

Human trophoblast responses to *Porphyromonas gingivalis* infection

S.D. Riewe¹, J.J. Mans¹, T. Hirano¹, J. Katz², K.T. Shiverick³, T.A. Brown¹ and R.J. Lamont¹

¹ Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, USA

² Department of Oral Medicine, College of Dentistry, University of Florida, Gainesville, FL, USA

³ Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, FL, USA

Correspondence: Richard J. Lamont, Department of Oral Biology, University of Florida, College of Dentistry, Gainesville, FL 32610-0424, USA Tel.: +1 352 273 8861; fax: +1 352 392 2361; E-mail: rlamont@dental.ufl.edu

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SUMMARY

Porphyromonas gingivalis is a periodontal pathogen that is also associated with preterm low-birthweight delivery. We investigated the transcriptional responses of human extravillous trophoblasts (HTR-8) to infection with *P. gingivalis*. Over 2000 genes were differentially regulated in HTR-8 cells by *P. gingivalis*. In ontology analyses of regulated genes, overpopulated biological pathways included mitogen-activated protein (MAP) kinase signaling and cytokine production. Immunoblots confirmed overexpression of the MAP kinase pathway components MEK3, p38 and Max. Furthermore, *P. gingivalis* infection induced phosphorylation and activation of MEK3 and p38. Increased production of interleukin (IL)-1 β and IL-8 by HTR-8 cells was demonstrated phenotypically by enzyme-linked immunosorbent assay of HTR-8 cell lysates and culture supernatants. Hence, infection of trophoblasts by *P. gingivalis* can impact signal transduction pathways and modulate cytokine expression, outcomes that could disrupt the maintenance of pregnancy.

INTRODUCTION

A leading cause of infant mortality and morbidity is preterm delivery of low-birth-weight (PTLBW) infants, which accounts for about 12% of births in the USA

(Hoyert *et al.*, 2006). Bacterial infection is a major cause of PTLBW delivery (Goldenberg & Thompson, 2003; Schloesser *et al.*, 2004; Bakardjiev *et al.*, 2005), and epidemiological evidence has indicated an association between periodontal disease and PTLBW delivery (Xiong *et al.*, 2006; Michalowicz & Durand, 2007). *Porphyromonas gingivalis*, a major periodontal pathogen, has been detected in the amniotic fluid in preterm labor (Leon *et al.*, 2007), and in the placenta in pre-eclampsia (Barak *et al.*, 2007). Furthermore, *P. gingivalis* infection results in pregnancy complications in rodent and rabbit animal models (Lin *et al.*, 2003; Boggess *et al.*, 2005; Belanger *et al.*, 2008). There are several possible mechanisms by which periodontal bacteria or their products could contribute to PTLBW delivery. For example, periodontal organisms and their antigens could initiate an inflammatory response with systemic consequences that could trigger preterm labor (Offenbacher *et al.*, 1998; Garcia *et al.*, 2001). Alternatively, direct oral-hematogenous spread of bacteria to the gestational tissues may occur. Bacteremias are common in patients with periodontal disease (Kinane *et al.*, 2005; Lockhart *et al.*, 2008; Perez-Chaparro *et al.*, 2008) and *P. gingivalis* is resistant to complement and neutrophil-mediated killing (Amano *et al.*, 1992; Mydel *et al.*, 2006; Slaney *et al.*, 2006; Perez-Chaparro *et al.*, 2008).

The placental-fetal barrier in the monohemochorial placenta of humans consists of a single layer of

