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ORIGINAL RESEARCH

Role of Toll-like receptor 4 in inflammation-induced preterm delivery

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ABSTRACT: The aim of the present study was to investigate the potential role of Toll-like receptor 4 (TLR4) in lipopolysaccharide (LPS)-induced preterm delivery. Intraperitoneal injection of LPS in the presence or absence of previous TLR4 blockade was performed to establish a murine model of preterm delivery. The incidences of preterm delivery and fetal death were calculated. Flow cytometry was performed to examine the percentages of blood $CD45^+CD86^+$, $CD3^+CD69^+$, $CD19^+CD69^+$ and $CD49b^+CD69^+$ cell subsets, and the percentages of placenta $CD45^+CD86^+$, $CD45^+CD49b^+$ and $CD49b^+CD69^+$ cell subpopulations. In our study, an inflammation-induced preterm delivery model was established by intraperitoneal injection of LPS. Blocking TLR4 significantly decreased LPS-induced preterm delivery and fetal death. LPS treatment markedly up-regulated the percentages of blood $CD45^+CD86^+$, $CD3^+CD69^+$ and $CD49b^+CD69^+$ cells, and of placenta $CD45^+CD86^+$, $CD45^+CD49b^+$ and $CD49b^+CD69^+$ cells. TLR4 blockade almost completely abrogated LPS-induced elevated cell proportions. These data demonstrate that TLR4 plays a critical role in inflammation-induced preterm delivery.

Key words: TLR4 / lipopolysaccharide / inflammation / preterm delivery

Introduction

Preterm delivery, defined as delivery occurring before 37 weeks of gestation, occurs in 12% of all births, and accounts for nearly half of long-term neurological morbidity and 60–80% of perinatal mortality, excluding infants with congenital anomalies (Wang *et al*., 2008). Despite the advances that have been made in obstetrics and neonatology, the rate of preterm delivery has not decreased throughout the past 20–30 years. In fact, the National Center for Health Statistics (Centers for Disease Control and Prevention) has reported a 20% increase (from 10.6% to 12.7%) in the percentage of preterm deliveries during the years 1990–2005 (Wang *et al*., 2008).

Infection is one of the major environmental factors leading to preterm delivery, with at least 40% of preterm births thought to be attributable to intrauterine infection (Garcia-Verdugo *et al*., 2007). Although broad-spectrum antibiotic therapy increased the likelihood of prolongation of the latency period of preterm delivery and improved neonatal health by reducing the risk of respiratory distress syndrome, early sepsis, severe intra-ventricular hemorrhage and severe necrotizing enterocolitis, there is little benefit in terms of the reduction in rate of preterm delivery from only antimicrobial administration (Li *et al*., 2007). Despite a growing association between inflammation and preterm delivery, the underlying mechanisms explaining the development of preterm delivery after infection are still poorly understood.

The innate immune response is a universal mechanism of host defense against infection. It functions on the basis of special receptors called pattern-recognition receptors which recognize conserved microbial structures called pathogen-associated molecular patterns. Due to pattern-recognition receptors, the human organism is able to discriminate between self- and non-self-antigens. Toll-like receptors (TLRs) are a group of pattern-recognition receptors that play a crucial role in 'danger' recognition and the induction of immune response.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is thought to play a key role in eliciting an inflammatory response including the activation of the immune cells and the release of enzymes involved in remodeling of the extracellular matrix leading to preterm delivery (Rey *et al*., 2008). There are a number of *in vivo* animal studies demonstrated the role of bacterial infection in inducing preterm delivery through TLR4 stimulation (Elovitz *et al*., 2003; Wang and Hirsch, 2003). However, the TLR4 signal transduction pathways responsible for preterm delivery in the setting of inflammation remain incompletely defined. In an attempt to increase our understanding of the clinical problem of preterm delivery, we have created a murine model of inflammation-induced preterm delivery by intraperitoneal injection of LPS with or without

previous TLR4 blockade with a specific anti-TLR4 monoclonal antibody to investigate the potential role of TLR4 in the pathogenesis of inflammation-induced preterm delivery.

