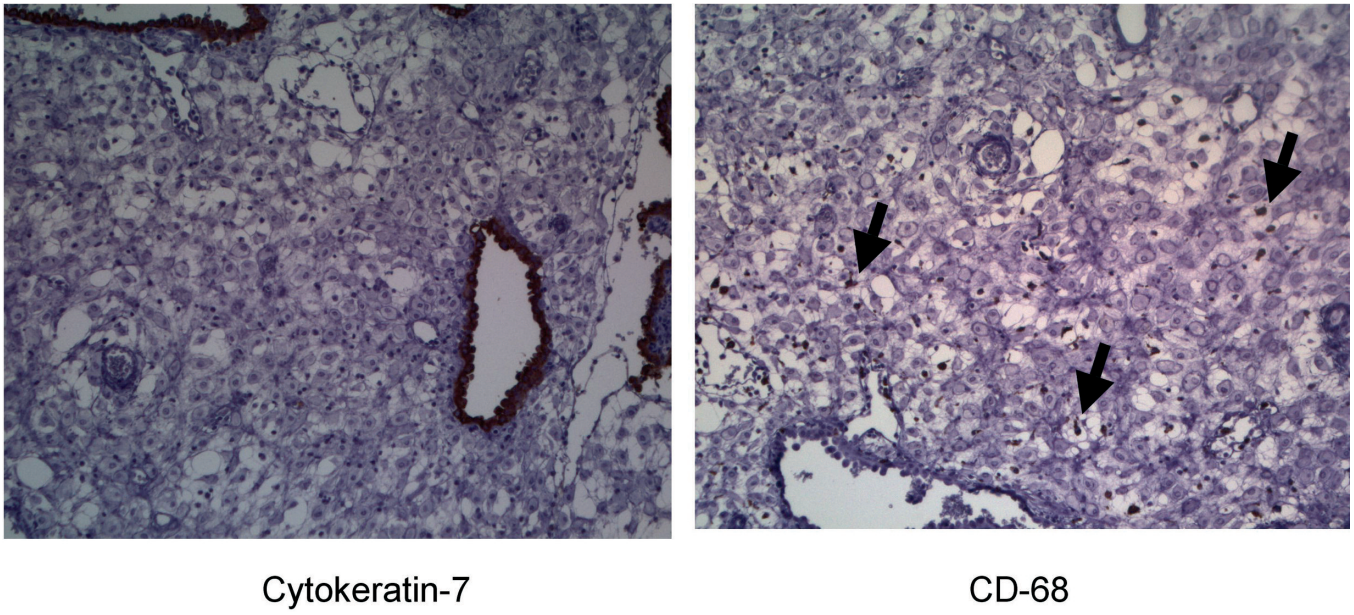

Fig. 3. Immunohistochemical staining for cytokeratin-7 and CD68 in first trimester decidua. Non sequential formalin fixed paraffin embedded sections of first trimester decidua were stained for cytokeratin-7 and CD68 as described in Methods. Black arrows points to selected macrophages. This is a representative photograph. x 20

first and third trimester decidual cells had the potential to be functional, we evaluated the expression of the two main signaling adapter proteins; MyD88 and TRIF by Western blot analysis. As can be seen in Fig. 5, first trimester decidual cells expressed both MyD88 and TRIF. Similar results were observed in term decidual cells (not shown). Addition of specific TLR activating peptides demonstrated that PG (TLR-2) or poly: (IC)

