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Differential host response to LPS variants in amniochorion and the TLR4/MD-2 system in *Macaca nemestrina*

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Abstract

OBJECTIVES—Microbial-specific factors are likely critical in determining whether bacteria trigger preterm labor. Structural variations in lipopolysaccharide (LPS), a component of gram-negative bacteria, can determine whether LPS has an inflammatory (agonist) or anti-inflammatory (antagonist) effect through Toll-like receptor 4 (TLR4). Our objective was to determine whether amniochorion can discriminate between LPS variants in a nonhuman primate model. We also cloned *Macaca nemestrina* TLR4 and MD-2 and compared this complex functionally to the human homologue to establish whether nonhuman primates could be used to study TLR4 signaling in preterm birth.

STUDY DESIGN—Amniochorion explants from *M. nemestrina* were stimulated with a panel of LPS variants for 24 hours. Supernatants were analyzed for IL-1 β , TNF- α , IL-6, IL-8 and prostaglandins E2 and F2 α . Tissue expression of TLR1, 2, 4, 6, MyD88 and NF- κ B was studied by RT-PCR. *M. nemestrina* TLR4 and MD2 genes were cloned and compared with their human counterparts in a recombinant TLR4 signaling system to determine LPS sensitivity.

RESULTS—LPS variants differentially stimulated cytokines and prostaglandins, which was not related to transcriptional changes of TLR4 or other TLRs. Nearly all elements of LPS binding and TLR4 leucine-rich repeats were conserved between humans and *M. nemestrina*. TLR4/MD-2 signaling complexes from both species were equally sensitive to LPS variants.

CONCLUSIONS—LPS variants elicit a hierarchical inflammatory response within amniochorion that may contribute to preterm birth. LPS sensitivity is similar between *M. nemestrina* and humans, validating *M. nemestrina* as an appropriate model to study TLR4 signaling in preterm birth.

Keywords

amniochorion; LPS; TLR4; TLR4 antagonist; *Macaca nemestrina*; nonhuman primate

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INTRODUCTION

Preterm birth, defined as birth before 37 completed weeks of gestation, remains a major challenge in obstetrics and is the most important direct cause of neonatal mortality [1]. Prematurity complicates 1 in 8 pregnancies with an annual economic burden of 26 billion dollars [2]. A large body of evidence suggests that up to 50% of very premature births (less than 30 weeks gestation) are caused by intrauterine infections [3]. Premature births have recently been associated with other infections such as periodontitis, a common bacterial infection of the dental periosteum characterized by a localized, chronic inflammatory response. Bacteria associated with periodontitis may also enter the bloodstream and infect the placenta [4–6]. However, the magnitude of the association between periodontitis and premature delivery is modest (odds ratios 2–3) and inconsistent. Further, randomized treatment trials of periodontitis in pregnancy have not consistently demonstrated reductions in preterm birth [7–9].

Lack of a consistent association between periodontitis and premature birth may be due to differences in bacterial pathogenicity. More than 300 bacterial species have been isolated from the oral cavity. [10,11] Principally, three gram negative species, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, have been associated with chronic periodontitis. Local and systemic inflammatory responses to these bacteria are modulated by differences in bacterial species and lipopolysaccharide (LPS) [12]. The bioactive component of LPS, lipid A, is recognized by the Toll-like 4 receptor (TLR4), which triggers an innate immune response through NF- κ B activation and cytokine gene transcription. While the response to the prototypical LPS structure (e.g. *E. coli*) is very potent, LPS structural variants have been reported for many bacteria, including *P. gingivalis*, for which the innate immune response is weak [13,14]. In addition, *P. gingivalis* is capable of synthesizing different LPS structures, in response to environmental conditions, which have differing abilities to weakly activate or antagonize TLR4 [15]. Modification of LPS represents a survival strategy that allows the bacterium to evade host immunity, replicate, and invade further into the host [16–18].

TLR4 is strongly expressed by the fetal membranes and its activation is critical for LPS-induced preterm birth [19,20]. We hypothesized that amniochorion can mount variable degrees of inflammation in response to the strength of TLR4 signaling. Since there is a link between periodontitis and preterm birth we decided to test LPS variants of *P. gingivalis*, a bacterium strongly implicated in periodontitis. These *P. gingivalis* LPS structural variants have been previously well characterized both structurally and functionally. In a nonhuman primate (*Macaca nemestrina*) model, we used an amniochorion explant system to test the ability of fetal membranes to discriminate between LPS variants that differentially activate TLR4. We also cloned *M. nemestrina* TLR4 and MD-2 and compared this complex functionally to the human homologue to establish our explant system as a model for studying TLR4 signaling in preterm birth.