Materials and Methods

Animals

Inbred strains of 8-week-old female and male BALB/c mice were purchased from Experimental Animal Center of Zhongshan University (Guangzhou, China) and subsequently maintained under pathogen-free conditions in the Laboratory Animal Facility of Guangzhou Medical College Affiliated Guangzhou First Municipal People's Hospital (Guangzhou, China). Animals were acclimated in our facility for at least 2 weeks before use in these experiments. All animal procedures followed the guidelines of the Chinese Council for Animal Care. Animals were housed at an ambient humidity and temperature of 63% and 20°C and a 12L:12D photoperiod. Animals had free access to food and water. Each female mouse was co-caged with one male. The point at which a vaginal plug was detected was designated Day 0 of gestation.

LPS-induced preterm delivery model

As described previously (Lin *et al.*, 2006), on Day 15 of gestation, the pregnant mice were injected intraperitoneally with 200 μl of LPS (Sigma, Saint Louis, MO, USA) saline solution with care not to enter the amniotic cavity at a dosage of 50 μg/kg of body weight. Control animals were received an intraperitoneal injection of equal volume of sterile phosphate-buffered saline (PBS). Animals were observed closely for any signs of morbidity (piloerection, decreased movement, vaginal bleeding and preterm delivery). Mice that delivered prematurely (defined as the finding of at least one pup in the cage or the lower vagina before Day 19 of gestation) or delivered at term underwent autopsy when fetuses were found in the cage. Fetal death was identified by white discoloration, markedly smaller fetal size and lack of blood flow in the umbilical cord. Some mice not delivered prematurely were sacrificed on Day 19 of gestation to determine fetal status. The incidence of preterm delivery and fetal death were calculated.

Blocking TLR4 in advance

TLR4 was blocked by multiple injection of monoclonal antibody (BioLegend, San Diego, CA, USA) against this receptor. In order to block TLR4 adequately, the injections were performed intraperitoneally at a dosage of 6 μg in 200 μl of PBS on Days 7, 10 and 13 of gestation consecutively, which were comparable to the second and the third trimesters of human gestation. Afterwards, LPS administration was performed.

Isolation of blood mononuclear cells

Because of the small size of BALB/c mouse, the mouse's ventricle was punctured to harvest enough blood. The blood was mixed with 2 mM ethylene diamine tetraacetic acid and incubated with red cell lysis buffer (GENMED, Arlington, MA, USA) to eliminate red blood cell contaminant. After two wash steps with PBS, the cells were re-suspended and adjusted to 1×10^6 cells/ml.

Isolation of placenta mononuclear cells

The detailed description of the procedure used to isolate placenta mononuclear cells has been reported elsewhere (Lin *et al.*, 2009). In brief, the uterine horns of pregnant mice were opened longitudinally, and the feto-placental unit was separated from the uterine implantation sites. The whole placental and decidual unit was separated individually from the respective embryo and its implantation site. After washing in cold PBS, the pooled placentas were cut into small pieces (1 mm^3). The obtained tissue was digested three times by 1 mg/ml Dispase® II (Roche, Mannheim, Germany) at 37°C for 20 min of each cycle in a shaking water bath. When single or bunches of cells were formed under microscopy, the released cells were separated from undigested tissue pieces by filtering through a 50-μm pore-size nylon mesh. Mononuclear cells were purified with Ficoll-Hypaque density medium (density 1.077 ± 0.002 g/ml) by centrifugation at 800 g for 20 min at 22°C. Similarly, any contaminating red blood cells that might have persisted in the single-cell suspension were eliminated by incubation with red cell lysis buffer (GENMED).