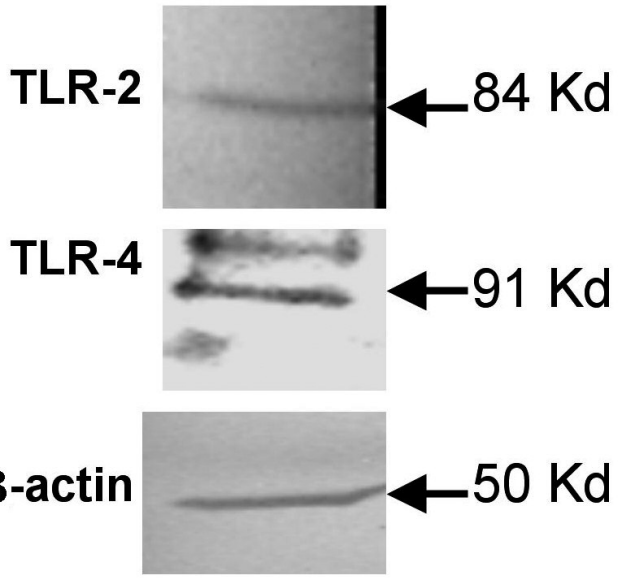

Fig. 4. Western blot analysis of TLR-2 and TLR-4. First trimester decidual cells were treated with E2 + MPA for 7 days. At the end of this period, cells were harvested and the protein expression of TLR-2 and TLR-4 was assessed by Western blotting as described in Methods. Membranes were stripped and re-probed for ß-actin as a loading control. (n=4)

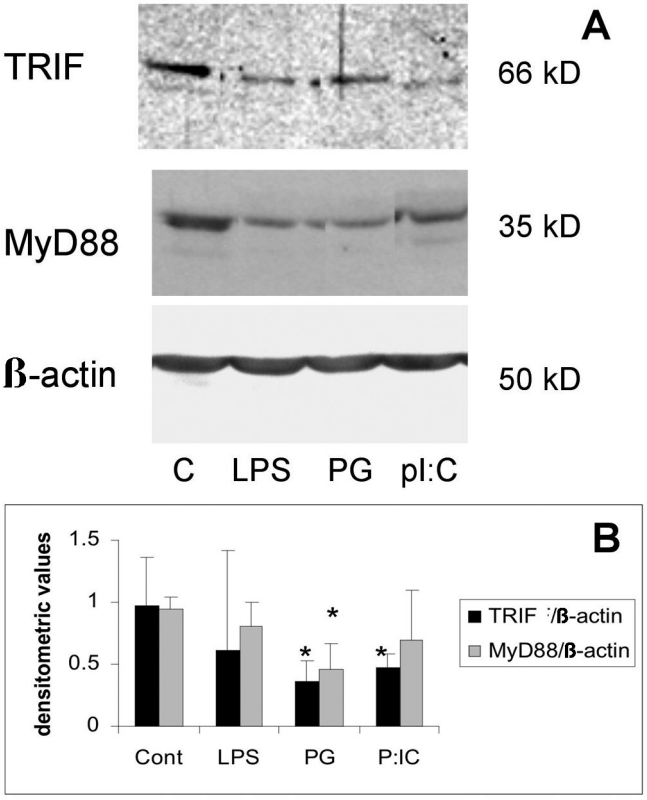

Fig. 5. Expression of TLR signaling proteins by first trimester decidual cells. **Panel A.** This is a typical experiment where cells were initially treated with E2 + MPA for 7 days. Cells were then treated for 6 hrs. with LPS, PG or poly (I:C) at a concentration of 10 µg/ml. Cells were harvested and the protein expression of TRIF, MyD88, and ß-actin was assessed by Western blotting as described in Methods. **Panel B.** Densitometric analysis of the blots (* n=4 p<0 .05 compared to controls).