fetally derived trophoblasts. This trophoblast barrier is formed from a highly proliferative, migratory and invasive subpopulation of trophoblasts, known as extravillous trophoblasts, which invade the uterus and its vasculature. The resulting trophoblast layer is in direct contact with maternal blood and is essential for the adequate exchange of key molecules between the maternal and fetal circulation. Trophoblasts produce pro- and anti-inflammatory cytokines (Griesinger *et al.*, 2001), which contribute to the immunological interplay between the fetal and maternal compartment and to the maintenance of pregnancy (Griesinger *et al.*, 2001; Hirsch & Wang, 2005). Organisms that commonly cause fetoplacental infections are often intracellular, and include viruses such as cytomegalovirus (Stagno *et al.*, 1986), protozoa such as *Toxoplasma gondii* (Jones *et al.*, 2001) and bacteria such as *Listeria monocytogenes* (Mylonakis *et al.*, 2002). *Listeria*, for example, invades trophoblasts both *in vitro* and *in vivo* and can induce apoptosis in placental cells (Disson *et al.*, 2008). In addition, bacteria that have been identified in intrauterine infections are capable of modulating cytokine expression from trophoblasts, and imbalances in these inflammatory mediators can act as a trigger for the initiation of infection-induced preterm labor (Griesinger *et al.*, 2001; Hirsch & Wang, 2005). *P. gingivalis* can invade trophoblasts and induce G1 arrest and apoptosis (Inaba *et al.*, 2009). G1 arrest is associated with activation of extracellular signal-regulated kinase (ERK) 1/2 and signaling through cyclins and Rb (retinoblastoma protein). In this study we used microarrays to uncover the global transcriptional responses of trophoblasts to *P. gingivalis*. Signaling pathways leading to cytokine expression, predicted by differential gene expression, were verified at the protein level, and the ability of *P. gingivalis* to modulate cytokine production from trophoblasts was confirmed phenotypically.

METHODS

Bacterial and cell culture

P. gingivalis ATCC 33277 was grown anaerobically at 37°C in trypticase soy broth supplemented with yeast extract (1 mg ml⁻¹), hemin (5 µg ml⁻¹) and menadione (1 µg ml⁻¹). The HTR-8/SVneo trophoblast cell line (henceforth called HTR-8 cells) was provided by Dr Charles Graham (Kingston, ON, Can-

ada). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 5% fetal bovine serum at 37°C in 5% CO₂.

Transcriptional profiling

The *P. gingivalis* cells were reacted with HTR-8 cells at a multiplicity of infection (MOI) of 200 for 2 h at 37°C in 5% CO₂. Cocultures were carried out in quadruplicate. The HTR-8 cells were lysed with Trizol (Invitrogen, Carlsbad, CA) before RNA extraction. RNA isolation, complementary DNA (cDNA) synthesis, labeled cRNA synthesis and chip hybridization were conducted as previously described (Handfield *et al.*, 2005). Briefly, total RNA was extracted from Trizol-lysed cells, treated with DNase I, purified and quantified according to standard methods (Qiagen, Valencia, CA; and Affymetrix, Santa Clara, CA). The cDNA synthesis was performed according to the Affymetrix protocol (SuperScript double-stranded cDNA synthesis kit; Invitrogen) with 8 µg total cellular RNA used as a template to amplify messenger RNA species for detection. Double-stranded cDNA was purified, and used as a template for labeled cRNA synthesis. *In vitro* transcription was performed using a BioArray high-yield RNA transcript labeling kit (T7) (Enzo Life Science, Farmingdale, NY), to incorporate biotinylated nucleotides. The cRNA was subsequently fragmented and hybridized onto Genechip Human Genome (HG) U133-A Plus 2.0 oligonucleotide arrays (Affymetrix) with proper controls. Each sample was studied in parallel, and the samples were not pooled. The microarrays were hybridized for 16 h at 45°C, stained with phycoerythrin-conjugated streptavidin and washed according to the Affymetrix protocol (EukGE-WS2v4) using an Affymetrix fluidics station, and scanned with an Affymetrix GeneChip 3000 scanner. Expression data can be accessed using accession number GSE19810 at the NCBI GEO database.

Microarray data analysis was performed as previously described (Mans *et al.*, 2006; Hasegawa *et al.*, 2008). Briefly, expression filters were applied to remove Affymetrix controls and probe-sets whose signal was undetected across all samples. The signal intensity values of the resulting dataset were variance-normalized, mean-centered and ranked by their coefficients of variation. Normalization was performed to give equal weight to all probe-sets in

the analysis, regardless of the order of magnitude of the raw signal intensity. To reduce the confounding effect of background signal variation on the analysis, only the half of the dataset demonstrating the most variation across samples was used to perform unsupervised hierarchical cluster analysis using CLUSTER software (Eisen *et al.*, 1998). The resulting heat-map and cluster dendrograms were visualized with TREEVIEW software to reveal the extent of characteristic host cell responses to each infection state, defined as identical treatments clustering together.

