Article

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Impact of Toll-like Receptor 4 Expression on Inflammatory Responses Related to Premature Membrane Rupture Induced by Lipopolysaccharide

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Objective: This study aimed to determine the mechanism through which the expression of Toll-like receptor 4 (TLR4) influences the lipopolysaccharide (LPS)-induced inflammatory response, a condition that is associated with premature rupture of membranes (PROM).

Methods: Human myeloid leukemia mononuclear cells (THP-1) were employed as the experimental model. These cells were treated with LPS and the TLR4 inhibitor CLI-095 and subsequently divided into three groups. A range of assays were utilized, including methyl thiazolyl tetrazole (MTT) assay, real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) for measuring TLR4 and tumor necrosis factor α (TNF-α) mRNA levels, double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for assessing monocyte chemoattractant protein 1 (MCP-1) and matrix metalloproteinase 9 (MMP-9), as well as secretion levels of interleukin (IL)-6 and IL-1β. And western blotting was used to detect the expression of extracellular signal-regulated kinase (ERK) and nuclear factor κB (NF-κB) p65, which are components of the TLR4 downstream signaling pathway.

Results: The LPS-induced proliferation of THP-1 cells was significantly inhibited ($p < 0.05$) when compared with normal THP-1 cells. Moreover, LPS also promoted TLR4 mRNA and protein expression levels, TNF-α mRNA expression, secretion of inflammatory factors, and phosphorylation of ERK and NF-κB p65 proteins ($p < 0.05$). On the other hand, administration of the TLR4 inhibitor CLI-095 significantly inhibited the expression of TLR4 mRNA and protein. It also effectively increased the proliferative activity of THP-1 cells and inhibited the secretion of TNF-α and inflammatory factors, as well as the phosphorylation of ERK and NF-κB p65 proteins ($p < 0.05$).

Conclusions: In summary, suppressing TLR4 expression can mitigate inflammatory responses, thereby reducing the likelihood of premature rupture of membranes during pregnancy, which is often triggered by such inflammation.

Keywords: toll-like receptor; lipopolysaccharide; inflammation; TNF-α; downstream signal molecule

Introduction

Premature rupture of membranes (PROM) is a significant risk factor for adverse maternal and infant outcomes, including fetal and neonatal morbidity or death [1]. If PROM occurs during delivery, the overall mortality rate for newborns can be high as approximately 12%, and the likelihood of developing acute respiratory distress syndrome is about 15% [2]. Research identifies reproductive tract infections as the primary cause of PROM [3,4]. The occurrence of PROM eradicates the natural protective barrier for both mother and infant, potentially leading to the development of complex infectious diseases. Chorioamnionitis, in particular, is a common complication following PROM [5].

The etiology of PROM is multifaceted. It's understood that membrane rupture can be due to a combination of normal physiological weakening and the shear force produced by uterine contractions. However, a host of other factors also contribute to PROM, such as reproductive tract infections, multiple pregnancies, exposure to stress, and genetic predisposition [6,7]. Toll-like receptor (TLR) forms a key part of the body's defense mechanism, playing a critical role in the inflammatory response triggered by cell stress, tissue damage, and infection [8]. Toll-like receptor 4 (TLR4), the inaugural member of the TLR family, is primarily found in placental and lung tissues, and tightly associated with preterm labor and PROM [9,10]. The ability of TLR4 to trigger cytokines synthesis paves the way for new approaches in studying the pathogenesis of PROM and the diagnosis and treatment of chorioamnionitis. Specific markers like tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-1β are sensitive predictors of intrauterine infection. Specifically, IL-6 has been validated as a diagnostic indicator for acute chorioamnionitis. Moreover, the application of CLI-095 to inhibit TLR4 expression can markedly suppress the phosphorylation of nuclear factor

κB (NF-κB) p65 and extracellular signal-regulated kinase (ERK). CLI-095 can lead to significant inhibition of TLR4 and its downstream signaling pathways, thereby playing a crucial role in mitigating the cellular inflammatory reaction (CIR).