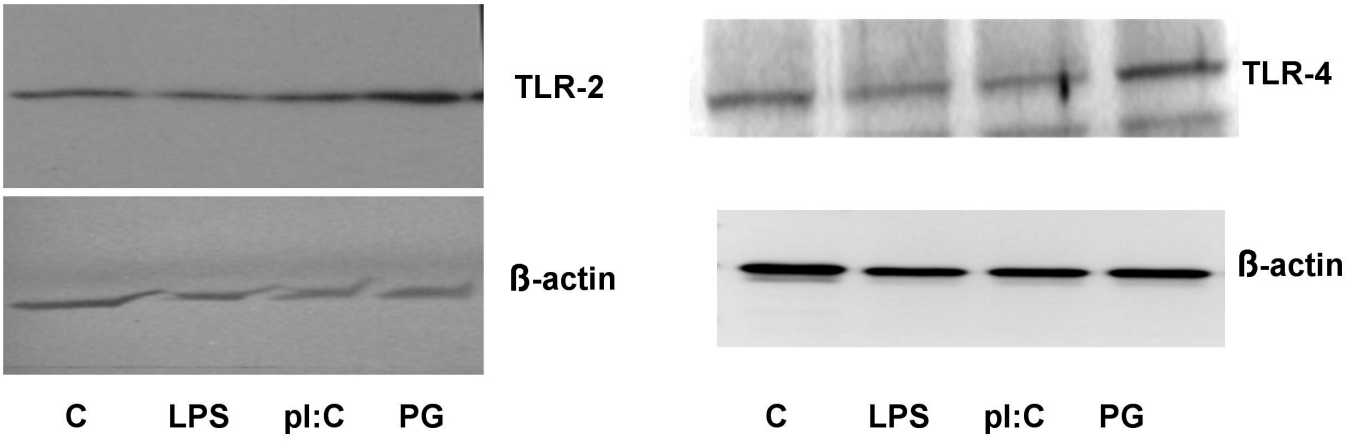

Fig. 6. Effect of TLR activating compounds on TLR protein expression. First trimester decidual cells were treated with E2 + MPA for 7 days. At the end of this period, cells were treated for 6 hrs. with LPS, PG or poly (I:C) as described in Methods. Cells were harvested and the protein expression of TLR-2 was analyzed by Western blotting as described in Methods. Membranes were stripped and re-probed for ß-actin as a loading control (n=3).

(TLR-3) significantly decreased the expression of TRIF and/or MyD88. Although a similar trend was observed for LPS treatment, statistical analysis did not reveal significance. Although one representative blot is shown in Figure 5 (panel A), the effects are more clearly distinguished in panel B which statistically analyzes 4 separate blots.

In contrast to the findings presented above, no significant changes in the expression of TLR-2 or TLR-4 were observed following the addition of LPS, PG or poly: (IC) to first trimester decidual cells (Fig. 6) following analysis by densitometry on 4 different experiments. Loading efficiencies were confirmed by re-probing the blots with ß-actin.

Cytokine production

TLRs signaling through intermediates such as TRIF and NFκB ultimately modulate the innate pro-inflammatory response through the production of cytokines and chemokines (Hopkins, 2005). The essential function of these factors is to direct cellular traffic along a concentration gradient and activate target cells to secrete cytokines, enzymes, chemokines and oxidative products (Kaplan, 2001; Fan and Malik, 2003; Puel et al., 2005). Since first and third trimester decidual

