Ureaplasma urealyticum-derived lipid-associated membrane proteins introduce IL-6, IL-8, and TNF- α cytokines into human amniotic epithelial cells via Toll-like receptor 2*

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Abstract: Objective: The purpose of this study was to determine the role of *Ureaplasma urealyticum*-derived lipid-associated membrane proteins (LAMPs) in the host innate immune system, specifically their effect on Toll-like receptors (TLRs). Methods: LAMPs were derived from *U. urealyticum* strains, and human amniotic epithelial cells (HAECs) were isolated from healthy full-term placentas. Cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA) and TLR2 mRNA by real-time PCR. Expression of TLR2 was confirmed by Western blotting and immunohistochemistry. Results: LAMPs induced HAECs to produce inflammatory cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α . Cytokine production was reduced after blocking TLR2 using TLR2 inhibitor (anti-hTLR2-IgA). Conclusions: LAMPs isolated from *U. urealyticum* induced TLR2-dependent up-regulation of inflammatory genes and cytokines in HAECs.

Key words: *Ureaplasma urealyticum*; Lipid-associated membrane protein; Human amniotic epithelial cell; Toll-like receptor 2

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1 Introduction

Intra-amniotic infection is a primary cause of preterm birth. Studies have shown that *Mycoplasma* infection is an important risk factor for adverse pregnancy outcomes and low birth weight in infants

(Viscardi, 2010; Capoccia et al., 2013). *Ureaplasma* spp., belonging to the Mycoplasmataceae family, are the most common organisms isolated from the urogenital tracts of women who present with preterm labor (Viscardi, 2010). *Ureaplasma* has 14 known serotypes and is divided into two biovars: *Ureaplasma parvum* and *Ureaplasma urealyticum*. Gerber et al. (2003) estimated that preterm birth occurs in 58.6% of *U. urealyticum*-positive women compared with only 4.4% in *U. urealyticum*-negative women.

Infection-induced preterm labor is an inflammatory process, where pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), sense microorganisms and their products. Chemokines (e.g. interleukin (IL)-1, IL-8, chemokine (C-C motif

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ligand 2 (CCL-2)), cytokines (e.g. IL-1 β , tumor necrosis factor (TNF)- α), prostaglandins, and proteases are produced, which lead to activation of the common pathway of parturition (Romero et al., 2001; Kannan et al., 2012). Triantafilou et al. (2013) demonstrated that TLR2, TLR6, and TLR9 are involved in *U. urealyticum*-induced inflammation of human amniotic epithelial cells (HAECs).

Mycoplasma lipid-associated membrane proteins (LAMPs) are cell-surface lipoproteins which induce potent inflammatory responses (He et al., 2009). Several reports have confirmed that *Mycoplasma*-derived LAMPs can induce nuclear transcription factor κ B (NF- κ B) activation in human acute monocytic leukemia cell lines (Shimizu et al., 2008) and embryonic bovine lung (EBL) cells (He et al., 2009).

Studies have revealed that it is possible to demonstrate the initial interaction between *Mycoplasma* membrane properties and host cells, such as leukocytes and epithelial cells (Shimizu et al., 2008; He et al., 2009; Choi et al., 2012). We hypothesized that *U. urealyticum* ligate TLRs can initiate inflammatory responses in HAECs. To test this hypothesis, we used *U. urealyticum*-derived LAMPs incubated with HAECs in culture ex vivo to examine inflammatory responses and to determine if the responses are mediated via TLR2 ligation.

2 Materials and methods

2.1 Preparation and culture of HAECs

Healthy full-term placentas were obtained with informed consent from women who had undergone a cesarean section at the Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China. The study was approved by the local research ethics committee.

HAECs were isolated from the placentas and cultured as follows (Liu et al., 2012). Briefly, the membrane was placed in Dulbecco's modified Eagle medium (DMEM; HyClone, USA) and cut with a razor to yield 0.5–1.0 cm² segments. The segments were digested with 0.25% (2.5 g/L) trypsin-ethylenediaminetetraacetic acid (EDTA) at 37 °C for 45 min. The resulting cell suspensions were cultured in RPMI 1640 (HyClone, USA) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, USA), 2 mmol/L L-glutamine, 100 U/ml penicillin G,

and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂ humid atmosphere.

2.2 Sample collection

The cultured HAEC cells were transferred to 12-well culture plates after the cell concentration was adjusted to 4 $\times$ 10⁵ cells/well. The group of HAECs treated with LAMPs was regarded as the LAMP test group, and the HAECs treated with *U. urealyticum* were regarded as the *U. urealyticum* test group. Aqueous phase from the preparation of LAMPs (see below) was regarded as the negative control group.