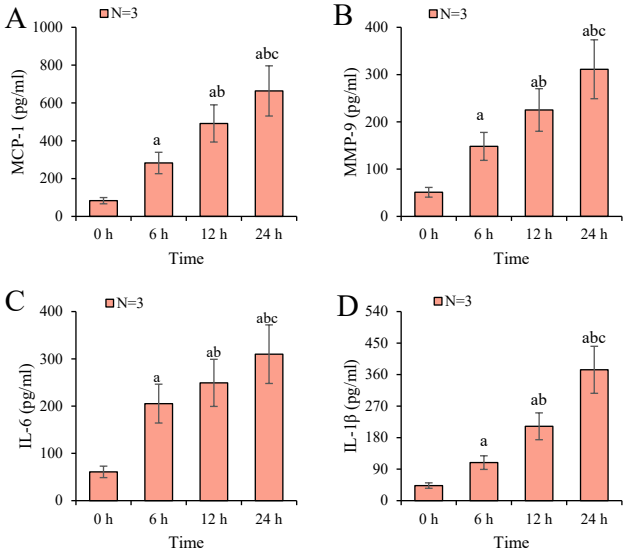

Fig. 1. Comparison of the level of inflammatory mediators secreted by human myeloid leukemia mononuclear cells (THP-1) cells after LPS induction. (A) The difference in MCP-1 levels. (B) The difference in MMP-9 levels. (C) The differences in IL-6 levels. (D) The difference in IL-1β levels. The labels a, b, and c denote a significant variation compared to the IL-1β levels measured at 0 hours, 6 hours, and 12 hours, respectively ($p < 0.05$).

In recent years, investigating the role of inflammatory factors in the onset of PROM has emerged as an important area of research. Although the connection between TLR4 and cellular inflammation has been extensively examined, its relationship with the specific inflammatory factors implicated in PROM has yet to be thoroughly explored. We chose THP-1 cells as our experimental model to address this research gap. We used lipopolysaccharide (LPS) as an inducer of CIR and applied a TLR4 inhibitor to uncover the specific mechanisms underpinning CIR. This study's objective is to explore the role of TLR4 in CIR, thereby providing experimental data that could inform clinical strategies for preventing and treating PROM and chorioamnionitis.

Materials and Methods

Materials

THP-1 cells (from Chinese Academy of Science, Shanghai, China) were cultured in RPMI (SH30809.01B, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (16000-044, Invitrogen, Carlsbad, CA, USA) at 37 °C. LPS, Fetal bovine serum (FBS), phosphate-buffered brine (PBS), polyvinylidene fluoride (PVDF), were purchased from Thermo Fisher Scientific, Waltham, USA. The TRIzol reagent, complementary deoxyribonucleic acid (cDNA) first-strand synthesis kit, and real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) detection kit were purchased from TaKaRa, Beijing, China; The cell protein extraction kit and bicinchoninic acid (BCA) protein quantitative detection kit was supplied by KGI Biotech, Jiangsu, China; The methyl thiazolyl tetrazolium (MTT) kit, TLR4, ERK and nuclear factor κB (NF-κB) p65, and β-actin antibodies were ordered from Abcam Trading Co., Ltd., Shanghai, China. CLI-095 and horseradish peroxidase-labeled IgG antibodies were purchased from Millipore, Billerica, MA, USA; An enhanced chemiluminescence (ECL) kit was purchased from Thermo Fisher Scientific, Waltham, USA; And enzyme-linked immunosorbent assay (ELISA) kits for monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinase 9 (MMP-9), secretion levels of IL-6 and IL-1β were ordered from (Proteintech, Wuhan, China).