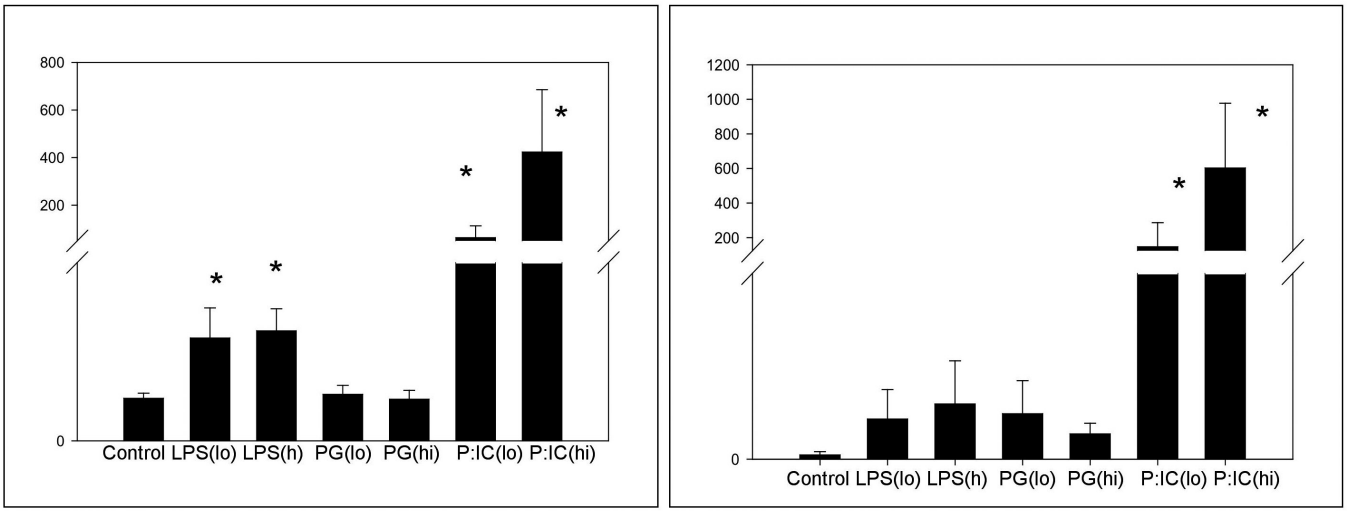

Fig. 7. IL-6 and IL-8 expression by first trimester decidual cells. Cells were treated as in Figure 3. At the end of the experiment, RNA was extracted and real time quantitative RT-PCR was carried out as described in Methods. The expression of IL-6 (*left*) and IL-8 (*right*) is reported as a ratio of the cytokine/ ß-actin (n=4 *p<0.05 vs control by the Kruskal-Wallis One Way Analysis of Variance on Ranks).

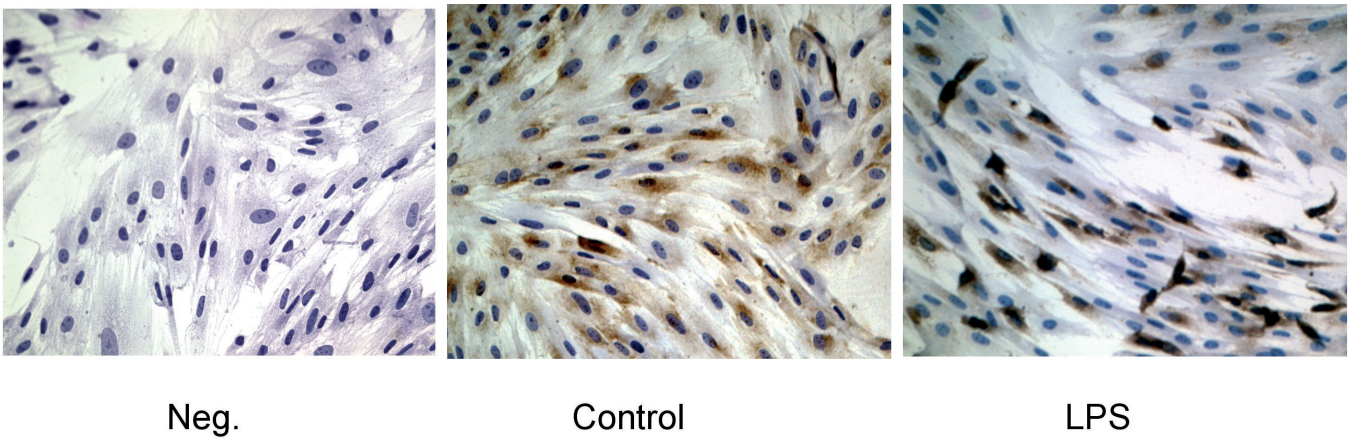

Fig. 8. Immunocytochemical staining for NFκB. First trimester decidual cells were grown in 6- well chamber slides and treated with either vehicle (E2+MPA) or E2+MPA + 10ug/ml LPS for 1 hr. Immunocytochemistry was then conducted as described in Methods. This is a representative experiment from 3 identically conducted ones.

cells express the signaling adapter proteins necessary for TLR functions, we analyzed the effects of TLR stimulation on decidual expression of the mRNA encoding the cytokines IL-6 and IL-8. First trimester DCs were treated with steroids as described in Methods and subsequently exposed to specific TLR agonists. As can be seen in Fig. 7, the TLR-3 agonist, poly(I:C) had a significant and prevailing induction of both cytokines (note break in axis). The TLR-4 agonist, LPS significantly induced IL-6 expression, while a similar trend for IL-8 was not significant. By contrast, the TLR-2 agonist peptidoglycan (PG) did not significantly induce the expression of either cytokine.