Following initial assessment of the host cell response to each condition, supervised analysis was performed to investigate differences in gene regulation among experimental conditions. For this analysis, the raw signal intensities were \log_2 -transformed for all probe-sets that passed the initial expression filters, and were correlated using BRB Array Tools (Simon and Peng-Lam, National Cancer Institute, Rockville, MD). In each supervised analysis, biological replicates were grouped into classes according to their infection state during coculture experiments and probe sets significant at the $P < 0.001$ level between classes were identified. To test the ability of these significant probe sets to truly distinguish between the classes, leave-one-out-cross-validation (LOOCV) studies were performed. In these LOOCV studies each array was left out in turn and a classifier was derived between the groups by selecting probe sets significant at $P < 0.001$. The significant probe sets were then used with several prediction models (compound covariate predictor, nearest neighbor predictor, and support vector machine predictor) to predict the class identity of the array that was left out and not included when the classification model was built. The significance ($P < 0.001$) of the LOOCV analysis was estimated using a Monte Carlo simulation with 2000 permutations of the dataset.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were populated using PATHWAY EXPRESS (Khatri *et al.*, 2005), available at <http://vortex.cs.wayne.edu/projects.htm>.

Immunoblotting

Infected (MOI 200) or control HTR-8 cells were washed three times with phosphate-buffered saline

and lysed in radioimmunoprecipitation assay buffer with proteinase inhibitors (Sigma). Proteins (25 μ g) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes (Millipore, Billerica, MA) and blocked with 5% skim-milk in Tris-buffered saline–0.1% Tween-20 (TBST). Membranes were incubated for 1 h with primary antibodies (Santa Cruz) diluted 1 : 200. After washing in TBST three times, membranes were reacted with the species-appropriate peroxidase-coupled secondary antibody (1 : 1000). Visualization was performed with the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ). Band intensities were scanned and quantified using the Kodak 1D IMAGE ANALYSIS SOFTWARE V.3.6.1.

Detection of cytokines

Supernatants and cell lysates from infected (MOI 200) or control HTR-8 cells were collected and filter-sterilized. Interleukin (IL)-1 β and IL-8 concentrations were investigated by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Experiments were conducted in triplicate.

RESULTS

Transcriptional profile of HTR-8 cells infected with *P. gingivalis*

All samples of uninfected HTR-8 cells and *P. gingivalis*-infected cells were used to determine the overall similarity of the transcriptional responses. After elimination of probe sets whose signals were not greater than background levels on all arrays, signal intensity data for the 29,598 probe sets that passed the initial expression filters were included in an unsupervised cluster analysis and supervised class prediction analysis. The unsupervised hierarchical cluster analysis revealed an infection state-dependent host cell transcriptional profile, as biological replicates clustered together (data not shown). In a supervised analysis based on the infection state, at a significance level of $P < 0.001$, 2045 probe sets were differentially expressed. Assuming normality of the dataset, 2045 significant genes are 68-fold greater than the 30 probe sets that would be expected by chance at a