THP-1 Cell Culture

THP-1 cells, which are round and transparent, were maintained in suspension. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at a constant temperature of 37 °C under a 5% CO_2 atmosphere. The medium was refreshed every 3 to 4 days, and the cells were passaged every 4 to 5 days at a passage ratio of 1:2. In this study, we ensured the THP-1 cells were free of mycoplasma contamination, and their identity was confirmed via short tandem repeat (STR) profiling.

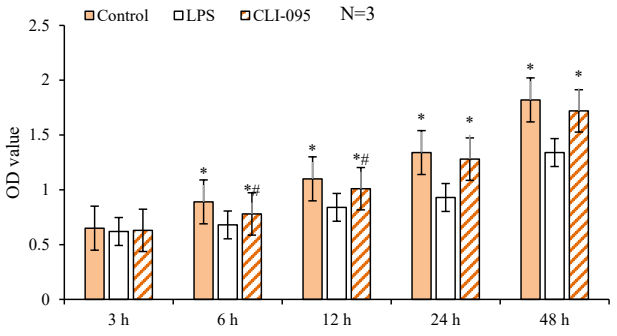

Fig. 2. Detection of TLR4 expression on the proliferation activity of LPS-induced THP-1 cells. Note: "*" represents significant statistical differences ($p < 0.05$) compared to LPS, while "#" represents significant statistical differences ($p < 0.05$) compared to the control group.

LPS-Induced Inflammation Test of THP-1 Cells

In this study, THP-1 cells were divided into 3 groups for comparison: A normal cell group (control group, re-

ferred to as 'controls'), a group induced with LPS (LPS group), and a group treated with LPS in combination with CLI-095 (CLI-095 group). THP-1 cells in the LPS and CLI-095 groups were incubated with LPS at a consistent concentration of 1 μg/mL. Meanwhile, the control group was treated only with an equivalent volume of PBS solution. In the CLI-095 group, cells were additionally administered 1 μg/mL CLI-095, following the LPS treatment. Twelve hours post-induction, cell pellets or supernatants from the THP-1 cultures were collected for subsequent functional assays. Each experimental group was replicated three times for robustness.

Cell Proliferation Test

The concentration of THP-1 cells was set to $1 \times 10^4 \cdot mL^{-1}$, and a 100 μL aliquot of the cell suspension was dispensed into each well of a 96-well plate. The cells were then cultured for 12 hours in serum-free RPMI 1640 medium. Following this, 50 $mg \cdot L^{-1}$ CLI-095 was added to the cells and incubated for another 12 hours. Subsequently, 1 μg/mL LPS was added, and the cells were incubated for 12 hours. The medium was then centrifuged using a centrifuge (Avanti JXN-26 Beckman Coulter International Trade, Shanghai, China) for 10 minutes at 3000 rpm. After centrifugation, 200 μL of MTT reagent was added to the cells, then placed under a microplate reader (SpectraMax iD5 Meigu Molecular Instrument, Shanghai, China) to measure the absorbance at 490 nm. The optical density readings were used to assess cell proliferation.

RT–qPCR Test

Post-treatment, the collected THP-1 cell pellets were subjected to lysis by adding 1 mL of pre-cooled TRIzol reagent. Subsequently, total RNA was isolated, and the cDNA template was synthesized as per the instructions provided with the kit. Then, RT-qPCR kit instructions were followed to detect the target gene *TLR4*, *TNF-*α, and *β-actin* messenger ribonucleic acid (mRNA) levels. The quantitative primer sequence was TLP4: (F) 5′-GTACCTGGGGAACAACCTCTT-3′, (R) 5′-GCAGCTTGACTAGACTCTCCA-3′, and the amplified fragment was 146 bp; TNF-α: (F) 5′-CCCAGGCAGTCAGATCATCTTCT-3′, (R) 5′-ATGAGGTACAGGCCCTCTGAT-3′, and the amplified fragment was 179 bp; β-actin: (F) 5′-: CACGAAACTACCTTCAACTCC-3′, (R) 5′-CATACTCCTCC'ITrGCTGATC-3′, and the amplified fragment was 266 bp. *β-actin* was used as the internal reference gene, and the target gene *TLR4* and *TNF-*α expression levels were detected according to the formula $2^{-\Delta\Delta Ct}$.

