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ORIGINAL ARTICLE

Placental Trophoblast Responses to *Porphyromonas gingivalis* Mediated by Toll-like Receptor-2 and -4

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ABSTRACT

Trophoblast participates in preventing allorecognition and controlling pathogens that compromise fetal wellbeing. Toll-like receptors recognize conserved sequences on the pathogens surface and trigger effector cell functions. *Porphyromonas gingivalis* is thought to spread to the umbilical cord and cause fetal growth restriction. **Objective:** To characterize expression and function of TLR-2 and TLR-4 in trophoblast cells from *Porphyromonas gingivalis*-infected pregnant rats. **Methods:** Live *Porphyromonas gingivalis* were challenged into the maxillary first molar subgingival sulcus of female rats before and/or during pregnancy and sacrified on gestational day (GD) 14 and 20. *Porphyromonas gingivalis* was detected by API-ZYM system in the maternal blood of the retro-orbital venous plexus and the umbilical cord. TLR-2 and TLR-4 expressions in trophoblast cells was detected by immunohistochemistry. **Results:** *Porphyromonas gingivalis* was first detected in the maternal blood and finally spread to the umbilical cord. Syncytiotrophoblast, spongitrophoblast and trophoblastic giant cell in treated groups had significantly higher expression of TLR-2 and TLR-4 than control group (p<0.05). **Conclusion:** Syncytiotrophoblast, spongitrophoblast and trophoblastic giant cell are able to recognize *Porphyromonas gingivalis* through TLR-2 and TLR-4 expression. The ligation of TLR-2 and TLR-4 promoted cytokine production and induced trophoblast cell death. These findings strengthen links between periodontal disease and fetal growth restriction.

ABSTRAK

Respons trofoblas terhadap *Porphyromonas gingivalis* yang dimediasi *Toll-Like Receptor*-2 dan -4. Trofoblas berpartisipasi mencegah *allorecognition* dan mengendalikan patogen berbahaya bagi kelangsungan hidup janin. *Toll-like receptor* mengenali urutan terkonservasi pada permukaan patogen dan memicu fungsi sel efektor. *Porphyromonas gingivalis* diduga menyebar ke tali pusat, dan menyebabkan restriksi pertumbuhan janin. **Tujuan:** Penelitian ini bertujuan untuk mengkarakterisasi ekspresi TLR-2 dan TLR-4 pada sel trofoblas dari tikus hamil yang terinfeksi *Porphyromonas gingivalis*. **Metode:** Tikus betina dipapar *live-Porphyromonas gingivalis* pada konsentrasi 10^9 *colony forming unit*/mL ke dalam sulkus subgingiva molar pertama maksilaris sebelum dan/atau selama kehamilan. Tikus dikorbankan pada *gestational day* 14 dan 20. *Porphyromonas gingivalis* dideteksi dengan sistem API-ZYM dalam darah vena pleksus retro-orbital maternal dan tali pusat. Ekspresi TLR-2 dan TLR-4 pada sel trofoblas dideteksi secara imunohistokimia. **Hasil:** *Porphyromonas gingivalis* pertama kali dideteksi dalam darah maternal dan akhirnya menyebar ke tali pusat. Sinsitiotrofoblas, spongitrofoblas dan *giant cell* trofoblas pada kelompok perlakuan memiliki ekspresi TLR-2 dan TLR-4 secara signifikan lebih tinggi daripada kelompok kontrol (p<0,05). **Simpulan:** Sinsitiotrofoblas, spongitrofoblas dan *giant cell* trofoblas mampu mengenali *Porphyromonas gingivalis* melalui ekspresi TLR-2 dan TLR-4. Ligasi TLR-2 dan TLR-4 mendorong produksi sitokin dan menginduksi kematian sel trofoblas. Temuan kami memperkuat hubungan antara penyakit periodontal dan restriksi pertumbuhan janin.