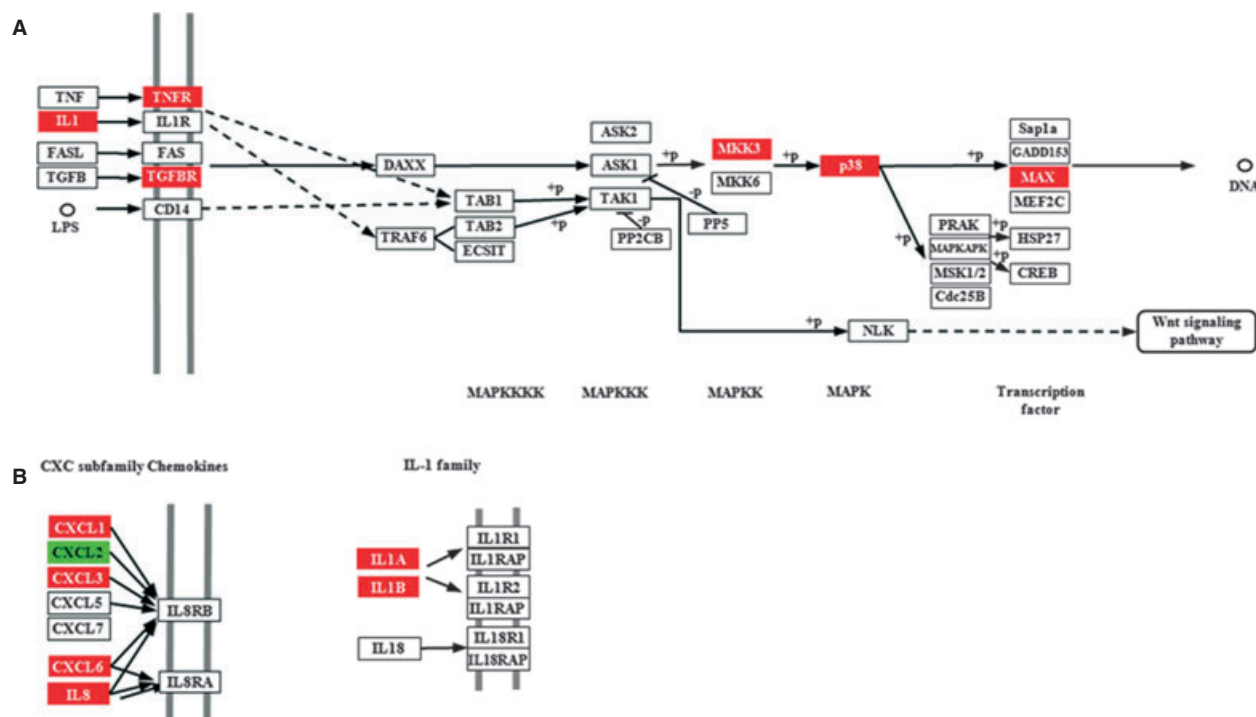


Figure 1 *Porphyromonas gingivalis* infection impacts gene expression in HTR-8 cells. Pathways containing genes differentially regulated by *P. gingivalis* at $P < 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature (see text for details). Red indicates upregulation, green indicates downregulation and white indicates no change in expression. (A) p38 branch of the mitogen-activated protein (MAP) kinase pathway. (B) CXC and interleukin-1 (IL-1) family cytokines.

significance threshold of $P < 0.001$, given that 29,598 probe sets passed the expression filter. To mine the array data for biologically relevant information, an ontology analysis of known metabolic pathways was performed using statistical algorithms in the PATHWAY EXPRESS software (Khatri *et al.*, 2005). Pathways significantly ($P < 0.05$) overpopulated with differentially regulated genes ($P < 0.001$) included mitogen-activated protein kinase (MAPK) Signaling, Cell Cycle, and Apoptosis. Differential expression of genes involved in the cell cycle and in apoptosis is consistent with our previous work showing that *P. gingivalis* can induce G1 arrest and apoptosis in HTR-8 cells. Genes upregulated by *P. gingivalis* in the MAPK pathway included MEK3 (MKK3), p38 and Max (Fig. 1A). The MEK3-p38 pathway can regulate the expression of inflammatory cytokines (Patil & Kirkwood, 2007; Schindler *et al.*, 2007), and accordingly interrogation of the cytokine–cytokine receptor interaction pathway showed upregulation of proinflammatory cytokines including IL-1 α and IL-1 β , along with CXC chemokines IL-8, CXCL1, CXCL3 and CXCL6 (Fig. 1B).

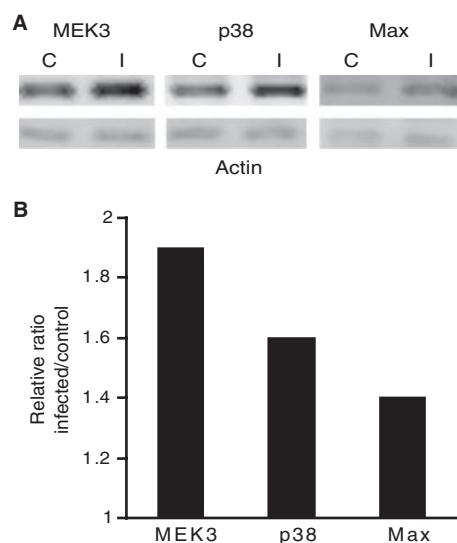


Figure 2 HTR-8 genes upregulated by *Porphyromonas gingivalis* show a corresponding increase in protein levels. (A) Immunoblots of control uninfected (C) and *P. gingivalis*-infected (I) HTR-8 cells. The blots were probed with antibodies to MEK3, p38 and Max. Duplicate samples were probed with antibodies to β -actin (lower panel). (B) Densitometric analyses of the ratio of test protein band intensity to β -actin band intensity relative to the uninfected control. Data are representative of three independent experiments.