NFκB translocation

As mentioned above, the activation of NFκB is one of the ultimate steps of the TLR signaling cascade. When NFκB is activated it translocates to the nucleus (Chen et al., 2001; Yamamoto and Gaynor, 2004). Hence, first trimester decidual cells were isolated, and seeded in 6-well chamber slides. The cells were then treated with steroids for 24 hours and subsequently for 1 hour with either vehicle control, LPS, PG or poly (I:C) Immunocytochemistry for NFκB was conducted as previously described (Krikun et al., 2004). As can be seen in Fig. 8, NFκB is largely localized in the cytoplasm of the decidual cells treated with media containing E2+MPA (Control), while additional treatment with LPS resulted in nuclear translocation of NFκB. Similar results were observed following treatment with PG or poly (I:C) (results not shown). No staining was observed in the negative control slides in which cells were incubated with isotype matched control antiserum.

Discussion

The human female reproductive tract is immunologically unique in that it must act in a paradoxical fashion, accepting the semi-allogenic placenta, while simultaneously detecting and responding to a broad diversity of pathogens.

During human implantation and placentation, blastocyst-derived cytotrophoblasts penetrate and traverse the underlying decidua. They then surround and breach decidual arteries and arterioles to become endovascular trophoblasts that transform the smooth muscle layer and replace the endothelium (Burrows et al., 1996; Gude et al., 2004). This process enhances vascular conductance and increases blood flow to the intervillous space requisite for fetal growth. Its success requires that the decidua become an immunologically distinctive site that allows the invasion and growth of the allogeneic trophoblast, while paradoxically maintaining host defense against an array of microbial pathogens. Modulation of inflammation at the maternal-fetal interface is thus critical for successful pregnancy.

Previous studies by Pioli et al. (2004) demonstrated that TLRs 1-6 as well as the signaling adapter MyD88 and the accessory molecule CD14 were detected in the non-pregnant female reproductive tract. The authors showed a quantitatively distinct mRNA expression profile in whole tissue derived from the fallopian tubes, endometrium, and cervix. In addition several studies have identified the role and expression of TLRs by the placenta. In first trimester trophoblasts, studies demonstrated that upon recognition of microbes through TLRs, trophoblasts coordinate an immune response by recruiting cells of the innate immune system to the maternal-fetal interface (Abrahams and Mor, 2005). In term placentas, one study showed expression of both TLR2 and TLR4 by intermediate trophoblasts found in free cell islands, in the cell columns and in the decidua (Holmlund et al., 2002).

In this study we investigated the expression of TLRs by first and term trimester decidua. We now show for the first time, that decidual cells express TLRs and critical TLR signaling adapter proteins. Moreover, we demonstrate changes in the production of IL-6 and IL-8 following treatment with TLR ligands. The viral component poly (I:C) was particularly effective in stimulating cytokine expression. Lastly, treatment of decidual cells with LPS, poly (I:C), and PG all resulted in the nuclear translocation of NFκB.

These findings are consistent with the involvement of TLR in the pathogenesis of adverse pregnancy outcomes. For example, in the placentas of pregnancies complicated by preeclampsia, TLR-4 protein expression is increased in interstitial trophoblasts, which may contribute to a local abnormal cytokine milieu (Kim et al., 2005). It is plausible that co-activation of specific TLR receptors in maternal and fetal derived tissues at the maternal-fetal interface leads to a predominant pro-inflammatory response which ultimately hinders trophoblast proliferation and adequate invasion of the decidua and myometrium, as noted in preeclampsia.

The specific role of decidual TLRs in complications of pregnancy remains unelucidated. Studies of decidual TLR function will further define their role in the pathogenesis of adverse pregnancy outcomes.

In summary, based on our current findings, we propose that abnormalities in decidual TLRs expression or function may be linked to abnormal placentation, inflammation and negative pregnancy outcomes.

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