Decreased Toll-like receptor-2 messenger ribonucleic acid and increased Toll-like receptor-4 in the tubal epithelium next to the infiltrated trophoblasts during tubal pregnancy

Yin Fen Ji, M.D.,[a] Jian Xu, M.D.,[a] Tao Zhang, M.M.,[a] and Li You Chen, M.M.[b]

[a] Department of Gynecology, Women's Hospital, School of Medicine, Zhejiang University; and [b] Center of Gynecological Laparoscopy, People's Hospital of Zhejiang Province, People's Republic of China

Objective: To explore the expression patterns of Toll-like receptor (TLR)2 and TLR4 in the tubal epithelial cells next to the infiltrated trophoblasts at the maternal–fetal interface during tubal pregnancy.
Design: Prospective, observational study.
Setting: University-based obstetrics and gynecology hospital.
Patient(s): Thirty-seven women undergoing salpingectomy for tubal ampullary pregnancy and nine nonpregnant patients with benign uterine or appendix disease.
Intervention(s): Oviduct tissues with ectopic gestations were separated into implantation site (group 1) and nonimplantation site (group 2). Tissues from ampullary fallopian tubes during mid-secretory phase (group 3) were collected as the control group. Immunohistochemistry and quantitative real-time polymerase chain reaction were performed.
Main Outcome Measure(s): Differences of TLR2 and TLR4 expression patterns between group 1 and group 2 and between the pregnant group (combined group 1 and group 2) and the nonpregnant group (group 3).
Result(s): Comparing the pregnant group with group 3, TLR4 messenger RNA (mRNA) and protein were both significantly up-regulated in the pregnant group. In contrast, TLR2 mRNA was significantly down-regulated, whereas TLR2 protein showed a tendency toward reduction. Detailed analysis between group 1 and group 3 revealed statistically significantly higher TLR2 and TLR4 protein in group 1. In terms of mRNA, TLR4 expression was still shown to be significantly increased in group 1, whereas TLR2 expression was markedly decreased in group 1.
Conclusion(s): Decreased TLR2 mRNA and increased TLR4 in the tubal epithelial cells next to the infiltrated trophoblasts may be associated with aspects of the pathophysiology of tubal ectopic pregnancy in immune defense. (Fertil Steril® 2017;107:282–8. ©2016 by American Society for Reproductive Medicine.)
Key Words: Fallopian tube, maternal–fetal interface, Toll-like receptor 2, toll-like receptor 4, tubal pregnancy

Toll-like receptors (TLRs), involved in the innate immunity of the human female reproductive tract, are transmembrane proteins with extracellular domains of leucine-rich repeat motifs that are evolutionarily conserved to recognize pathogen-associated molecular patterns in bacteria, viruses, fungi, and parasites (1, 2). There already exists extensive literature confirming that the innate immune system, mediated by TLRs that are implicated in the response to microbial pathogens during pregnancy, is activated in the maternal–fetal interface (3–10).

At the maternal–fetal interface, TLRs are widely expressed not only in immune cells but also in nonimmune cells, such as fetal membranes (4),

Received July 23, 2016; revised September 10, 2016; accepted September 13, 2016; published online October 25, 2016.
Y.F.J. has nothing to disclose. J.X. has nothing to disclose. T.Z. has nothing to disclose. L.Y.C. has nothing to disclose.
Supported by the National Nature Science Foundation of China (grant nos. 81000233 and 81270659); a project funded by Key research project of traditional Chinese medicine of Zhejiang province (grant no. 2014ZZ008).
Reprint requests: Li You Chen, M.M., People's Hospital of Zhejiang Province, Center of Gynecological Laparoscopy, No. 148 Shangtang Road, Hangzhou, Zhejiang 310004, People's Republic of China (E-mail: mendac@sina.cn).

Fertility and Sterility® Vol. 107, No. 1, January 2017 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.09.026

trophoblasts (5–8), and decidual cells (4, 9, 10). Their expression patterns also vary according to the stage of pregnancy with spatial difference (1, 3). Further studies using the TLRs mutational mouse intrauterine injection model have shown that overstimulation of TLR2, -3, -4, and -9 can induce preterm labor (11–15). Recent clinical studies also have linked TLRs to pregnancy complications, such as chorioamnionitis (4), abortion, preterm labor, pre-eclampsia, and even fetal complications (16–18). All of the above suggested that TLRs in these cells regulate the inflammatory response to microbial pathogens at the maternal–fetal interface during pregnancy (3) and that inadequate or exaggerated activity may be involved in the pathology of pregnancy complications (19, 20).