2.3 Preparation and culture of *U. urealyticum*

U. urealyticum (ATCC 27618) was cultivated in modified *Mycoplasma* liquid broth (Oxoid, England) at 37 °C in a 5% CO₂ humid atmosphere. Cell density was measured and a concentration of 1 $\times$ 10⁸ color forming units (CCU) per milliliter was used.

CCU was used for measuring *U. urealyticum* concentration. According to the metabolic activity of microbes in the culture medium, the relative content of microbes is counted. The method is described briefly: (1) 1.8 ml of the liquid *Mycoplasma* medium was added to each of 12 aseptic small tubes; (2) a 10-time gradient dilution was based on 1 $\times$ 10⁰–1 $\times$ 10⁻¹¹; (3) all tubes were cultured at 37 °C for 14 d. The highest dilution showing a color change in the culture medium, which corresponds to the maximum metabolic activity of *Mycoplasma*, was used as the CCU of the *Mycoplasma* to be tested.

2.4 Preparation of LAMPs

LAMPs were prepared as described previously (He et al., 2009).

U. urealyticum was cultured in modified *Mycoplasma* solid medium (Oxoid, England) at 5% CO₂, at 37 °C for 3–7 d until “fried egg” colonies were visible at low magnification. A single “fried egg” colony was then cultured in 500 ml of modified *Mycoplasma* liquid medium (Oxoid, England) at 5% CO₂, at 37 °C for 3–7 d. When the medium color changed from red to yellow and was clear, it was regarded as indicating the logarithmic phase of *U. urealyticum*.

Cells of *U. urealyticum* in the logarithmic phase were centrifuged at 12000g for 10 min and then the sediment was resuspended in 5 ml TBSE solution (50 mmol/L Tris (pH 8.0), 0.15 mmol/L NaCl, 1.0 mmol/L EDTA). A final concentration of 2% of

Triton X-114 (TX-114) was added to lyse *U. urealyticum* at 4 °C for 1 h. Phase separation of the lysates of *U. urealyticum* occurred at 37 °C for 10 min. The upper aqueous phase separation, which was regarded as the negative control, was stored at −80 °C after 12000g centrifugation for 20 min. The TBSE solution (4 °C) with an equal volume of TX-114 phase was added and mixed with the TX-114 phase at 4 °C for 10 min. The phase separation process was carried out three times.

The TX-114 phase was suspended with TBSE (4 °C) to the initial volume. After adding 2.5 times the volume of anhydrous alcohol, the whole suspension was left overnight at −20 °C to precipitate the membrane protein components. The precipitate was suspended in phosphate-buffered saline (PBS) after centrifugation. LAMPs were obtained after ultrasonic treatment. The protein concentration of the suspension was measured by the Bradford method (Coomassie blue protein assay).

2.5 HAECs stimulated with *U. urealyticum* or LAMPs

HAECs were seeded into 12-well plates at a concentration of 4×10^5 cells/well until the cells reached about 70%–80% confluence. HAECs were stimulated for 2, 6, or 24 h with the different concentrations (1.0–7.0 µg/ml) of LAMP or with *U. urealyticum* (1×10^8 CCU/ml). The supernatants were collected and frozen until the cytokine assays were performed. The expression of TLR2 mRNA was detected by real-time PCR after the cells were collected. Immunohistochemistry was used to detect the location of TLR2 in the cell.

2.6 TLR2 expression blocked by TLR2 inhibitor in HAECs

Anti-hTLR2-IgA (InvivoGen, USA), a TLR2 inhibitor (10 µg/ml), was added to the culture medium of HAECs for culture at 37 °C for 2 h, and then *U. urealyticum* (1×10^8 CCU/ml) or LAMPs (4 µg/ml) were added for 24 h. The culture solution was collected to detect cytokines. Immunohistochemistry was used to detect the location of TLR2 in the cell and the expression of TLR2 was detected by Western blotting.

2.7 Cytokine assays

Concentrations of cytokines in culture supernatants after stimulation were measured using enzyme-

linked immunosorbent assay (ELISA) kits (eBioscience, USA) according to the manufacturer's instructions. High-sensitivity ELISA kits were used to determine IL-6, IL-8, and TNF-α concentrations (the limits of detection were 0.10, 0.05, and 0.13 pg/ml, respectively).