Key words*:* bacteremia, fetal growth restriction, periodontitis, *Porphyromonas gingivalis*, toll-like receptors

INTRODUCTION

The growth and survival of fetus is dependent on placenta, which forms the interface of feto-maternal circulation, facilitates metabolism, gas exchange, and waste disposal of the fetus. In addition, the placenta produces hormones that altered maternal physiology during pregnancy and forms a barrier against the maternal immune system.[1] Murine placenta consists of three layers, decidual zone, juntional zone and labyrinth zone. Labyrinth zone consists of branched villi designed for the efficient exchange of nutrients.[2] Maternal and fetal blood flows are adversely in the labyrinth to maximize the transport of nutrients.[3] If thelabyrinthdoes not obtain the proper pattern, branching and dilatation of vascularization, then perfused placenta will be disrupted, resulted in poor oxygen and nutrients diffusion.[4]

Placental growth and development relies highly on trophoblast cells. Apoptosis and the factors involved in the regulation are associated with almost all stages of development and trophoblast differentiation.[5] One of the factors that led to the increase apoptosis of trophoblast during pregnancy is intrauterine infection.[6] The exact mechanism of infection resulted the progression of this disorder is not clearly understood, but recent studies suggested that bacterial products have a direct effect on the trophoblast. Trophoblast can respond to infection through the expression of a family of natural immune receptors, Toll-like receptor (TLR).[7]

Periodontal disease is a multifactorial chronic infection resulted to destruction of the periodontium, the tissues that surround and support teeth. The primary periodontal pathogenic bacteria is *Porphyromonas gingivalis* which has potential virulence factors such as proteolytic enzymes, endotoxin (lipopolysaccharide/LPS), evasion of host responses, invasion of host tissues, and induction of inflammatory mediators.[8] The possible correlation of periodontal disease and intrauterine growth restriction (IUGR) have been explored in several experimental animal models, but those studies in experimental animals only supported the concept of the relationship of bacterial infection subcutaneously and the risk of pregnancy adverse, and did not examine the *Porphyromonas gingivalis* dissemination from periodontal tissues via the blood circulation which affect fetal growth restriction.[9] Hence, it is required a model animal to examine the relationship between local infection and fetal growth, as well as to better understand host-pathogen interactions. This study will make a model of chronic periodontal infections caused by live-*Porphyromonas gingivalis* exposure in gingival tissues of rats. The model developed chronic infection with periodontal pathogens similar to that observed in humans. Furthermore, this study aimed to characterize the expression of TLR-2 and TLR-4 in trophoblast cells from *Porphyromonas gingivalis*-infected pregnant rats.

METHODS

All procedures were approved by the Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia. This study had taken female *Sprague-Dawley* rats, adult, 2 months, 150-250 g and primiparous. The rats were maintained on the controlled and standardized conditions. The subjects of study were consisted of two blocks, they were sacrificed on gestational day (GD) 14 and GD 20. Each block was subdivided into four groups, which consisted of (1) the control group, no *Porphyromonas gingivalis* infection; (2) the Pg-BD group, an infection of *Porphyromonas gingivalis* before and during pregnancy; (3) the Pg-B group, an infection of *Porphyromonas gingivalis* before pregnancy; and (4) the Pg-D group, an infection of *Porphyromonas gingivalis* during pregnancy. Each group consisted of five pregnant rats.

Before injecting *Porphyromonas gingivalis*, all female rats were given antibiotic to prevent infection from other bacterias. Induction of experimental periodontitis was performed by injection of 0.05 mL live-*Porphyromonas gingivalis* ATCC 33277 with a concentration of $2x10^9$CFU/mL that was dissolved in saline into the distopalatal and distobuccal gingival sulcus area of maxillary first molar. Injection was repeated every 3 days for 30 days. For infection after pregnancy, it was also performed by a repeated injection every 3 days for 19 days. Control group rats were injected saline 0.05mL as the treatment schedule of the treated group rats. Then, the female rats were mated with the same strain of male rat overnight ratio 2:1. The next morning, female rats were removed from the cages and examined the vaginal plug. If the vaginal plug was found, the day was recorded as GD 1.

Blood of the maternal retro-orbital venous plexus and the umbilical cord were conducted immediately to bacteremia test, such as colony morphology, Gram staining and API-ZYM enzymatic assay. Each fetus was taken post-mortem from the chorioamniotic sac. Furthermore, the placental immunohistochemical analysis was undertaken to determine the expression of TLR-2 and TLR-4. Samples were incubated overnight at 4^oC with primary antibody, rabbit polyclonal anti-TLR-2 and TLR4 antibody dilution 1:300 and 1:500, respectively (Abbiotec, San Diego, CA), whereas negative control was incubated with secondary antibody as a substitute for the primary antibody. TLR-2 and TLR-4 were expressed on the cell wall and cytoplasm. Data were presented as mean number of cells expressing TLR-2 and TLR-4 in each type of cell. These specimens were evaluated in macrophages of labyrinth zone (LM), junctional zone (SM) and decidua zone (DM), as well as syncytiotrophoblast (LS), spongitrophoblast (ST) and trophoblastic giant cell (DG).