Fetal membranes, trophoblasts, decidual cells, and the local neighboring epithelium cells next to the infiltrated trophoblast cells are in close contact at the maternal–fetal interface. Although TLRs have been studied extensively in the three former, to our knowledge, only one report study (10) has described that TLR4 expression was also evident in glandular epithelial cells of the endometrium at the maternal–fetal interface without any further characterization. Therefore, little is known about the TLRs expression patterns and their possible roles in the local neighboring epithelial cells at the maternal–fetal interface. To better understand the possible role of TLRs at the maternal–fetal interface, it is necessary to elucidate the expression patterns of TLRs in the local neighboring epithelial cells next to the infiltrated trophoblast cells during pregnancy.

During pregnancy, the epithelial cells are destroyed when the trophoblast cells infiltrate. Therefore, it is very difficult to collect samples which include both the trophoblast cells and their adjacent epithelial cells during intrauterine pregnancy terminated by artificial abortion. We instead focused on the fallopian tubal pregnancy, where in which salpingectomy is usually performed to protect maternal health. We separated the fallopian tube into implantation site (the area within 5 mm of the gestational mass) and nonimplantation site (more than 10 mm beyond the mass) (21) to explore the intrinsic expression patterns of TLRs in the local neighboring epithelial cells next to the infiltrated trophoblast cells. The existing literature has illustrated the importance of TLR2 and -4, which are involved in the innate immunity of the fallopian tube, with the most extensive expression in the tube along the female reproductive tract (2, 22) during the nonpregnant state. However, there is no information on TLRs in the fallopian tube with tubal pregnancy. In the present study we compared TLR2 and TLR4 messenger RNA (mRNA) and protein expression in the human tubal epithelial cells of the implantation and nonimplantation sites during tubal pregnancy with TLR2 and TLR4 mRNA and protein expression in ampullary fallopian tube during the secretory phase of the menstrual cycle from nonpregnant women, to provide a better understanding of the initial mechanism of innate immune response at the maternal–fetal interface during tubal pregnancy.

MATERIALS AND METHODS

Tissue Collection

Fallopian tubes from 37 women with ampullary, tubal pregnancies (mean age 31 years; range, 23–42 years) were sampled at both the implantation sites (group 1) and the nonimplantation sites (group 2). During the process of collecting implantation site tissues, we removed the macroscopic villi as much as possible to avoid the impact of the trophoblast cells on the results of TLR expression. As the nonpregnant group (group 3), nine normal ampullary fallopian tubes during mid-secretory phase (from 19 to 24 days of the menstrual cycle) were also obtained from women undergoing salpingectomy and hysterectomy simultaneously for benign uterine or appendix disease (mean age 35 years; range, 30–43 years); and in parallel the group's endometriums were biopsied for histologic dating to identify the precise menstrual cycle.

All enrolled women with normal intrauterine pregnancy history underwent the operations at Women's Hospital of Zhejiang University between December 2010 and July 2011. They had no previous history of ectopic pregnancy, had not taken any hormonal preparations in the 3 months before surgery, and had regular menstrual cycles (about 28 days). For tubal pregnant patients (less than 7 pregnant weeks) there was no co-intrauterine pregnancy as established by ultrasound scans of the pelvis, and no methotrexate treatment before the operation.

Each tubal sample was divided into two parts: one was snap-frozen in liquid nitrogen and stored at −80°C for real-time polymerase chain reaction (PCR), and the other was placed in 10% buffered formalin (pH 7.4) for 16–24 hours, then embedded in paraffin for immunohistochemistry and confirmed by subsequent pathology. Hematoxylin and eosin–stained tissue sections from the implantation site (group 1) show anchoring villus penetrating into the mucosa and the villus's adjoining luminal epithelium of the fallopian tube; that is, tissues in group 1 contained anchoring villus as well as tubal mucosa and stroma; whereas in the nonimplantation site samples there were only tubal tissues, without any trophoblast cells (group 2). The endometrial biopsies, which underwent hematoxylin and eosin staining, were examined by a gynecological pathologist. Ethical approval (20120046) for this study was obtained from the institutional review board at the Women's Hospital, Zhejiang University. Written and informed consent was obtained from all patients before sample collection.