MATERIALS AND METHODS

LPS structural variants

LPS variants from *E. coli* K-12 strain JM83 and *P. gingivalis* have been previously isolated, purified, and characterized (Figure 1) [14,15,21]. The lipid A structures have been elucidated by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry [14,22]. The strong TLR4 agonist was an *E. coli*-derived LPS (EcLPS). The weak TLR4 agonist was produced by *P. gingivalis* grown under low haemin conditions (PgLPSweak) [14,23]. We used two TLR4 antagonists. The first was an *E. coli* mutant with an altered LPS which down-

regulates the host response to LPS (LPSmsbB) [24]. The second was derived from *P. gingivalis* grown under high haemin conditions (PgLPSantag) [15,25].

Amniochorion explant system and LPS stimulation

Pregnant *M. nemestrina* (n=6) underwent cesarean section at term prior to the onset of labor. Amniochorion from these placentas were cleared of decidua and blood using gauze and washed in Hank's Balanced Salt Solution. A single six millimeter amniochorion punch biopsy was placed in each cell culture insert within a 24-well tissue culture plate (Falcon). The explants were cultured in Dulbecco's modified Eagle's medium Ham's F12 nutrient mixture (1:1) with 15% heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and Amphotericin B (0.025 ug/ml) for 48 hours at 37 °C in 5% CO₂; culture media and room air was changed daily. The explants were cultured for 48 hours prior to initiation of the experiment to allow for a return to baseline of inflammatory mediators stimulated by the physical manipulation of tissue during initial preparation.[26] Light microscopy was performed daily to inspect each well for bacterial contamination. After 48 hours, each explant was treated for 24 hours with culture medium (control) or 10 ng/ml of EcLPS, LPSmsbB, LPSweak, or LPSantag. At 24 hours, supernatants from each well were collected and stored at -80°C until processing. Explants were then placed in RNeasy lysis buffer (Qiagen, Valencia, CA) and incubated at 4°C prior to storing at -80°C.

Quantitation of cytokines and prostaglandins

Supernatants were tested for IL-1 β , TNF- α , IL-6 and IL-8 using a nonhuman primate cytokine multiplex immunoassay (Millipore, Billerica, MA) with Luminex technology (Luminex Corp, Austin, TX). Supernatants were also assayed for PGE₂ and PGF₂ α using commercially available ELISA kits (Cayman Chemical, Ann Arbor, MI) known to cross-react with *M. nemestrina* antigens.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extractions were performed using a standard TRIzol (Invitrogen, Carlsbad, CA, USA) method and concentrations ascertained using a NanoDrop-1000 (Thermo Scientific, Waltham, MA, USA). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Complementary DNAs (cDNA) were made with SuperScript III First-Strand Synthesis System (Invitrogen Inc., Carlsbad, CA) following the manufacturer's protocol for random hexamers.

Primers and probes to quantitate gene expression of TLR1 (Rh02787277_s1), TLR2 (Rh02787279_s1), TLR3 (Rh02845008_m1), TLR4 (Rh01564846_m1), TLR6 (Rh00414981_m1), and 18S rRNA (Rh00414981_m1) were ordered through Applied Biosystem's Assay-on-Demand (Applied Biosystems Inc.). MyD88 and NF- κ B cDNA primers and probes were designed based on published sequences for *Macaca mulatta* with the following sequences: MyD88 – FWD: 5'CCTGCAGAGCAAGGAATGTGA3', REV: 5'ACCTGGAGAGAGGCTGAGT3', 5'-FAM-TTCCAGACCAAATTTG-NFQ-3'. NF- κ B – FWD: 5'CACGTGGCCGCTACCTA3', REV: 5'GGACCCCGGAGTTAAGCA3', 5'-FAM-CTCCCAGGAGTTCTC-NFQ-3'. For all genes, probes were labeled with [6-carboxy-fluorescein] and a Nonfluorescent Quencher (NFQ).

qRT-PCR was performed on ABI PRISM 7900HT system (Applied Biosystems Inc., Foster City, CA, USA) using the FAST system in a 12 ul reaction consisting of 20 ng cDNA, 0.5 $\times$ TaqMan Gene Expression Fast Universal Master Mix (Applied Biosystems Inc.), and 0.5 $\times$ Assay Mix (Applied Biosystems Inc.). The reaction profile consisted of denaturing at 95°C for 15 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Data analysis was completed using the Sequence Detection Systems V2.2.2 software (Applied Biosystems Inc.).