Chi-Square test was performed to compare frequencies between categorical variables, such as maternal and fetal bacteremia. One-way analysis of variance (ANOVA) with post hoc test was performed to compare the TLR-2 and TLR-4 expression in trophoblast cells of maternal periodontal infection. Value of significance was determined as $p<0.05$. Numerical data were presented in mean±standard deviation.

RESULTS

All procedures were approved by the Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia. This study had taken female *Sprague-Dawley* rats, adult, 2 months, 150-250g and primiparous. The rats were maintained on the controlled and standardized conditions. The subjects of study were consisted of two blocks, they were sacrificed on gestational day (GD) 14 and GD 20 (Table 1). Each block was subdivided into four groups, which consisted of the control group, no *Porphyromonas gingivalis* infection; the Pg-BD group, an infection of *Porphyromonas gingivalis* before and during pregnancy; the Pg-B group, an infection of *Porphyromonas gingivalis* before pregnancy; and the Pg-D group, an infection of *Porphyromonas gingivalis* during pregnancy. Each group consisted of five pregnant rats (Table 2).

Before injecting *Porphyromonas gingivalis*, all female rats were given antibiotic to prevent infection from other bacterias. Induction of experimental periodontitis was performed by injection of 0.05 ml live-*Porphyromonas gingivalis* ATCC 33277 with a concentration of $2x10^9$ CFU/ml that was dissolved in saline into the distopalatal and distobuccal gingival sulcus area of maxillary first molar. Injection was repeated every 3 days for 30 days. For infection after pregnancy, it was also performed by a repeated injection every 3 days for 19 days. Control group rats were injected saline 0.05mL as the treatment schedule of the treated group rats. Then, the female rats were mated with the same strain of male rat overnight ratio 2:1. The next morning, female rats were removed from the cages and examined the vaginal plug. If the vaginal plug was found, the day was recorded as GD 1 (Table 3).

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Chi-Square test was performed to compare frequencies between categorical variables, such as maternal and fetal bacteremia. One-way analysis of variance (ANOVA) with post hoc test was performed to compare the TLR-2 and TLR-4 expression in trophoblast cells of maternal periodontal infection. Value of significance was determined as $p<0.05$. Numerical data were presented in mean ± standard deviation.

DISCUSSION

During pregnancy, placenta is not only exposed to the maternal immune system, but also to microorganisms. This study indicated that the placenta responsed to *Porphyromonas gingivalis* infection, which was characterized by expression of TLR-2 and TLR-4 in placental trophoblast cells and macrophages on GD 14, further expression of TLR-2 and TLR-4 was increased on GD 20. These results are consistent with a study which found that TLR-4 expression was higher in the human placenta a term than the first trimester.[10] This suggests that placenta in early pregnancy is less response to pathogenic stimulation than the end of pregnancy, but the mechanisms of TLR regulation based on gestational age should be studied in more detail.

This study suggested that *Porphyromonas gingivalis* could be expected to proliferate intracellularly in decidua and placenta of maternal bacteremia positive, then breach the fetomaternal barrier membrane and spread into the fetal umbilical cord. The direct contact of two areas between maternal and fetal cells is the maternal blood-syncytiotrophoblast and extravilloustrophoblast-uterus. *Porphyromonas gingivalis* could be expected to reach decidua carried by phagocytic and non-phagocytic cells. On reaching decidua, *Porphyromonas gingivalis* can spread intracellularly into decidua and trophoblast cells, or lyse host cells and subsequent, these bacteria invade spongiotrophoblast and trophoblastic giant cell. After entering spongiotrophoblast and trophoblastic giant cell, *Porphyromonas gingivalis* still alive and multiply, and able to survive and colonize. Hence in infected-placenta, *Porphyromonas gingivalis* can spread to the fetus. Pathways of bacterial dissemination to fetus may occur through the fetal capillaries in villous stroma.
Porphyromonas gingivalis may penetrate to syncytio-

Tabel 1. Maternal and fetal bacteremia in *Porphyromonas gingivalis*-infected pregnant rats GD 14 and GD 20