Immunohistochemistry

Immunohistochemical localization of TLR2 and TLR4 was performed on fallopian tube sections using biotinylated secondary antibodies and peroxidase-conjugated detection systems. In brief, tissue sections were dewaxed in xylene and rehydrated in descending grades of alcohol. For antigen retrieval, sections were put in 0.01 M sodium citrate (pH 6.0) at 126°C for 2.5 minutes by autoclave. Unless stated otherwise after each step the sections were thoroughly washed three times for 5 minutes each with phosphate-buffered saline (10 mM sodium phosphate containing 0.1 M

NaCl, pH 7.4). The endogenous peroxidase was quenched by 3% hydrogen peroxidase, and nonspecific binding was reduced by 1.5% normal mouse serum in phosphate-buffered saline. Sections were then incubated with monoclonal mouse anti-human TLR2 (ab9100) and TLR4 antibody (ab22048) (both from Abcam; dilution 1:2,000 for TLR2 and 1:400 for TLR4), respectively, at room temperature for 2 hours, and subsequently with biotin-conjugated secondary antibody and horseradish-labeled streptavidin–peroxidase (Zymed Laboratories) at 37°C for 30 minutes, respectively. The reaction visualization was done with 3, 3′-diaminobenzidine, and the slides were counterstained with Harris' hematoxylin and mounted in Pertex (Cellpath Technologies).

Two independent blind observers graded the sections semi-quantitatively, and the H score was calculated using the following equation: H score $= \Sigma$ Pi $(i + 1)$, where $i =$ intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively), and Pi is the percentage of stained epithelial cells for each intensity, varying from 0 to 100% (21).

RNA Extraction, Reverse Transcription, and Quantitative Real-time–PCR of TLR2, TLR4

Using an RNeasy Mini kit (Qiagen), total RNA was extracted from tissue samples. All RNA samples were treated with DNase to eliminate genomic DNA contamination. One microgram of total RNA was reverse-transcribed in a 20-μL volume using a reverse transcription–PCR kit (Toyobo). Reverse transcription reactions and real-time PCR were performed according to the manufacturer's protocols (TaKaRa). All reverse transcription reactions, including no-template controls, were run in a PTC-200 Peltier Thermal Cycler (MJ Research). Messenger RNA levels were quantified with the ABI Prism 7900HT sequence detection system (Applied Biosystems). Primer sequences used to amplify TLR2, TLR4, and β-actin, an internal reference, are listed in Supplemental Table 1 (available online). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the 2- Δct method (Δct = CT of objective − CT of β-actin).

Statistical Analysis

Values that follow a nonnormal distribution were expressed as median and range. Data were analyzed using the Statistical Package for Social Sciences (SPSS 11.0 for Windows, IBM). Differences between the implantation group (group 1) and the nonimplantation group (group 2) were analyzed using the paired t test, and the Mann-Whitney U test was used for comparisons between the other groups. The significance level was set at $P<.05$, and two-tailed tests were used for all hypothesis tests.

RESULTS

Expression of TLR2 and TLR4 Protein in Human Fallopian Tube

TLR2 and TLR4 proteins have similar localization, both being found mainly in the cytoplasm of fallopian tubal epithelial cells (Fig. 1A–E). An apocrine distribution of TLR2 expression was observed in all specimens of the implantation site group (group 1) (Fig. 1A). However, this phenomenon was not seen in other groups with TLR2 expression or in any groups with TLR4 expression. The staining of TLR2 and TLR4 at the implantation site (3.68 [2.00–4.00], 2.22 [0.84–3.56], respectively) was stronger than that in the matched, nonimplantation site (2.00 [0.24–4.00], 0.90 [0.30–2.00], respectively).