Double Antibody Sandwich ELISA Test

Supernatants from treated THP-1 cells in each group were collected and mixed with 100 μL samples and varying concentrations of standard substances in the reaction wells, which were then sealed and then incubated at 37 °C for 30 minutes. Subsequently, 350 μL of wash solution was injected into each well, with a 20 seconds interval set between injection and washing. Next, 100 μL of biotinylated antibody was added to each well. The wells were sealed and incubated at 37 °C for one hour. After four washes, 100 μL of enzyme conjugate working solution was added to each well. The wells were sealed again and incubated at 37 °C for 30 minutes. Following four more washes, 100 μL of the color-developing solution was added to each well and incubated at 37 °C in the dark for 15 minutes. Following this, 100 μL of stop solution was added to halt the reaction. After mixing, the absorbance was measured at 450 nm to detect the levels of inflammatory markers MCP-1, MMP-9, IL-6, and IL-1β.

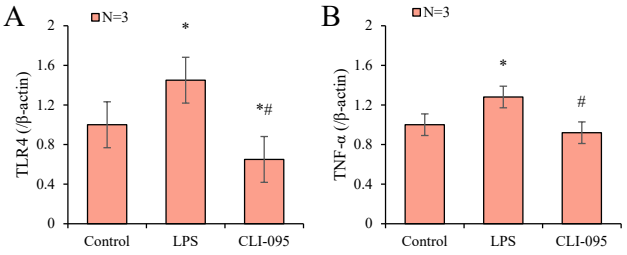

Fig. 3. Comparison of differences in the levels of TLR4 and TNF-α mRNA. (A) The difference in TLR4 mRNA. (B) The difference in TNF-α mRNA. * and # suggest $p < 0.05$ compared to the controls and the LPS group.

Western Blot Test

Post-treatment, the THP-1 cells were lysed with RIPA buffer to extract the total protein. The protein concentration was then quantified using a BCA assay kit. After the sample protein was taken and reacted with buffer, it was denatured at 94 °C for 5 minutes, and then a 12% SDS-PAGE separation gel was prepared for protein separation. Following electrophoresis, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. This membrane was then incubated in TBST (prepared fresh) with 5% non-fat milk for an hour to block non-specific binding sites. After the addition of rabbit anti-human TLR4 (1:1000), ERK (1:1000), p-ERK (1:1000), NF-κB p65 (1:1000), p-NF-κB p65 (1:1000), and mouse anti-human β-actin (1:2500) primary antibodies, the cells were incubated at 4 °C overnight. After the membrane was rinsed with TBST, horseradish peroxidase-labeled goat anti-rabbit IgG (1:2000) was added for culturing at 37 °C for one hour. After the membrane was rinsed with TBST, the ECL color development kit was adopted to develop the color of the protein band, and the quantitative analysis of the gray value of the target band was applied in the gel imager.

Statistical Methods

SPSS 26.0 (IBM-SPSS Statistics, Armonk, NY, USA) was adopted to process the data statistically, and all experimental data were given as the mean ± SD. The index data between each treatment group were statistically analyzed by one-way ANOVA, and $p < 0.05$ indicated that the difference was statistically significant.

Results

Changes in Inflammatory Substances Produced by LPS-Induced THP-1 Cells

The alterations in the secretion of MCP-1, MMP-9, IL-6, and IL-1β by THP-1 cells after being stimulated by LPS for 0, 6, 12, and 24 hours were observed. As shown in Fig. 1, there was a gradual increase in the levels of MCP-1, MMP-9, IL-6, and IL-1β secreted by THP-1 cells as the induction period lengthened. Statistically significant differences were noted in the secretion of MCP-1, MMP-9, IL-6, and IL-1β by THP-1 cells across varied induction durations ($p < 0.05$).