2.8 Real-time PCR assay

Total mRNA was extracted from infected and non-infected HAECs using TRIzol reagent (Invitrogen Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop ND-2000 spectrophotometer (Life Technologies, USA). TLR2 mRNA in HAECs was determined by conducting quantitative SYBR[®] Green-based real-time quantitative reverse transcription PCR (real-time qRT-PCR). All treatments were performed in triplicate. β-Actin was used as an internal control; two negative controls were also used in all RT-PCR experiments. The sequences of the primers used for real-time PCR were as follows: TLR2 sense, 5'-GGCCAGCAAATTACCTGTGTG-3'; TLR2 antisense, 5'-AGGCGGACATCCTGAACC-3'; TNF-α sense, 5'-TTCTCCTTCCTGATCGTGGC-3'; TNF-α antisense, 5'-TTCTCCTTCCTGATCGTGGC-3'; IL-8 sense, 5'-CTGGCCGTGGCTCTCTTG-3'; IL-8 antisense, 5'-CCTTGGCAAACTGCACCTT-3'; IL-6 sense, 5'-CCACTCACCTCTTCAGAACG-3'; IL-6 antisense, 5'-CATCTTTGGAAGGTTTCAGGTG-3'; β-actin sense, 5'-GCTCTGGCTCCTAGCACCAT-3'; β-actin antisense, 5'-GCCACCGATCCACACAGAGT-3'.

2.9 Western blotting

Compared with control β-actin, the amount of TLR2 protein in HAECs was detected both by antibodies against TLR2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and by Western blotting using enhanced chemiluminescence as specified by the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA).

2.10 Immunohistochemistry

HAECs infected with *U. urealyticum* or LAMPs were mounted on glass slides for 24 h. The cells were routinely processed and stained overnight with an appropriate concentration of a primary monoclonal antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody.

2.11 Statistical analysis

Statistical comparisons were made using the *t*-test. The results are reported as mean±standard deviation (SD). *P*-values of <0.05 were considered statistically significant. All statistical analyses were performed with SPSS 20.0 software (IBM, USA).

3 Results

3.1 TLR2 mRNA expression in HAECs

TLR2 mRNA expression in HAECs varied depending on the concentration of LAMPs (1.0–7.0 µg/ml). Expression reached a maximum at a concentration of 4.0 µg/ml, and then remained largely unchanged at higher concentrations (Fig. 1).

To determine whether TLR2 mRNA expression in HAECs stimulated with *U. urealyticum* (1×10^8 CCU/ml) or LAMPs (4.0 µg/ml) varied in relation to incubation time, HAECs were collected after incubation for 2, 6, or 24 h and tested by the following methods. Compared with the control group, expression of TLR2 mRNA was higher in HAECs infected with *U. urealyticum* or LAMPs for 6 or 24 h ($P < 0.01$). After 2 h, TLR2 mRNA expression was higher in HAECs incubated with *U. urealyticum* than in the control or in HAECs incubated with LAMPs ($P < 0.05$; Fig. 2).

3.2 TLR2 expression in HAECs induced by *U. urealyticum* or LAMPs

Immunohistochemical analysis showed that expression of TLR2 occurred after stimulation with *U. urealyticum* or LAMPs, and was repressed following

Fig. 1 TLR2 mRNA expression in HAECs stimulated with different concentrations of LAMPs

treatment with TLR2 inhibitor, suggesting that expression of TLR2 was associated with stimulation by *U. urealyticum* or LAMPs (Fig. 3).

Compared to the control, TLR2 expression was significantly higher in HAECs after infection with *U. urealyticum* or treatment with LAMPs for 24 h ($P < 0.01$). TLR2 expression in HAECs was inhibited by TLR2 inhibitor in the *U. urealyticum* group ($P < 0.05$) and the LAMP group ($P < 0.01$) (Fig. 4).

3.3 Cytokine production induced by *U. urealyticum* and LAMPs in HAECs

As HAECs are commonly used as models for the human amnion, we initially tested the ability of *U.*

Fig. 2 TLR2 mRNA expression after different incubation time, measured by qPCR

Data are expressed as mean±SD ($n=6$). * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$ vs. LAMPs

Fig. 3 Immunohistochemical staining results of TLR2 in HAECs

(a) Negative control groups (as infection control). (b) Negative control groups (as inhibitor control). (c) TLR2 expression after *U. urealyticum* infection for 24 h (arrow). (d) TLR2 expression after LAMP treatment for 24 h (arrow). (e) TLR2 expression after *U. urealyticum* infection for 24 h (pretreatment with TLR2 inhibitor, arrow). (f) TLR2 expression after LAMP infection for 24 h (pretreatment with TLR2 inhibitor, arrow)

urealyticum and LAMPs to stimulate the production of various cytokines in HAECs. HAECs were incubated with *U. urealyticum* (1×10^8 CCU/ml) or LAMPs (4.0 $\mu\text{g/ml}$) for up to 24 h. Compared with the control group, HAECs produced significantly more cytokines (IL-6, IL-8, TNF- α) after stimulation with *U. urealyticum* or LAMPs for 24 h ($P < 0.01$; Fig. 5).