Semi-quantitative analysis showed that the expression of TLR2 and TLR4 proteins in the tubal epithelial cells was markedly higher in group 1 than in group 2 or group 3, and this difference was significant (group 1 vs. group 2, $P<.0001$, $P<.0001$; group 1 vs. group 3, $P=.046$, $P<.0001$ for TLR2, TLR4, respectively). Yet there were no statistically significant differences of TLR2 and TLR4 protein expression between group 2 and group 3 ($P=.207$, $P=.413$, respectively). The expression levels of TLR2 and TLR4 protein in the epithelial cells are shown in Table 1. We did further analysis by combining the implantation group and the nonimplantation group as one "pregnant group" and explored the protein expression patterns of TLR2 and TLR4 in the tubal epithelial cells in comparison with the nonpregnant group (group 3). The TLR4 protein was significantly increased in the pregnant group in comparison with group 3 ($P=.007$). In contrast, there was no significant difference in TLR2 protein expression, with only slightly lower levels detected in the pregnant group ($P=.713$) (Table 2).

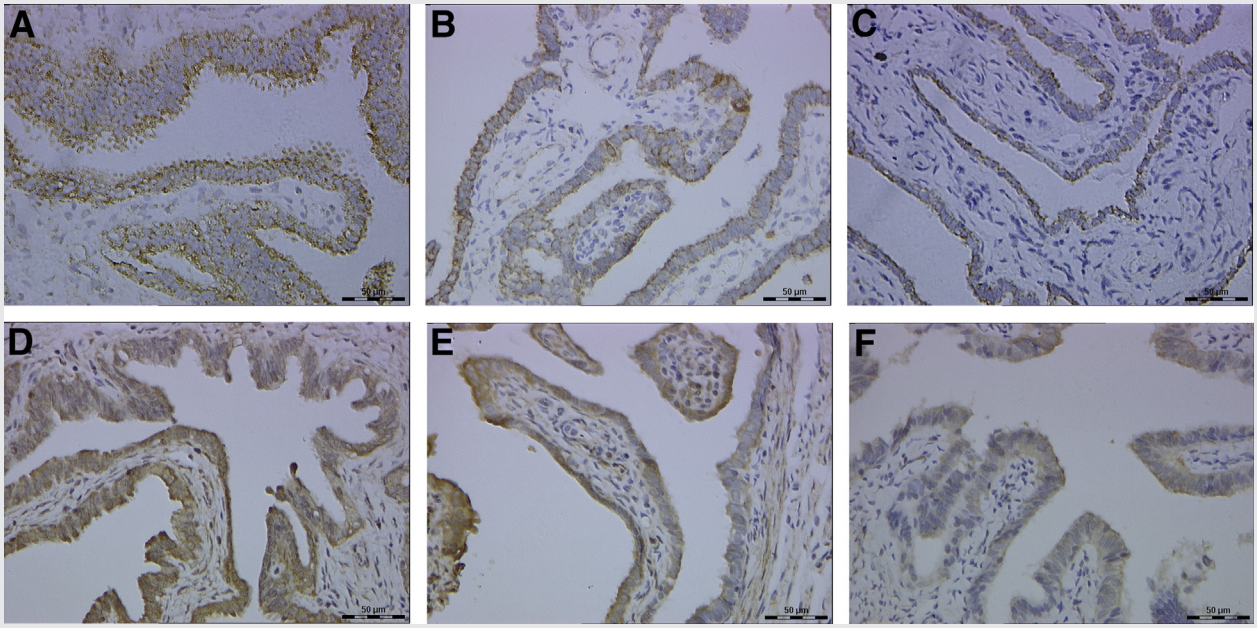
mRNA Expression Levels of TLR2 and TLR4 in Human Fallopian Tube

We observed distinct expression patterns of TLR2 and TLR4 mRNA in our study groups. Compared with group 3, in the pregnant group TLR4 mRNA was significantly up-regulated ($P<.0001$), whereas the TLR2 mRNA was significantly down-regulated ($P=.039$) (Table 2). Similarly, increased TLR4 mRNA expression combined with decreased TLR2 mRNA expression was also found and reached statistical significant difference when comparing group 1 with group 3 (group 1 vs. group 3, $P<.0001$, $P=.022$ for TLR4, TLR2, respectively). We then explored the differences between group 2 and group 1 or group 3. The TLR4 mRNA expression levels in group 2 were similar to those in group 1 (group 1 vs. group 2, $P=.187$) but higher than in group 3 (group 2 vs. group 3, $P<.0001$); as for TLR2 mRNA expression levels, there were no significant differences (group 2 vs. group 1, $P=.736$; group 2 vs group 3, $P=.105$), although levels were slightly higher in group 2 compared with group 1 (Table 3).