Flow cytometry analysis

Every 10^6 blood cells in 50 μl PBS were incubated with PE/Cy5-conjugated CD45 0.25 μg, APC-conjugated CD49b 0.5 μg, PE/Cy7-conjugated CD69 0.25 μg, PE-conjugated CD86 0.25 μg, FITC-conjugated CD3 0.5 μg and APC/Cy7-conjugated CD19 0.5 μg for 30 min at 4°C. Every 10^6 placenta cells in 50 μl PBS were incubated with PE/Cy5-conjugated CD45 0.25 μg, APC-conjugated CD49b 0.5 μg, PE/Cy7-conjugated CD69 0.25 μg and PE-conjugated CD86 0.25 μg for 30 min at 4°C. After being washed twice with PBS, these cells were fixed in 10 g/l paraformaldehyde. Immunostained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton–Dickinson, San Jose, CA, USA). Ten thousand cells were detected in each sample. Staining with respective fluorescence-labeled isotype matched control antibodies was also induced for each condition to detect non-specific background staining. All of the fluorescence-labeled antibodies were purchased from BioLegend.

The percentage of blood $CD86^+$ cells in $CD45^+$ cell population was calculated as: percentage $CD86^+$ cells in $CD45^+$ cell population = $CD86^+CD45^+$ cell number/$CD45^+$ cell number × 100. The percentages of blood $CD69^+$ cells in $CD3^+$ cell population, $CD69^+$ cells in $CD19^+$ cell population and $CD69^+$ cells in $CD49b^+$ cell population were also calculated. In the same way, the percentages of placenta $CD86^+$ cells in $CD45^+$ cell population, $CD49b^+$ cells in $CD45^+$ cell population and $CD69^+$ cells in $CD49b^+$ cell population were calculated.

Statistical analysis

The rate of preterm delivery and fetal death were analyzed using χ^2 text. The cell percentages were determined by Student's *t*-test. Results were given as mean ± SD. $P < 0.05$ was considered as the significant difference between the analyzed groups.

Results

Comparison of preterm delivery rate

With our model of intraperitoneal infusion of LPS, we obtained a 50% (8/16) preterm delivery rate within 48 h, which was significantly higher than that of PBS control group whose preterm delivery rate was zero (0/16) ($P < 0.01$). All offspring delivered preterm were dead. We observed no maternal mortality in this model. Further, we investigated whether LPS induced preterm delivery via the activation of TLR4. Blocking TLR4 with anti-TLR4 monoclonal antibody before administrating the same amount of LPS as used before, we observed a 6.3% (1/16) preterm delivery rate in the anti-TLR4 group, which was significantly lower than that of LPS group ($P < 0.01$) (Table I).

Table I. Comparison of preterm delivery rate and fetal mortality.

Group	Stimulator	Blocking TLR4	N1	Preterm delivery rate	N2	Fetal mortality
A	PBS	−	16	0 (0/16)	16	3.1% (5/163)
B	LPS	−	16	50% (8/16)[a]	815	11.0% (9/82)[c]
C	LPS	+	16	6.3% (1/16)[b]		3.9% (6/154)[d]

[a]$P < 0.01$ versus Group A, [b]$P < 0.01$ versus Group B, [c]$P < 0.05$ versus Group A, [d]$P < 0.05$ versus Group B.

Comparison of fetal death rate

Among animals treated with PBS, intrauterine demise was observed in only 3.1% of fetuses (158 live fetuses and 5 dead fetuses in a total of 16 mothers killed on Day 19 of gestation). Among LPS-stimulated mice which did not deliver preterm, fetal death was observed in 11.0% of fetuses (73 live fetuses and 9 dead fetuses in a total of 8 mothers killed on Day 19 of gestation), which was significantly higher than that of PBS control group ($P < 0.05$). Among LPS-stimulated mice with previous TLR4 blockade, fetal mortality was 3.9% (148 live fetuses and 6 dead fetuses in a total of 15 mothers killed on Day 19 of gestation), which was significantly lower than that of LPS-treated group ($P < 0.05$) (Table I).