Correlation between messenger RNA, protein levels and activation status of MAPK signaling pathway

As messenger RNA levels do not necessarily reflect protein levels or activity, we examined protein levels of MEK3, p38 and Max in cells stimulated by *P. gingivalis* under the same conditions as the microarray experiment. Immunoblots (Fig. 2) showed increased expression of MEK3, p38 and Max, consistent with the transcription data. Furthermore, because the activity of the MAPK pathway depends on phosphorylation/dephosphorylation of the components, we investigated the ability of *P. gingivalis* to induce activation of MEK3 and p38. As shown in Figs 3 and 4, *P. gingivalis* infection of HTR-8 cells resulted in increased phosphorylation of MEK3 and p38, indicating that *P. gingivalis*

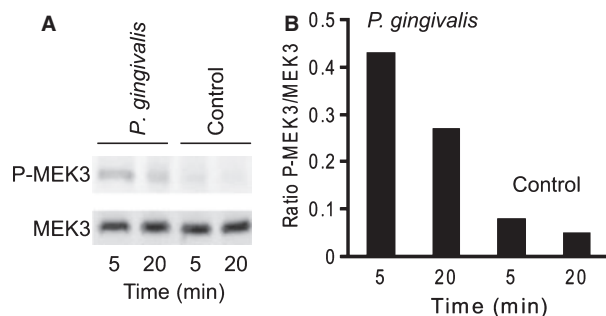


Figure 3 MEK3 is phosphorylated in response to *Porphyromonas gingivalis* infection. (A) Immunoblots of control and infected HTR-8 cells probed with antibodies to MEK3 and phospho- (P) MEK3. (B) Densitometric analysis of the ratio of the P-MEK3 band to the MEK3 band. Data are representative of three independent experiments.

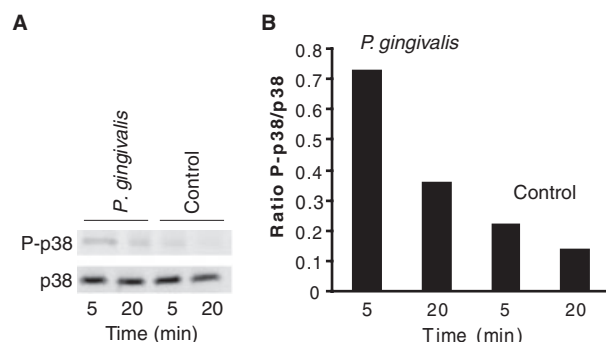


Figure 4 p38 is phosphorylated in response to *Porphyromonas gingivalis* infection. (A) Immunoblots of control and infected HTR-8 cells probed with antibodies to p38 and phospho- (P) p38. (B) Densitometric analysis of the ratio of the P-p38 band to the p38 band. Data are representative of three independent experiments.

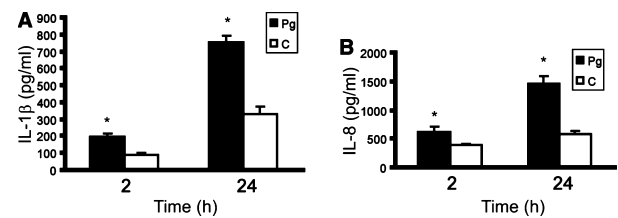


Figure 5 *Porphyromonas gingivalis* induces interleukin (IL)-1 β and IL-8 production in HTR-8 cells. Enzyme-linked immunosorbent assay of IL-1 β in HTR-8 lysates (A) and IL-8 in HTR-8 cell supernatants (B) following coculture with *P. gingivalis* for 2 or 24 h. Control (C) cells were uninfected. Error bars indicate standard deviation ($n = 3$). * $P < 0.05$ (Student's *t*-test) compared with control.

enhances both the level and activation status of components of the MAPK signaling pathway.

HTR-8 cytokine responses to *P. gingivalis*

Activation of p38 can lead to stimulation of inflammatory cytokine secretion, and IL-1 β and IL-8 were up-regulated at the transcriptional level in the array experiment. However, control of cytokine secretion is hierarchical and can occur at the transcriptional and posttranscriptional levels. Hence we assessed the phenotypic significance of the ontology analysis by directly assaying at the protein level the proinflammatory cytokines IL-1 β and IL-8. Production of IL-1 β and IL-8 by HTR-8 cells following stimulation with *P. gingivalis* was monitored over time (Fig. 5). Levels of both cytokines were elevated in response to *P. gingivalis* after 2 h of infection and continued to rise over a 24-h period, indicating that infection with *P. gingivalis* induces a proinflammatory phenotype in placental cells.