DISCUSSION

Previous studies have suggested that TLRs, activated at the maternal–fetal interface, are responsible for the response to pathogens and regulation of infection-related inflammation during pregnancy (1, 19, 22–25). However, there are almost no data about these TLRs' expression levels in the tubal epithelial cells next to the infiltrated trophoblasts at the maternal–fetal interface during tubal pregnancy. In this investigation we explored TLR2 and TLR4 expression

FIGURE 1



Expression of TLR2 and TLR4 protein in epithelial cell of human fallopian tube, determined by immunohistochemistry. The proteins were mainly localized in the epithelial cells. Increased immunostaining of TLR2 (**A**) and TLR4 (**D**) was observed in the implantation site, where it was obviously stronger than in the corresponding nonimplantation site (**B** and **E** for TLR2 and TLR4, respectively) and in the normal group (**C** and **F** for TLR2 and TLR4, respectively). Notably, there was apparent apocrine secretion of TLR2 protein in the implantation site (**A**). The negative control section has no staining (not shown).

Ji. TLR2, -4 at tubal maternal–fetal interface. Fertil Steril 2016.

patterns in the tubal epithelial cells next to the infiltrated trophoblasts in the implantation sites and found the different expression patterns of TLR2 and TLR4 during tubal pregnancy.

Toll-like receptors 2 and 4 are expressed at the highest levels in the female fallopian tube along the female reproductive tract (with the overwhelming majority of studies measuring mRNA expression) and play a crucial role in the tubal immune defense (2, 26–28). In theory, TLR2 and TLR4, as transmembrane proteins, are primarily expressed on the host plasma membrane and immediately detect invasive pathogens' membrane components (29–32). However, in this investigation TLR2 and TLR4 were detected mainly in the epithelial cells' cytoplasm. This is consistent with what Ghosh et al. (33) reported. They found there was no response of human fallopian tubal epithelial cells (with measurable TLR2 and TLR4 expressions) to TLR2 and 4 agonists, and presumed that one possible explanation was the intracellular location of TLR2 and -4 responding only to intracellular pathogens in the upper female reproductive tract. Intracellular expression was also shown by Ueta et al. (34) in corneal epithelial cells to build an immuno-silent environment at the ocular mucosa. Furthermore, altered microenvironment can also change the localization of TLRs. One study suggested that an infection during chorioamnionitis may induce the translocation of TLR4 from apical to basal membrane to decrease TLR signaling but maintain the competence of amniotic epithelium to invasive bacteria during early infection (35). Therefore, it is reasonable to presume that the translocation of TLR2 and TLR4 in the cytoplasm of tubal

TABLE 1

Expression of TLR2 and TLR4 protein in the epithelium cells of the fallopian tubes determined by immunohistochemistry.

Group	n	TLR2 protein	*P* value (TLR2)	TLR4 protein	*P* value (TLR4)
Implantation	37	3.68 (2.00–4.00)	< .0001[a,*]	2.22 (0.84–3.56)	< .0001[a,*]
Nonimplantation	37	2.00 (0.24–4.00)	.207[b]	0.90 (0.30–2.00)	.413[b]
Nonpregnant	9	3.04 (1.20–3.20)	.046[c,*]	1.00 (0.28–1.20)	< .0001[c,*]

Note: Values are expressed as median (range). Implantation: the implantation site (group 1); nonimplantation: the nonimplantation site (group 2); nonpregnant: the nonpregnant group (group 3).
[a] *P*: Group 1 vs. group 2.
[b] *P*: Group 2 vs. group 3.
[c] *P*: Group 1 vs. group 3.
* Significant difference.

Ji. TLR2, -4 at tubal maternal–fetal interface. Fertil Steril 2016.

TABLE 2

Comparison of TLR2 and TLR4 expression in the tubal epithelium between the pregnant group and the nonpregnant group.