Effect of TLR4 Expression on THP-1 Cell Proliferation Following LPS Induction

THP-1 cell proliferation activity changes after LPS induction and TLR4 inhibitor CLI-095 treatment were compared and analyzed. As the treatment time increased, the proliferation activity of THP-1 cells in each group gradually increased. Except for 3 hours after treatment, the cell proliferation activity of the LPS group was always observably lower than that in the other two groups ($p < 0.05$). After 6 and 12 hours of treatment, the cell proliferation activity of the CLI-095 group was decreased to that of the controls ($p < 0.05$). Still, no visible differences were found between them after 24 and 48 hours ($p > 0.05$). The data are illustrated in Fig. 2.

Effect of LPS on TLR4 and TNF-α mRNA Expressions

In THP-1 cells, TLR4 and TNF-α mRNA expression differences were observed following LPS induction and subsequent treatment with the TLR4 inhibitor CLI-095, as detailed in Fig. 3. TLR4 and TNF-α mRNA expressions significantly increased post-LPS induction ($p < 0.05$). In comparison to the group with only LPS induction, TLR4 inhibitor CLI-095 treatment resulted in a significant reduction in TLR4 and TNF-α mRNA expressions ($p < 0.05$). Furthermore, relative to controls, TLR4 mRNA expression was significantly lowered in THP-1 cells treated with the TLR4 inhibitor CLI-095 ($p < 0.05$), but there was no significant change observed in TNF-α mRNA ($p > 0.05$).

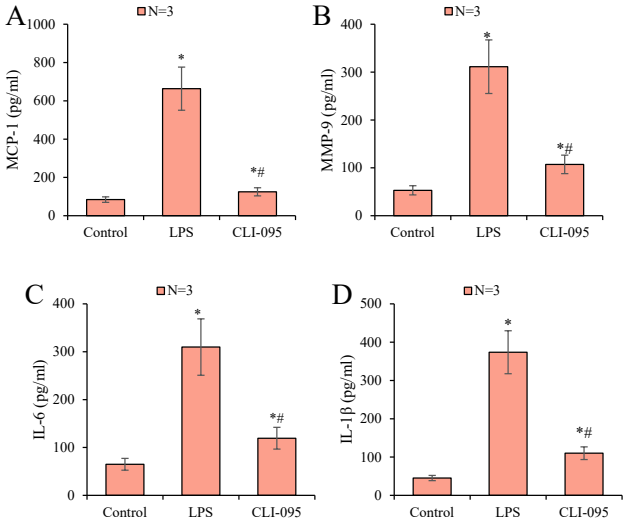

Fig. 4. Comparison of TLR4 expression on the level of inflammatory mediators. (A) The difference in MCP-1 levels. (B) The difference in MMP-9 levels. (C) The differences in IL-6 levels. (D) The difference in IL-1β levels. * and # suggest that the difference was statistically significant compared to the controls and LPS groups ($p < 0.05$).

Effect on TLR4 Expression of the Secretion of Inflammatory Substances

We assessed the differences in the secretion of MCP-1, MMP-9, IL-6, and IL-1β by THP-1 cells after their exposure to LPS induction and treatment with the TLR4 inhibitor CLI-095. The results are presented in Fig. 4. We observed that the levels of MCP-1, MMP-9, IL-6, and IL-1β secreted by THP-1 cells increased significantly after LPS induction ($p < 0.05$). Nonetheless, a significant reduction ($p < 0.05$) in the secretion of these molecules was observed when the cells underwent LPS induction in the presence of the TLR4 inhibitor CLI-095. Notably, after treatment with CLI-095, the MCP-1, MMP-9, IL-6, and IL-1β were significantly higher than those observed in untreated control cells ($p < 0.05$).