Cloning of *M. nemestrina* TLR4 and MD-2

M. nemestrina genes encoding TLR4 and MD-2 were cloned from RNA isolated from peripheral blood mononuclear cells (PBMC). PBMC were extracted from *M. nemestrina* whole blood by Ficoll Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation (1.077 g/ml density). RNA was isolated from 10^6 cells using RNeasy (Qiagen). Since the *M. nemestrina* genome has not been sequenced, primers were designed using the sequenced and annotated genome of *M. mulatta*, the closest sequenced relative. Using *M. mulatta* as the reference strain, primers (Table 1) were designed to amplify *M. nemestrina* *tlr4* and *md-2* genes by RT-PCR using extracted RNA as the template. Both *tlr4* and *md-2* genes were amplified and sequenced twice from independently isolated RNA samples. The *md-2* gene was amplified using primers SJ110 and SJ123, digested with NheI and PmlI, and cloned into the NheI and EcoRV sites of the plasmid vector pcDNA3.1-, generating the plasmid pSJ96. The *tlr4* gene was amplified in three fragments utilizing the internal EcoRI and HinDIII sites. The first fragment was amplified using primers SJ116 and SJ113, digested with NheI and EcoRI, and cloned into the NheI and EcoRI sites of pcDNA3.1-. The middle fragment was added as an EcoRI-HinDIII fragment and amplified using primers SJ112 and SJ115. The last piece was added on a HinDIII-PmeI fragment and amplified using primers SJ114 and SJ117. The final plasmid, pSJ90, contained the full length *M. nemestrina* *tlr4* gene. Sequence data have been submitted to the GenBank database under accession number GU969614 (*tlr4*) and GU969615 (*md-2*).

Recombinant TLR4 Signaling System

Human embryonic kidney cells (HEK293) were transfected with plasmids encoding either *M. nemestrina* or human TLR4 and MD-2. These cells were also transfected with reporter plasmids pNF- κ B-TA-luciferase and p β -actin-*Renilla*-luciferase which are part of the dual luciferase assay system used to measure transcription of a reporter plasmid by NF- κ B, a transcription factor activated by LPS stimulation of TLR4 [25].

HEK293 was maintained in Dulbecco's Modified Eagle Medium (Invitrogen Life Technologies) and supplemented with 10% heat-inactivated Fetal Bovine Serum (HyClone), penicillin (100 U/ml) and streptomycin (100 ug/ml). HEK293 cells were plated in 96-well plates at a density of 4×10^4 cells/well and transfected the following day by a standard calcium phosphate precipitation method. The amounts of plasmids transfected were as follows: pNF- κ B-TA-Luc (2 ng), p β -actin-*Renilla*-Luc (0.3 ng), pTLR4 (2 ng), and pMD-2 (2.5 ng). The empty expression vector, pDisplay, was used to adjust the total amount of DNA transfected per well up to 0.1 ug. After 24 h, the transfected cells were stimulated in triplicate for 4 h with LPS. Stimulation medium contained 10% human serum, which is rich in soluble CD14 required for LPS transfer to TLR4/MD-2. After stimulation, the cells were lysed with 50 ul of passive lysis buffer (Promega). Luciferase activity of 10 ul of each lysate was measured using the Dual Luciferase Assay Reporter System (Promega). Data are expressed as the ratio of NF- κ B-dependent firefly luciferase activity to the constitutive β -actin promoter-dependent *Renilla* luciferase activity. Fold-increase of the above ratio from LPS-stimulated samples was compared to unstimulated samples.

Statistical analysis

Data were log transformed and then analyzed using ANOVA with a Bonferroni correction for multiple comparisons using Intercooled STATA 9.0 (StataCorp LP, College Station, Texas). Comparisons between experimental conditions in the explant model were between explants cultured in LPS or media (control) for 24 hours after the initial 48 hours in culture. To compare human and *M. nemestrina* activation of the reporter assay in the recombinant TLR4 signaling system, a Wilcoxon rank sum test with Bonferroni correction was performed using Intercooled STATA 9.0.