3.4 *U. urealyticum*- or LAMP-mediated production of cytokines inhibited by TLR2 inhibitor

HAECs were treated with TLR2 inhibitor for 2 h before incubation with *U. urealyticum* or LAMPs,

Fig. 4 HAECs expressing TLR2 after treatment with inhibitor, LAMPs or *U. urealyticum*

Data are expressed as mean $\pm$ SD ($n=6$). ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. *U. urealyticum*

Fig. 5 Mean productions of IL-6, IL-8, and TNF- α after 24 h incubation with *U. urealyticum*- or LAMP-induced HAECs

Data are expressed as mean $\pm$ SD ($n=6$). ** $P < 0.01$ vs. control

and cytokines in the supernatants were measured after incubation for 24 h (Fig. 6). The control group of HAECs was treated with PBS or TLR2 inhibitor. The results showed that after treatment with TLR2 inhibitor, cytokine production by HAECs infected with *U. urealyticum* or treated with LAMPs was significantly lower than that by HAECs treated with PBS ($P < 0.01$).

4 Discussion

To our knowledge, this is the first report of *U. urealyticum*-derived LAMPs stimulating HAECs to express TLR2. A growing body of evidence suggests that the presence of *U. urealyticum* in the amniotic cavity correlates with an adverse effect on pregnancy outcomes and complications of preterm birth. Previous studies (Abele-Horn et al., 2000; Kwak et al., 2014) have demonstrated that the incidence of preterm delivery, low Apgar scores, neonatal intensive care unit (NICU) admission, and histologic chorioamnionitis are directly correlated with the level of *U. urealyticum* colonization. High levels of cytokines in the amniotic fluid may serve as an indicator of bacterial infection and can predict subsequent pregnancy loss (Daskalakis et al., 2009). A shift in signaling from anti-inflammatory to pro-inflammatory pathways plays an important role in adverse outcomes of preterm birth (Wenstrom et al., 1996).

In this study, *U. urealyticum*-derived LAMPs and *U. urealyticum* were capable of inducing secretion of pro-inflammatory cytokines IL-6, IL-8, and TNF- α after the host cells were treated for 24 h. These results are similar to those of Wang et al. (2016) who found that *Mycoplasma bovis*-derived LAMPs can activate IL-1 β production via TLR2. He et al. (2009) reported that *Mycoplasma genitalium*-derived LAMPs can activate the immune system of the cell. This study built on previous research which determined that *U. urealyticum* or *Ureaplasma* spp. trigger the secretion of pro-inflammatory cytokines (Triantafyllou et al., 2013). Since LAMPs are the major surface proteins of *U. urealyticum*, it seemed likely that they may play a role in the induction of cytokine expression.

We confirmed that *U. urealyticum*-derived LAMPs can induce cytokine expression in HAECs and that TLR2 was involved in triggering pro-inflammatory responses in response to them. There is

Fig. 6 Cytokine production in the supernatants after incubation for 24 h by HAECs treated with TLR2 inhibitor for 2 h before incubation with *U. urealyticum* or LAMPs

(a) Mean production of IL-6. (b) Mean production of IL-8. (c) Mean production of TNF- α . Data are expressed as mean $\pm$ SD ($n=6$). ** $P<0.01$ vs. HAECs treated with PBS

accumulating evidence (Aalaei-Andabili and Rezaei, 2013) that intrauterine infection-induced preterm labor is the result of an inflammatory cascade initiated by bacterial interaction with host PRRs such as the

TLRs 2, 6, and 9. Following engagement with PRRs, there is a sequential rise in inflammatory cytokines such as IL-1 β , TNF- α , IL-6, and IL-8 followed by leukocyte recruitment, and increases in prostaglandins such as prostaglandin E2 (PGE2) and prostaglandin F2 α (PGF2 α) and matrix metalloproteinases. It is increasingly reported that non-immune cells, including HAECs, can regulate the secretion of inflammatory cytokines via the TLR signaling pathway (Triantafyllou et al., 2013). Our experiments confirmed that TLR2 is involved in activation of the inflammatory cascade. While LAMPs did not induce cytokine expression as strongly as whole cell *U. urealyticum*, which stimulated HAECs to express TLR2 within 2 h, they activated TLR2 after 6 h (Fig. 2). TLR2 has been identified as a receptor that is important to the innate immune response and transmits signaling pathways via IL-1 receptor (IL-1R)-associated signal molecules, such as myeloid differentiation factor 88 (MyD88), mitogen-activated protein kinases (Sweet and Schorey, 2006; Lyu et al., 2017). Further research is needed to confirm which intracellular pathway is involved in this interaction.