Effect of TLR4 Expression on the Signaling Pathway of THP-1 Cells after LPS Induction

Fig. 5 shows the detection results of LPS induction and TLR4 inhibitor CLI-095 treatment of THP-1 cell TLR4 and downstream ERK and NF-κB protein expression. ERK, NF-κB, and β-actin in THP-1 cells showed no observable difference among the three groups. In the LPS group, TLR4 protein levels and the phosphorylation of p-ERK and p-NF-κB, p65 in THP-1 cells significantly increased compared to the controls ($p < 0.05$). Yet, compared to the CLI-095 group, these measures showed no significant difference ($p > 0.05$). TLR4 protein and p-ERK and p-NF-κB p65 phosphorylation in THP-1 cells in the CLI-095 group were sharply reduced compared to those in the LPS group ($p < 0.05$).

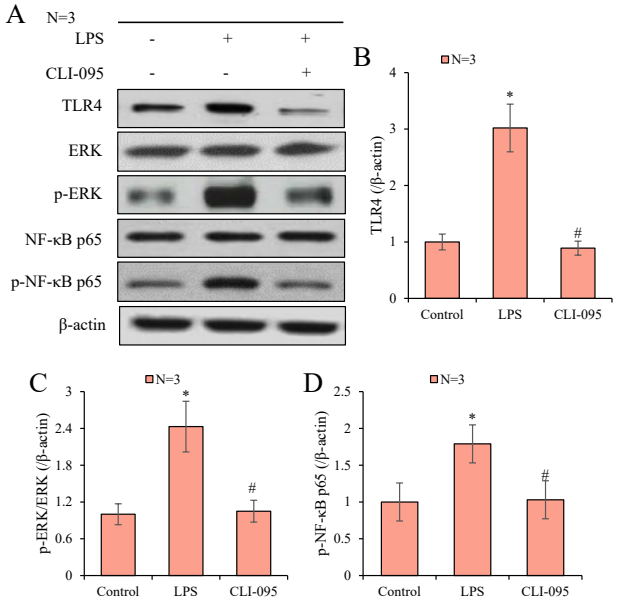

Fig. 5. Effect of TLR4 expression on the signaling pathway of THP-1 cells after induction with LPS. (A) Western blot test results. (B) The difference in TLR4 protein. (C) Differences in p-ERK/ERK. (D) The difference in phosphorylated p-NF-κB p65. Note: "*": significant differences were observed compared to the control group with statistical significance ($p < 0.05$); "#": significant differences were observed compared to the LPS group with statistical significance ($p < 0.05$).

Discussion

PROM which may occur at all stages of pregnancy poses significant risks to both mother and child, contributing to fetal and neonatal morbidity and mortality. Notably, the incidence of PROM in full-term pregnancies is alarmingly high, reaching up to 10%. Furthermore, when PROM occurs during delivery, neonatal mortality, and acute respiratory distress syndrome risk escalate considerably [11]. Existing research suggests a close link between PROM and infection-induced inflammation [12]. Importantly, the duration of PROM globally correlates with an increased risk of adverse complications for both mother and baby [13,14]. Consequently, current research focuses on predicting PROM with chorioamnionitis and devising early therapeutic interventions to optimize prenatal and postnatal outcomes [15]. Therefore, it is critical to understand the underlying mechanisms of the inflammatory reaction associated with PROM.