Variable	Maternal periodontal infection				p
	Control	Pg-BD	Pg-B	Pg-D	
GD 14:					
Maternal bacteremia positive, n(%)	0(0)	5(100)	4(80)	5(100)	0.000*
Maternal bacteremia negative, n(%)	5(100)	0(0)	1(20)	0(0)	
Fetal bacteremia positive, n(%)	0(0)	17(39.5)	11(25.6)	15(34.9)	0.000*
Fetal bacteremia negative, n(%)	38(38.8)	14(14.3)	24(24.5)	22(22.4)	
GD 20:					
Maternal bacteremia positive, n(%)	0(0)	5(100)	4(80)	5(100)	0.000*
Maternal bacteremia negative, n(%)	5(100)	0(0)	1(20)	0(0)	
Fetal bacteremia positive, n(%)	0(0)	22(45.8)	12(25)	14(29.2)	0.000*
Fetal bacteremia negative, n(%)	30(32.3)	21(22.6)	25(26.9)	17(18.3)	

Pg-BD : periodontal infection before and during pregnancy
Pg-B : periodontal infection before pregnancy
Pg-D : periodontal infection during pregnancy
: mean difference was significant at 0.05

Table 2. TLR-4 expression in the placenta by the periodontal maternal-infected *Porphyromonas gingivalis* GD 14 and GD 20

Variable	Maternal periodontal infection				p
	Control (n=23)	Pg-BD (n=10)	Pg-B (n=13)	Pg-D (n=10)	
GD 14:					
TLR4-LM	1.13±0.92*	2.50±1.84*	2.62±1.19*	2.50±1.08*	0.001
TLR4-LS	1.57±0.79*	3.30±1.16*	2.92±1.04*	3.30±1.95*	0.000
TLR4-SM	1.48±0.79*	2.60±0.97*	2.15±0.38*	2.20±1.03*	0.002
TLR4-ST	1.65±1.03*	6.70±1.89*	4.92±1.85*	4.80±1.48*	0.000
TLR4-DM	1.35±0.65*	2.60±1.65*	2.46±0.66*	1.90±1.10	0.002
TLR4-DG	1.74±0.92*	3.90±0.88*	3.08±0.64*	3.60±1.35*	0.000
GD 20:					
TLR4-LM	2.00±0.95*	3.70±1.70*	2.31±1.18	3.60±2.07*	0.003
TLR4-LS	2.78±1.09*	7.50±1.72*	3.85±1.41*	6.90±1.73*	0.000
TLR4-SM	2.22±1.04*	2.60±1.17	2.23±0.83	3.30±0.95*	0.037
TLR4-ST	2.96±1.07	3.80±1.39	3.38±1.33	3.40±0.52	0.249
TLR4-DM	2.35±1.03	2.50±1.18	2.00±0.71	2.90±1.19	0.224
TLR4-DG	2.26±1.05*	3.40±0.69*	2.85±0.69*	3.30±0.48*	0.001

Pg-BD : periodontal infection before and during pregnancy
Pg-B : periodontal infection before pregnancy
Pg-D : periodontal infection during pregnancy
* : mean difference was significant at 0.05

Table 3. TLR-2 expression in the placenta by the periodontal maternal-infected *Porphyromonas gingivalis* GD 14 and GD 20

Variable	Maternal periodontal maternal				p
	Control (n=23)	Pg-BD (n=10)	Pg-B (n-13)	Pg-D (n=10)	
GD 14:					
TLR2-LM	1.17±0.39*	2.60±1.43*	1.85±0.80*	2.20±1.03*	0.000
TLR2-LS	1.48±0.67*	3.60±1.35*	2.00±0.71	3.40±2.79*	0.000
TLR2-SM	1.48±0.67*	2.80±0.63*	1.46±1.05	2.40±1.34*	0.000
TLR2-ST	1.96±0.98*	6.30±2.16*	3.38±1.81*	4.70±2.06*	0.000
TLR2-DM	1.22±0.67*	3.40±1.43*	1.85±0.80*	2.00±0.82*	0.000
TLR2-DG	1.39±0.89*	6.60±1.51*	3.85±2.67*	4.50±2.22*	0.000
GD 20:					
TLR2-LM	2.17±1.43	4.00±3.31	3.31±1.49	4.10±2.23	0.105
TLR2-LS	3.39±1.95*	7.20±3.29*	6.38±3.25*	7.90±2.08*	0.000
TLR2-SM	2.48±0.89	2.90±0.88	2.77±1.54	2.90±0.57	0.606
TLR2-ST	3.35±1.69	4.00±1.05	4.08±2.14	4.40±2.50	0.443
TLR2-DM	2.39±1.08	2.60±1.51	2.85±1.52	3.00±1.24	0.586
TLR2-DG	2.78±1.24*	4.40±0.97*	3.08±0.92	3.80±1.28*	0.003