Analysis of co-stimulatory molecule CD86 expression on blood leukocytes

Results from our flow cytometry experiment revealed that LPS treatment resulted in a 2-fold increase in CD86 expression on blood leukocytes as CD45 as the leukocyte common antigen compared with control mice. To determine whether the effect of LPS on CD86 expression is via TLR4, we investigated the expression of CD86 on blood $CD45^+$ cells with previous TLR4 blockade by intraperitoneal injection of neutralizing antibody against TLR4. Our results demonstrated that previous TLR4 blockade almost completely abrogated LPS-induced elevated CD86 expression on blood $CD45^+$ cells (Table II).

Analysis of blood T cell activation

In order to determine whether LPS-induced preterm delivery was through blood T cell activation, flow cytometry was performed to examine the expression of early activation marker CD69 on blood $CD3^+$ cells. LPS exposure caused a significant increase in CD69 expression on blood $CD3^+$ cells compared with that in control mice ($P < 0.01$). Similarly, LPS treatment with previous TLR4 blockade resulted in a marked down-regulation of CD69 expression on blood $CD3^+$ cells in comparison with LPS-treated mice ($P < 0.01$) (Table II).

Analysis of blood B cell activation

We also wanted to know whether inflammation-induced preterm delivery and intrauterine demise were associated with blood B cell activation. Flow cytometry was performed to analyze CD69 expression on blood $CD19^+$ cells using CD19 as a specific marker of B cells. As shown in Table II, LPS treatment did not change CD69 expression on blood B cells. Pretreatment with a TLR4 antibody did not change the expression of CD69 on blood $CD19^+$ cells as well.

Analysis of blood NK cell activation

Blood NK cell activation was also examined by using CD49b as a specific marker of mouse NK cells. LPS exposure significantly up-regulated the expression of CD69 on blood $CD49b^+$ cells. In order to determine LPS-induced blood NK cell activation and mobilization is owing to TLR4, we administered an anti-TLR4 monoclonal antibody before administration of LPS. Our experiments revealed that pretreatment with a TLR4 antagonist could abolish LPS-caused elevated CD69 expression on blood NK cells (Table II).

Analysis of CD86 expression on placenta leukocytes

To better understand the potential roles of maternal–fetal interface in the induction of inflammation-induced preterm delivery, a flow cytometry analysis of placenta immune cell populations post-infection was performed. To examine whether changes in placenta dendritic cells (DCs) following LPS exposure were responsible for the inflammation-induced preterm delivery, an analysis of the expression of DC co-stimulatory marker CD86 on placenta $CD45^+$ cells was

Table II. Comparison of the percentages of mouse blood $CD45^+CD86^+$, $CD3^+CD69^+$, $CD19^+CD69^+$ and $CD49b^+CD69^+$ cell populations (%, mean ± SD, $n = 16$).

Group	Stimulator	Blocking TLR4	$CD45^+CD86^+$ $CD45^+$	$CD3^+CD69^+$ $CD3^+$	$CD19^+CD69^+$ $CD19^+$	$CD49b^+CD69^+$ $CD49b^+$
A	PBS	−	8.0 ± 0.3	10.1 ± 0.5	5.7 ± 0.2	7.2 ± 0.4
B	LPS	−	24.7 ± 1.7[a]	21.7 ± 2.1[a]	6.7 ± 0.3	25.7 ± 1.6[a]
C	LPS	+	9.3 ± 0.6[b]	10.4 ± 0.6[b]	6.1 ± 0.3	8.0 ± 0.3[b]

[a]$P < 0.01$ versus Group A, [b]$P < 0.01$ versus Group B.

Table III. Comparison of the percentages of mouse placenta $CD45^+CD86^+$, $CD45^+CD49b^+$ and $CD49b^+CD69^+$ cell populations (%, mean ± SD, $n = 16$).