Parameter	Pregnant group	Nonpregnant group	*P* value
TLR2 protein	2.66 (0.24–4.00)	3.04 (1.20–3.20)	.713
TLR2 mRNA	12.21 (1.86–5,032.20)	33.49 (9.91–1,797.15)	.039*
TLR4 protein	1.72 (0.30–3.56)	1.00 (0.28–1.20)	.007*
TLR4 mRNA	9.35 (2.08–48.13)	1.34 (1.19–4.03)	<.0001*

Note: Values are expressed as median (range).
* Significant difference.

Ji. TLR2, -4 at tubal maternal–fetal interface. Fertil Steril 2016.

epithelial cell may be important for avoiding a state of unnecessary inflammation that could disrupt the epithelial barrier and cause irreversible tubal scarring; only when a pathogen breaches the epithelial barrier, then an obvious response can be mounted.

Another interesting observation in the present study was the presence of the granule-like objects, which were positive for TLR2 and secreted into the tubal lumen in all specimens of group 1. This was not seen in other groups for TLR2 and in none of the groups for TLR4. Other similar instances, such as a secretory form of TLR4 in the human endocervical glands (26) and soluble TLR2 in amniotic fluid (36), have been reported. It has been suggested that these special TLRs may work as a feedback mechanism (26) or interfere with the recognition of homologous TLRs ligands by TLRs (36) to prevent unnecessary activation of the inflammatory process. Our findings also support this hypothesis. In this study we found that in comparison with the nonpregnant group (group 3), the mRNA expression of TLR2 in the pregnant group was statistically significantly decreased, especially in group 1; in contrast, TLR2 protein expression levels were the highest in group 1 compared with all other groups, whereas the levels in the overall pregnant group were slightly decreased (but not significantly) when compared with group 3. This variance can be explained by the negative feedback mechanism; that is, the uniquely abundant TLR2 apocrine secretion in group 1 inhibits the local expression of TLR2 mRNA in return. Because the generous apocrine expression of TLR2 protein in group 1 is the trigger for local decreased TLR2 mRNA, the change in TLR2 protein expression lags behind the mRNA, and we observe a divergent expression levels. Conversely, both the mRNA and protein of TLR4 expression in the pregnant group were significantly up-regulated during the tubal pregnancy. Then the quite different expression patterns between the TLR2 and TLR4 begs for an explanation, which is probably because that they have obviously different ligands.

Growing evidence shows that TLR ligands also can be endogenous substances, such as fibrinogen (37), surfactant protein A (38), and breakdown products of the extracellular matrix (39), etc. which are mostly released by injured or dying cells especially accompanied by tissue remodeling during pregnancy, and can stimulate TLRs to be involved in non-infection-related conditions associated with pregnancy (32, 40–42). Furthermore, TLR4 is the primary mediator of the host response against those endogenous ligands, despite the absence of infection (3, 43, 44). During early pregnancy, extensive tissue remodeling occurs at the implantation site, accompanied by apoptosis, breakdown of epithelium cell, and extracellular matrix degradation, which produces an abundance of endogenous ligands. Under the stimulatory effect of the increased ligands for TLR4, we found the highest expression levels of TLR4 in the implantation site (group 1), which also influenced the expression of TLR4 at the nonimplantation site (group 2), with significantly up-regulated results in comparison with the nonpregnant group (group 3). On the other hand, Takeuchi et al. (45) reported that down-regulated expressions of particular TLRs during the gestation period would make the female reproductive organ tolerate the fetus and maintain the pregnant state. It is likely that in this investigation the concomitant opposite expression patterns of TLR2 and TLR4 mRNA may also maintain a hospitable environment for the fetus; however, the exactly regulatory mechanisms between TLR2 and TLR4 need further confirmation.

TABLE 3

Expression of TLR2 and TLR4 mRNA in the tubal epithelium, determined by quantitative real-time PCR.

Group	n	TLR2 mRNA (2-Δct × 104)	*P* value (TLR2)	TLR4 mRNA (2-Δct × 104)	*P* value (TLR4)
Implantation	37	10.1959 (1.86–1,891.76)	.736[a]	8.5721 (2.08–18.20)	.187[a]
Nonimplantation	37	14.4175 (2.29–5,032.20)	.105[b]	9.5160 (2.20–48.13)	<.0001[b,*]
Nonpregnant	9	33.4890 (9.91–1,797.15)	.022[c,*]	1.3435 (1.19–4.03)	<.0001[c,*]

Note: Values are expressed as median (range). Implantation: the implantation site (group 1); nonimplantation: the nonimplantation site (group 2); nonpregnant: the nonpregnant group (group 3).
[a] *P*: Group 1 vs. group 2.
[b] *P*: Group 2 vs. group 3.
[c] *P*: Group 1 vs. group 3.
* Significant difference.