Inhibition of the interaction between TLRs and lipoproteins may result in a reduction of the inflammatory response induced by LAMPs. After HAECs were treated with TLR2 inhibitor, cytokine production was significantly reduced in HAECs treated with either LAMPs or *U. urealyticum* (Fig. 2). Pre-treatment with inhibitors offers promise as a protective means to lower the activity of cytokines. Glushkova et al. (2013) found that inhibitors of TLR4 signaling reduced the toxic effect of lipopolysaccharide. The results of our study might help in the search for new TLR-based therapeutic targets for LAMPs and *U. urealyticum*-induced chorioamnionitis.

Our data showed that TLR2 mRNA expression continued to rise to some extent as the concentration of LAMPs increased. The fact that LAMPs and *U. urealyticum* engage TLR2 might reveal the reasons why *U. urealyticum* can cause the development of chronic, low-level inflammation of amniotic epithelial cells leading to irreversible injury of fetal membranes. Activation via TLR2 results in a soft inflammatory response allowing the LAMPs to establish a chronic foothold in amniotic epithelial cells.

Taken together, our data indicate that *U. urealyticum*-derived LAMPs have potent inflammatory properties and can mediate changes in gene

expression in HAECs. Our studies suggest that using the inhibitor anti-hTLR2-IgA to inhibit the signaling pathway can result in a reduction in the downstream expression of several pro-inflammatory cytokines. Our data also sustain the notion that *U. urealyticum* may have additional mechanisms for inducing inflammatory responses in HAECs beyond those mediated by LAMPs.

There were several limitations inherent in this study. First, we detected only three kinds of cytokines; more chemokines should be investigated in further studies. Second, although we confirmed that cytokine expression was reduced after blocking TLR2, other TLRs may play a similar role and should be investigated.

5 Conclusions

This is the first investigation into the effects of *U. urealyticum*-derived LAMPs on the expression of cytokines IL-6, IL-8, and TNF- α in HAECs via TLR2.

Contributors

Guang-yong YE and Ke-yi WANG participated in the design. Guang-yong YE wrote this article. Guang-yong YE, Ke-yi WANG, and Qiao-di GUI collected and screened references. Min WANG checked and approved the final version.

Compliance with ethics guidelines

Guang-yong YE, Ke-yi WANG, Qiao-di GUI, and Min WANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要

题目: 解脲脲原体脂质相关膜蛋白经 Toll 样受体 2 信号通路调控人羊膜上皮细胞诱导 IL-6、IL-8 和 TNF- α 的产生

目的: 探讨解脲脲原体 (*Ureaplasma urealyticum*) 及其脂质相关膜蛋白 (LAMPs) 作用于人羊膜上皮细胞 (HAECs) 过程中白介素 6 (IL-6)、IL-8 和肿瘤坏死因子 α (TNF- α) 的变化情况, 阐明 Toll 样受体 2 (TLR2) 的调控机制, 明确解脲脲原体潜在的致病性。

创新点: 从解脲脲原体诱导炎症反应的分子机制入手, 提出 TLR2 信号通路在其中的关键作用。

方法: 经 TX-114 处理萃取解脲脲原体获得 LAMPs, 将 LAMPs 和解脲脲原体分别感染 HAECs, 用酶联免疫吸附试验 (ELISA) 测定炎症细胞因子 (IL-6、IL-8 和 TNF- α); 采用实时聚合酶链反应 (real-time PCR) 测定 TLR2 mRNA 水平, 用蛋白质印迹 (Western blot) 检测 TLR2 的表达量; 经 TLR2 阻断剂 (anti-hTLR2-IgA) 处理后, 测定相应炎症细胞因子。

结论: 解脲脲原体 LAMPs 能诱导 HAECs 的 TLR2 表达上调和炎症因子增加, 从而发生炎症反应; TLR2 受阻断后, 炎症因子表达减少, 炎症水平下降。TLR2 在解脲脲原体 LAMPs 感染 HAECs 过程起关键作用。

关键词: 解脲脲原体; 脂质相关膜蛋白; 人羊膜上皮细胞; Toll 样受体 2