Pg-BD : periodontal infection before and during pregnancy
Pg-B : periodontal infection before pregnancy
Pg-D : periodontal infection during pregnancy
* : mean difference was significant at 0.05

trophoblast or cause destruction, it can colonize in the fetal blood vessels. On GD14 and GD20, TLR-2 and TLR-4 were expressed by syncytiotrophoblasts and spongiotrophoblasts. This indicated that TLR-2 and TLR-4 in them were able to respond *Porphyromonas gingivalis* that previously have been through the decidual compartment. Thus, *Porphyromonas gingivalis* will only pose a threat to fetus if the syncytiotrophoblast layer was breached, so that *Porphyromonas gingivalis* can enter the fetal blood vessels.

The expressions of TLR-2 and TLR-4 in syncytiotrophoblasts and spongiotrophoblasts of rat suggested that the ability to respond pathogens invasion is not only by immune cells but also by placental trophoblasts. It may be involved in the physiological protection of the placenta, similar to the natural immune system. In addition, this study also found that TLR-2 and TLR-4 were expressed by trophoblastic giant cells and decidual macrophages. These results are consistent with the idea that microorganisms will be pathogenic after breached physical barrier and accessed tissue compartments where the pathogen recognition receptor (PRR) expressed.[7]

This study also showed that TLR-2 and TLR-4 is expressed on the cell surface and intracellular trophoblast. Expression in the cytoplasm may provide rapid mobilization of additional receptors to the cell surface of bacteria after the initial recognition.[11-13] Thus the expression of TLR-2 and TLR-4 in the cytoplasm serves to facilitate the recognition and intracellular responses.[14,15]

Toll-like receptor-2 expressed in macrophages and trophoblasts may respond to other components of *Porphyromonas gingivalis* such as PDG, lipoproteins and LTA. The increased expression of TLR-2 probably will contribute to the accelerated immune response by macrophages and trophoblasts. The regulation of TLR-2 expression may be one mechanism of immune regulation that is often involved in host defense against various strains of bacteria. This implied that if *Porphyromonas gingivalis* invade the placenta, the macrophages and trophoblasts at first recognize LPS via TLR-4 which expressed constitutively. Later, TLR-2 is induced directly by LPS or indirectly through secondary cytokines. Thus, macrophages and trophoblasts were more responsive to LPS or other components of *Porphyromonas gingivalis*. Ligation of TLR-2 and TLR-4 by *Porphyromonas gingivalis* LPS in macrophages and trophoblasts appeared to improve regulation of pro-inflammatory and anti-inflammatory cytokines both on GD 14 and GD 20. These results imply that *Porphyromonas gingivalis* LPS is functionally interact with TLR-2 and TLR-4.

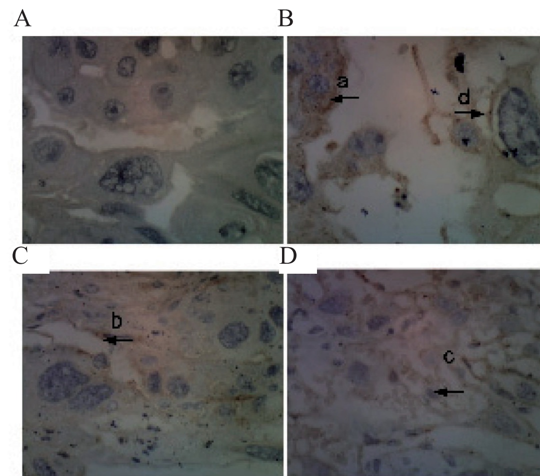

Figure 1. TLR-4 expression on placenta of control (A), Pg-BD (B), Pg-B (C) and Pg-D (D) groups at GD 20. TLR-4 was expressed weakly (A) and strong (B-D) on the wall and cytoplasm of macrophage (a), syncytiotrophoblast (b), spongiotrophoblast (c) and trophoblastic giant cell (d). Magnification 400x