Ji. TLR2, -4 at tubal maternal–fetal interface. Fertil Steril 2016.

In summary, the divergent expression levels between TLR2 mRNA and TLR4 mRNA, and also between TLR2 protein and TLR2 mRNA, in the tubal epithelium next to the infiltrated trophoblast cell at the maternal–fetal interface during tubal pregnancy suggest complicated regulatory mechanisms. Decreased TLR2 mRNA and increased TLR4 in the tubal epithelial cells next to the infiltrated trophoblast cells during tubal pregnancy may contribute to a hospitable environment for the fetus and also contribute to aspects of the pathophysiology of tubal ectopic pregnancy.

Acknowledgments: The authors thank Caiyun Zhou for technical assistance; and the pathology department of their hospital for providing endometrial histologic analysis and semiquantitatively grading the sections.

REFERENCES

1. Koga K, Mor G. Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy disorders. Am J Reprod Immunol 2010;63:587–600.
2. Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM. Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract. Infect Immun 2004;72:5799–806.
3. Koga K, Izumi G, Mor G, Fujii T, Osuga Y. Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy complications. Am J Reprod Immunol 2014;72:192–205.
4. Koga K, Mor G. Expression and function of toll-like receptors at the maternal-fetal interface. Reprod Sci 2008;15:231–42.
5. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. Placenta 2005;26:540–7.
6. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol 2002;168:554–61.
7. Holmlund U, Cebers G, Dahlfors AR, Sandstedt B, Bremme K, Ekstrom ES, et al. Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta. Immunology 2002;107:145–51.
8. Kumazaki K, Nakayama M, Yanagihara I, Suehara N, Wada Y. Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. Hum Pathol 2004;35:47–54.
9. Krikun G, Lockwood CJ, Abrahams VM, Mor G, Paidas M, Guller S. Expression of Toll-like receptors in the human decidua. Histol Histopathol 2007;22:847–54.
10. Schatz F, Kayisli UA, Vatandaslar E, Ocak N, Guller S, Abrahams VM, et al. Toll-like receptor 4 expression in decidual cells and interstitial trophoblasts across human pregnancy. Am J Reprod Immunol 2012;68:146–53.
11. Wang H, Hirsch E. Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4. Biol Reprod 2003;69:1957–63.
12. Elovitz MA, Wang Z, Chien EK, Rychlik DF, Phillippe M. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. Am J Pathol 2003;163:2103–11.
13. Ilievski V, Lu SJ, Hirsch E. Activation of toll-like receptors 2 or 3 and preterm delivery in the mouse. Reprod Sci 2007;14:315–20.
14. Koga K, Cardenas I, Aldo P, Abrahams VM, Peng B, Fill S, et al. Activation of TLR3 in the trophoblast is associated with preterm delivery. Am J Reprod Immunol 2009;61:196–212.
15. Scharfe-Nugent A, Corr SC, Carpenter SB, Keogh L, Doyle B, Martin C, et al. TLR9 provokes inflammation in response to fetal DNA: mechanism for fetal loss in preterm birth and preeclampsia. J Immunol 2012;188:5706–12.
16. Calleja-Agius J, Jauniaux E, Pizzey AR, Muttukrishna S. Investigation of systemic inflammatory response in first trimester pregnancy failure. Hum Reprod 2012;27:349–57.
17. Redman CW, Sargent IL. Preeclampsia and the systemic inflammatory response. Semin Nephrol 2004;24:565–70.
18. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. Obstet Gynecol 2010;116:393–401.
19. Gervasi MT, Chaiworapongsa T, Pacora P, Naccasha N, Yoon BH, Maymon E, et al. Phenotypic and metabolic characteristics of monocytes and granulocytes in preeclampsia. Am J Obstet Gynecol 2001;185:792–7.
20. Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. Am J Obstet Gynecol 1998;179:80–6.
21. Ji YF, Chen LY, Xu KH, Yao JF, Shi YF. Locally elevated leukemia inhibitory factor in the inflamed fallopian tube resembles that found in tubal pregnancy. Fertil Steril 2009;91:2308–14.
22. Girling JE, Hedger MP. Toll-like receptors in the gonads and reproductive tract: emerging roles in reproductive physiology and pathology. Immunol Cell Biol 2007;85:481–9.
23. Gonzalez JM, Xu H, Ofori E, Elovitz MA. Toll-like receptors in the uterus, cervix, and placenta: is pregnancy an immunosuppressed state? Am J Obstet Gynecol 2007;197:296, e1–6.
24. King AE, Kelly RW, Sallenave JM, Bocking AD, Challis JR. Innate immune defences in the human uterus during pregnancy. Placenta 2007;28:1099–106.
25. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG 2006;113(Suppl 3):17–42.
26. Fazeli A, Bruce C, Anumba DO. Characterization of Toll-like receptors in the female reproductive tract in humans. Hum Reprod 2005;20:1372–8.
27. Aflatoonian R, Fazeli A. Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. J Reprod Immunol 2008;77:7–13.
28. Darville T, O'Neill JM, Andrews CW Jr, Nagarajan UM, Stahl L, Ojcius DM. Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. J Immunol 2003;171:6187–97.
29. Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. Pattern recognition receptors and the innate immune response to viral infection. Viruses 2011;3:920–40.
30. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
31. Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity 2010;32:305–15.
32. Kannaki TR, Shanmugam M, Verma PC. Toll-like receptors and their role in animal reproduction. Anim Reprod Sci 2011;125:1–12.
33. Ghosh M, Schaefer TM, Fahey JV, Wright JA, Wira CR. Antiviral responses of human Fallopian tube epithelial cells to toll-like receptor 3 agonist poly(I: C). Fertil Steril 2008;89:1497–506.
34. Ueta M, Nochi T, Jang MH, Park EJ, Igarashi O, Hino A, et al. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. J Immunol 2004;173:3337–47.
35. Adams KM, Lucas J, Kapur RP, Stevens AM. LPS induces translocation of TLR4 in amniotic epithelium. Placenta 2007;28:477–81.
36. Dulay AT, Buhimschi CS, Zhao G, Oliver EA, Mbele A, Jing S, et al. Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. J Immunol 2009;182:7244–53.
37. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. J Immunol 2001;167:2887–94.
38. Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M. Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. J Immunol 2002;168:5989–92.
39. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001;276:10229–33.
40. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805–20.