DISCUSSION

Trophoblasts are essential for the adequate exchange of key molecules between the maternal and fetal circulation, and form an important physical barrier that protects the fetus from microbial intrusion. Successful hematogenously derived fetal pathogens are capable of traversing the placenta, in some cases by invading and damaging trophoblasts (Disson *et al.*, 2008). Accumulating evidence implicates *P. gingivalis* and other periodontal pathogens in fetal complications such as PTLBW delivery (Xiong *et al.*, 2006; Michalowicz & Durand, 2007). The physiological status of the trophoblasts is important for normal

pregnancy progression. Trophoblasts regulate transcriptional activity on a global scale according to environmental conditions (Fukushima *et al.*, 2008), and following bacterial stimulation (Carvalho Neta *et al.*, 2008). In the current study we found that stimulation of trophoblasts with *P. gingivalis* differentially regulated expression of over 2000 genes, indicating that *P. gingivalis* has a major affect on normal trophoblast function with the consequent potential for adverse placental function. The differentially regulated genes could be assembled into 18 significantly impacted KEGG pathways. These pathways include cell cycle and apoptosis, consistent with the ability of *P. gingivalis* to induce G1 arrest and apoptosis in trophoblasts (Inaba *et al.*, 2009). Another pathway significantly impacted was MAPK, and upregulation of one branch of this pathway (MEK3, p38 and Max) was confirmed at the protein level. For many signaling pathways, such as MAPK, the level of constituent proteins is less important for information flow than is phosphorylation status. *P. gingivalis* induced phosphorylation, and therefore activation, of both MEK and p38, indicating that *P. gingivalis* infection of trophoblasts does stimulate signal transduction by the MAPK pathway.

The activation of the MEK3–p38 component of the MAPK pathway is predicted to lead to increased production of proinflammatory cytokines (Patil & Kirkwood, 2007). This has relevance to PTLBW delivery, because adverse pregnancy outcomes can result from disruption of normal immune and inflammatory status (Goldenberg *et al.*, 2000; Hirsch & Wang, 2005). Gestational tissues synthesize a variety of inflammatory mediators including cytokines and prostaglandins, which play important roles in normal pregnancy processes such as uterine contraction. Trophoblasts also express pattern recognition receptors and hence bacterial infection can evoke elevated levels of cytokines and prostaglandins, such as IL-1, IL-6, IL-8, IL-10, tumor necrosis factor- α and prostaglandin E₂. Imbalances in these inflammatory mediators act as a trigger for the initiation of infection-induced preterm labor (Griesinger *et al.*, 2001; Hirsch & Wang, 2005). Furthermore, proinflammatory cytokines can recruit and activate neutrophils, resulting in the release of matrix metalloproteinases that damage chorioamniotic membranes (Goldenberg *et al.*, 2000). *P. gingivalis* infection induced higher levels of IL-1 β and IL-8. Elevated production of both

of these cytokines has been associated with PTLBW infants. Serum or vaginal IL-1 β and IL-8 levels are also predictive of preterm delivery (Leitich, 2005; Torbe *et al.*, 2007a,b; Bogavac & Brkic, 2009).

The interaction between *P. gingivalis* and trophoblasts differs from that between *P. gingivalis* and gingival epithelial cells in the oral cavity. The latter interaction results in antagonism of IL-8 production (Darveau *et al.*, 1998) and suppression of apoptosis (Nakhjiri *et al.*, 2001; Yilmaz *et al.*, 2004; Mao *et al.*, 2007) in an overall less disruptive interaction that may reflect a long-term evolutionary balanced relationship (Handfield *et al.*, 2008). In contrast, trophoblasts and *P. gingivalis* have not had the opportunity to adapt to each other over evolutionary time and hence the organism may be a more acute pathogen in placental tissues. Recent evidence indicates that *P. gingivalis* is common in placental tissues both in pregnancies that proceed to term and in those that terminate prematurely (Katz *et al.*, 2009). As a consequence, the presence of *P. gingivalis* alone may be insufficient to induce PTLBW delivery; nonetheless the ability of the organism to disrupt the transcriptome, signal transduction and cytokine production are characteristics that would make *P. gingivalis* a potent contributory factor in PTLBW delivery.

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