Group	Stimulator	Blocking TLR4	$CD45^+CD86^+$ $CD45^+$	$CD45^+CD49b^+$ $CD45^+$	$CD49b^+CD69^+$ $CD45^+$
A	PBS	−	7.0 ± 0.4	15.1 ± 0.9	5.4 ± 0.1
B	LPS	−	28.5 ± 1.6[a]	35.2 ± 2.3[a]	38.5 ± 2.6[a]
C	LPS	+	6.7 ± 0.3[b]	14.5 ± 0.8[b]	6.5 ± 0.3[b]

[a]$P < 0.01$ versus Group A, [b]$P < 0.01$ versus Group B.

performed. As shown in Table III, there was a marked increase in the expression of CD86 on placenta $CD45^+$ cells upon LPS stimulation when compared with PBS control mice ($P < 0.01$). In contrast, blocking TLR4 with neutralizing antibody prior to LPS treatment resulted in a significant reduction of CD86 expression on placenta $CD45^+$ cells compared with LPS-treated mice ($P < 0.01$).

Analysis of the percentage of NK cells in placenta leukocytes

On the basis of the data suggesting that NK cells are important in the pathogenesis of recurrent pregnancy loss (Dosiou and Giudice, 2005) and fighting intrauterine infection (Szekeres-Bartho, 2008), we monitored the percentage of $CD49b^+$ cells in placenta $CD45^+$ cells. LPS treatment in the absence of previous TLR4 blockade caused a significant elevated proportion of $CD49b^+$ cells in placenta $CD45^+$ cells in comparison with control mice ($P < 0.01$). However, LPS exposure in the presence of previous TLR4 blockade counteracted LPS-induced elevated CD49b expression on placenta $CD45^+$ cells (Table III).

Analysis of placenta NK cell activation

To further dissect the mechanism regulating inflammation-induced preterm delivery, placenta NK cell activation was assessed. In LPS-treated mice, the NK cell activation marker, CD69, was significantly up-regulated on placenta $CD49b^+$ cells when compared with PBS controls ($P < 0.01$). In TLR4-blocked mice, the proportion of $CD69^+$ cells in placenta $CD49b^+$ cells was dramatically down-regulated when compared with LPS-treated mice ($P < 0.01$) (Table III).

Discussion

A special interaction is established during pregnancy between the maternal immune system and fetal cells to allow the survival and the normal growth of the fetus. Fetal cells expressing paternal alloantigens are not recognized as foreign by the mother because of an efficient anatomic barrier and a local immunosuppression determined by the interplay of locally produced cytokines, biologically active molecules and hormones. Accumulating evidence suggests that parturition represents an inflammatory process (Dubicke *et al.*, 2008; Shynlova *et al.*, 2009; Vega-Sanchez *et al.*, 2009). Disruption of the delicate balance of cytokines by bacteria or other factors increases the production of proinflammatory cytokines at the maternal–fetal interface and activates the parturition mechanism prematurely.

Confirming our hypothesis that TLR4 is a significant mediator of inflammation-induced preterm delivery, we demonstrated that TLR4-neutralizing monoclonal antibody was able to significantly reduce the incidence of inflammation-induced preterm delivery and fetal death. In consistent with our study, it has been reported that pretreatment with TLR4 antagonist inhibits LPS-induced preterm uterine contractility, cytokines and prostaglandins in rhesus monkeys (Adams Waldorf *et al.*, 2008).

TLR4 has been identified in both first trimester and term placenta, suggesting that the placenta may recognize pathogens through this receptor and induce an immune response (Beijar *et al.*, 2006; Canavan and Simhan, 2007; Ma *et al.*, 2007). Trophoblast cells, upon recognition of LPS through TLR4, may coordinate an immune response by recruiting cells of the innate immune system to the site of an infection at the maternal–fetal interface. Moreover, intrauterine inflammation up-regulated TLR4 messenger RNA in uterus, cervix and placenta. The observed up-regulation of TLR4 in the uterus in response to intrauterine LPS may be a mechanism to augment the inflammatory response and may serve to promote parturition in the setting of inflammation (Elovitz and Mrinalini, 2005).