41. Zhang Z, Schluesener HJ. Mammalian toll-like receptors: from endogenous ligands to tissue regeneration. Cell Mol Life Sci 2006;63:2901–7.
42. Yang Z, Kong B, Mosser DM, Zhang X. TLRs, macrophages, and NK cells: our understandings of their functions in uterus and ovary. Int Immunopharmacol 2011;11:1442–50.
43. Matzinger P. The danger model: a renewed sense of self. Science 2002;296:301–5.
44. Kim YM, Romero R, Oh SY, Kim CJ, Kilburn BA, Armant DR, et al. Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia? Am J Obstet Gynecol 2005;193:921–7.
45. Takeuchi T, Yoshida M, Shimizu T, Asano A, Shimokawa T, Nabeta M, et al. Differential expressions of toll-like receptor genes in the vagina of pregnant mice. J Vet Med Sci 2013;75:561–5.

SUPPLEMENTAL TABLE 1

Sequences of primer sets for quantitative real-time PCR.

Gene	Primer (5′–3′)	Product size (bp)	Accession no.
TLR2	Sense: CCTCCAATCAGGCTTCTCTG Antisense: TGGAGGTTCACACACCTCTG	172	NM_003264.3
TLR4	Sense: AATCCCCTGAGGCATTTAGG Antisense: CCCCATCTTCAATTGTCTGG	100	U88880.1
Actin	Sense: CAGTCGGTTGGAGCGAGCAT Antisense: GGATGGCAAGGGACTTCCTGTA	126	NM_001101.3

Ji. TLR2, -4 at tubal maternal–fetal interface. Fertil Steril 2016.