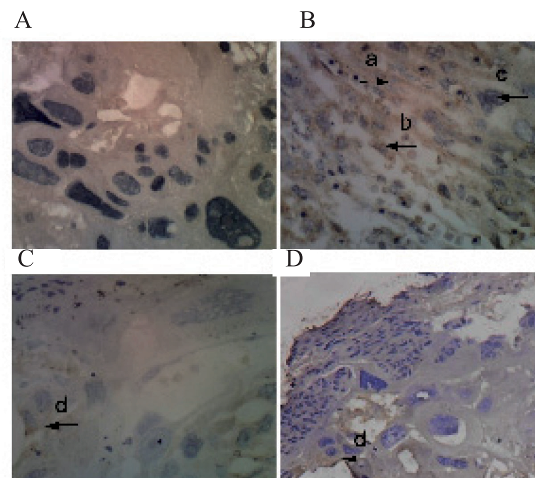

Figure 2. TLR-2 expression in the placenta of control (A), Pg-BD (B), Pg-B (C) and Pg-D (D) groups at GD 20. TLR-2 was expressed weakly (A) and strong (B-D) on the wall and cytoplasm of syncytiotrophoblast (a), spongiotrophoblast (b), macrophage (c) and trophoblastic giant cell (d). Magnification 400x.

TLR-2 and TLR-4 were strong expressed in placental on GD 14 and 20, especially the trophoblasts in labyrinth zone and junctional zone which are a frontal barrier between maternal and fetus. Trophoblast was expected to have important functions in regulating the host immune response against bacterial infection. Co-stimulation of TLR-2 and TLR-4 is required for the reliable signaling to synergize LPS binding with TLR-2 and TLR-4 in TNF release from macrophages and trophoblasts. Thus, co-expression of TLR-2 and TLR-4 in trophoblast of the labyrinth and junctional

zone could potentially be a guardian against destructive infection. It is assumed that expressions of TLR-2 and TLR-4 are increased their regulation in placenta as a defense mechanism that can be easily mobilized to protect the fetus from infection during pregnancy. TLR-2 and TLR-4 may be an important regulator of the immune system from placental infection, but also may be required for the maturation of fetal immune response.

Porphyromonas gingivalis exposure in trophoblastic giant cell can increase the expression of TLR-2 and TLR-4. Trophoblastic giant cell has been reported as a differentiated trophoblast precursor cells. It is possible that *Porphyromonas gingivalis* can cause cells of maternal-fetal interface, such as decidual cells and spongiotrophoblasts, to degenerate and die, thus it stimulates trophoblasts to perform phagocytosis and eliminate damaged cells. Phagocytosis acts as a biological mechanism for the elimination of dead or degenerated cells. It was expected that the degeneration and deterioration of trophoblastic giant cells eventually lead to reduced trophoblasts. Therefore, TLR-2 and TLR-4 serve as an important sensor for trophoblasts, which makes it possible to coordinate the local immune response and to enhance cell invasion and placenta formation. TLR-2 and TLR-4 also can provide a bridge for the introduction of placenta to the danger signal and the produced type of response can have harmful consequences for the pregnancy.

Analysis of the biological functions of TLR-2 and TLR-4 in human trophoblast cells showed that first trimester trophoblast cells express MyD88, an adapter protein that allows for all TLR signaling through intracellular pathways. After ligation with TLR ligands, MyD88 was recruited to the TLR intracellular domains.[16,17] Furthermore, MyD88 recruits and activates IL-1R-associated kinase (IRAQ).[17] IRAQ then dissociates from the receptor complex and becomes associated with TNFR-associated factor-6, resulting in activation of downstream signaling pathways of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK).[18,19] TLR signals produce an inflammatory immune response characterized by the production of pro-inflammatory cytokines and anti-inflammatory.[20] In the first trimester trophoblast cells, ligation of TLR-4 by LPS seems to trigger a classic response, characterized by increased regulation of pro-inflammatory cytokines and anti-inflammatory.[21] However, ligation of TLR-2 are expressed by first trimester trophoblast cells have a very different effect. Treatment of first trimester trophoblast cells with TLR-2 ligand, such as peptidoglycan (PDG) and lipoteichoid acid (LTA), only increases the expression of regulation of IL-6 and IL-8, while the levels of other cytokines or down regulation does not change. In addition, ligation of TLR-2 causes the first trimester trophoblast cells undergoing apoptosis, this is consistent with reports of TLR-mediated apoptosis-2 in another cell type.[21,22-24] Trophoblast cell apoptosis induced PDG and LTA can be through TLR-2 homodimers or heterodimers TLR-1/TLR-2.[21]

CONCLUSION

It can be concluded that syncytiotrophoblast, spongi-trophoblast and trophoblastic giant cell are able to recognize *Porphyromonas gingivalis* through the expression of TLR-2 and TLR-4. The ligation of TLR-2 and TLR-4 promoted cytokine production and induced trophoblast cell death. The maternal periodontal health during pregnancy affects maternal status implied by changes in placental function and fetal growth.

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