Firstly, we utilized the LPS-induced CIR of THP-1 cells to mimic the cell model of PROM in combination with chorioamnionitis. We then examined the potential inhibitory effect of TLR4 expression on this response. TLR4 is a type I transmembrane glycoprotein primarily found in damaged tissues and inflammatory byproducts. It recognizes and responds swiftly to microorganisms, activating the human immune response [16]. TLR4 is highly expressed in the uterus, placenta, and amniotic membrane at the maternal-fetal interface, and its expression is closely associated with the onset of inflammation [17,18]. Studies have shown that LPS can stimulate and activate the MAPK/NF-κB signaling pathway, regulating the phosphorylation of the ERK/MEK pathway [19,20]. After LPS recognition, TLR4 can activate NF-κB and MAPK, thereby regulating gene transcription and participating in the pathological processes of diseases such as inflammation and tumors [21,22]. Our results showed that the phosphorylation of TLR4 and TNF-α mRNA, TLR4 protein and p-ERK, and p-NF-κB p65 was significantly elevated compared to the control group ($p < 0.05$). We also found that following LPS induction, the levels of secreted MCP-1, MMP-9, IL-6, and IL-1β in THP-1 cells, as well as the mRNA expression levels of TNF-α and TLR4, were significantly increased. These results indicated that LPS-induced THP-1 cells could significantly induce CIR, effectively stimulating the inflammatory response process of PROM combined with Chorioamnionitis [23]. Among the cytokines and chemokines examined, MCP-1 is a soluble basic protein that regulates the systemic transport of immune cells during inflammation and plays a crucial role in maintaining pregnancy status and immune regulation [24,25]. Other studies have shown that TNF-α and MCP-1 levels in patients significantly increased with Chorioamnionitis [26]. MMP-9, a member of the zinc ion-dependent protease family, promotes the rapid disintegration of the extracellular matrix of type IV collagen in the amniotic basement membrane, inducing apoptosis [27]. The levels of MMP-9 in amniotic fluid significantly increase following childbirth, membrane rupture, and amniotic fluid cavity infection [28]. Finally, TNF-α, IL-6, and IL-1β are sensitive indicators for predicting intrauterine infection, with IL-6 recognized explicitly as the diagnostic indicator for acute chorioamnionitis [29,30].

To further explore the interaction between TLR4 expression and CIR, the TLR4 inhibitor CLI-095 was administered to LPS-stimulated THP-1 cells. The expression levels of TLR4 and TNF-α mRNA, MCP-1, MMP-9, IL-6, and IL-1β, TLR4 protein p-ERK, and p-NF-κB p65 phosphorylation in THP-1 cells in the CLI-095 group were significantly lower than those in the LPS group ($p < 0.05$). It has been reported that limiting TLR4 can effectively inhibit the inflammatory response of LPS-stimulated THP-1 cells [31]. Inhibition of TLR4 expression using CLI-095 resulted in significant suppression of NF-κB p65 and ERK phosphorylation levels. These results showed that CLI-095 significantly inhibited TLR4 and its downstream signaling pathway, thus significantly inhibiting CIR. Our findings confirm that suppressing TLR4 expression can mitigate the inflammatory response, potentially preventing PROM, often triggered by inflammation during pregnancy. Nonetheless, the absence of corroborating clinical evidence suggests that further investigation into this topic is necessary.

Conclusions

Our study aimed to produce experimental findings that could assist in the early diagnosis of PROM and chorioamnionitis, with the overarching goal of enhancing maternal and neonatal health outcomes. The study results showed that the LPS induction in THP-1 cells triggered a significant CIR, providing a viable simulation of the inflammatory process linked to PROM and chorioamnionitis. It is important to note, however, that the focus of this study was specifically on examining the pro-inflammatory effects of TLR4 in monocytes. Hence, additional clinical data are required to validate further the relationship between TLR4 expression and inflammation in PROM and chorioamnionitis.

Availability of Data and Materials

Data sharing are not applicable.

Author Contributions

Conceptualization: JZ; Data Curation: FW; Formal Analysis: JZ, FW, ZL; Funding Acquisition: JZ; Investigation: YW, LQ; Methodology: JZ, GZ, HL; Project Administration: JZ; Resources: JZ; Software: JZ, FW; Supervision: JZ, FW, ZL; Validation: JZ; Visualization: JZ; Writing – Original Draft Preparation: JZ; Writing – Review & Editing: JZ, FW, ZL, YW, LQ, GZ, HL. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The patients and their accompanying patients volunteered to participate in this study and signed the informed consent forms. The research had been approved by the relevant medical ethics committee (batch number: 201915073).

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Conflict of Interest

The authors declare no conflict of interest.

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