To determine the signaling mechanism by which TLR4 mediate inflammation cascades, the expression of the co-stimulatory factor CD86 and activation molecule CD69 in systemic and local immune cell subpopulations were investigated.

We have demonstrated that LPS exposure increases CD86 expression on both blood and placenta $CD45^+$ cells. During pregnancy, a delicate balance of innate and adaptive immune responses at the maternal–fetal interface promotes survival of the semi-allogeneic embryo and, at the same time, allows effective immunity to protect the mother from environmental pathogens. Maternal DCs are scattered throughout the decidualized endometrium during all stages of pregnancy and appear to play a crucial role in mediating the balance between immunity and tolerance (Laskarin *et al.*, 2007; Bachy *et al.*, 2008; Ban *et al.*, 2008; Iijima *et al.*, 2008). Driven by pathogens and inflammatory signals, DCs undergo a complex maturation process, which not only leads to enhanced expression of co-stimulatory molecules and increased formation of stable MHC-peptide complexes but also to cytokine secretion modulating T cell activation and expansion, synthesis of chemokines and chemokine receptors, and regulation of T cell and DC trafficking (Kopcow and Karumanchi, 2007). Binding of CD86 with CD28 antigen at the cell surface of T cells is a co-stimulatory signal for activation of the T cells and rejection of the semi-allogeneic embryo. As expected, our experiments demonstrated that LPS treatment caused a significant increase in CD69 expression on blood $CD3^+$ cells. Meanwhile, CD69

expression on blood CD49b$^+$ cells was also up-regulated upon LPS stimulation.

In addition to the effects of LPS on systemic immunity, it also caused up-regulation of NK cell proportion and activated NK cell proportion at maternal–fetal interface. NK cells are lymphocytes characterized by high cytolytic potential against virus-infected and tumor-transformed cells without prior sensitization and without restriction by HLA antigens (Dosiou and Giudice, 2005). NK cells are thus part of the innate immune system. NK cell activation can lead to cytokine secretion or cytotoxicity of targets presenting ligands for NK cell-activating receptors (Kopcow and Karumanchi, 2007; Riley and Yokoyama, 2008). The immunological function of uterine NK cells is still unknown, but uterine NK cells are correlated with the angiogenesis in the decidua by production of angiogenesis factors and have critical function in pregnancy that promotes decidual health, the appropriate vascularization on implantation sites and placental size (Hanna *et al*., 2006; Tabiasco *et al*., 2006; Bilinski *et al*., 2008; Lightner *et al*., 2008; Kalkunte *et al*., 2009; Nakamura, 2009). However, at the same time, they are equipped with cytotoxic potential to fight intrauterine infections (Szekeres-Bartho, 2008). Elevated NK cell infiltration and inappropriate activation may be an underlying cause of pregnancy complications, such as embryo resorption (Lin *et al*., 2009) and recurrence pregnancy loss (Dosiou and Giudice, 2005).

Our findings demonstrated that infection causes maturation of DCs, systemic T and NK cell activation, and infiltration of activated NK cells at the maternal–fetal interface. The activated lymphocytes could secret proinflammatory cytokines and chemokines and mediate cervical ripening, weakening and rupture of the membranes, and the initiation of rhythmic contractions of increasing amplitude and frequency, and ultimately the production of full-blown labor and delivery. A specific TLR4-neutralizing monoclonal antibody almost completely abrogated LPS-induced systemic and local co-stimulatory molecule expression, systemic T and NK cell activation, and the activation of NK cells at the maternal–fetal interface, suggesting that TLR4 plays a critical role in inflammation-induced preterm delivery.

Despite years of searching, there is still no broadly effective strategy for preventing preterm delivery and most therapies are directed at inhibiting myometrial contractions and improving neonatal outcome. The identification of TLR4 as an upstream mediator of inflammation may offer alternative target for preventing inflammation-induced preterm delivery. Effort will be needed to develop antagonists for TLR4 in counteracting the harmful proinflammatory response that complicates systemic microbial infections without compromising fetal survival.

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