RESULTS

A strong TLR4 agonist is necessary to stimulate cytokine and prostaglandin synthesis in fetal membranes

To determine if LPS variants elicit a hierarchical inflammatory response in fetal membranes from a nonhuman primate, we used an amniochorion explant system from term *M. nemestrina* (N=6) [26,27]. Unstimulated membranes secreted low or undetectable levels of all cytokines and prostaglandins tested (Figure 2). Compared to controls, EcLPS induced significantly higher levels of TNF- α , IL-6, and IL-8 (all $p < 0.01$); IL-1 β levels were elevated, but not significantly ($p = 0.05$). EcLPS stimulated significantly higher levels of TNF- α than the weak TLR4 agonist (PgLPSweak) or TLR4 antagonists (LPSmsbB, PgLPSantag; all $p < 0.01$). Levels of cytokines stimulated with TLR4 antagonists or weak agonist were generally similar to unstimulated controls, except in the case of LPSmsbB which induced higher IL-8 than present in controls ($p < 0.05$).

EcLPS induced significantly greater levels of PGE2 ($p < 0.05$) and PGF2 α ($p < 0.01$) compared to controls (Figure 2). EcLPS also induced higher PGE2 compared to PgLPSweak ($p < 0.05$). In comparison to all other LPS variants, EcLPS stimulated the greatest levels of PGF2 α ($p < 0.05$ for LPSmsbB, PgLPSantag; $p < 0.01$ for PgLPSweak). PGE2 and PGF2 α levels were similar after stimulation by culture media alone, a weak TLR4 agonist, or TLR4 antagonists.

LPS structural variants do not upregulate TLR4 mRNA in amniochorion

The effect of differential TLR4 signaling on transcription of several TLRs (TLR1, 2, 4, 6) and mediators of TLR4 signaling (MyD88, NF- κ B) was evaluated using quantitative reverse transcriptase-polymerase chain reaction assays after 24 hours of LPS stimulation (Figure 3). TLR4 mRNA was not altered in response to any LPS variant and levels remained similar to unstimulated controls. Although no comparisons were statistically significant, slightly higher TLR2 mRNA levels were found under all experimental conditions. Compared to the unstimulated control, TLR2 mRNA was increased 2.4-fold after stimulation with EcLPS.

M. nemestrina and human TLR4 and MD-2 are structurally similar with a nearly identical dimerization interface

In order to determine whether the *M. nemestrina* TLR4 and co-receptor MD-2, is functionally similar to that of human, we cloned them from the RNA of *M. nemestrina* peripheral blood. Sequence analysis revealed that the *M. nemestrina* *tlr4* cDNA is 2481 base pairs (bp) and, at the protein level, differs in only six amino acids (aa) from the *M. mulatta* TLR4 (99.3% identical; Supplementary Figures 1 and 2). The human *tlr4* cDNA is 2520 bp with an extra 13 aa at the carboxy-terminus of the protein. Over the aligned portions of 826 aa, *M. nemestrina* TLR4 differs from the human TLR4 at 48 aa residues (92.6% identical, 95.4% similar). The MD-2 protein sequence between *M. nemestrina* and *M. mulatta* is identical and both differ from human MD-2 by 11 aa residues scattered over the 160 aa protein (93.2% identical, 96.9% similar).

We further analyzed whether differences in aa residues might affect the TLR4-MD-2-LPS dimerization interface as recently revealed by the crystal structure of the human complex [28]. All TLR4 residues important for hydrophobic interactions (F440, L444, and F463) and charge interactions with LPS phosphates (K388, R264, K341, K362) are conserved. Three of the four TLR4 aa residues important for hydrogen bonding between TLR4 and MD-2 are identical (S416, N417, Q436) in *M. nemestrina* and human; only a single residue changed from glutamic acid (E439) to glutamine. The leucine-rich repeat modules in the main TLR4 dimerization interface (15–17) are also identical in human and *M. nemestrina* [29]. The MD-2 residues important for hydrophobic interactions (F126, I124, L87, M85, V82) and hydrogen

bonding (G123, K125, R90) are conserved between human and *M. nemestrina*; for the residues involved in charge interactions with phosphates, two are conserved (S118, K122) and a third is a conservative change (K58R). These results suggest that the *M. nemestrina* TLR4-MD-2-LPS complex has a nearly identical protein structure to that of the human in the regions critical for dimerization.

LPS Stimulation of *M. nemestrina* and human TLR4 recombinant signaling system

To compare human and *M. nemestrina* TLR4 activation in response to *E. coli* and multiple LPS variants, we used a recombinant TLR4 signaling system that has previously been established [25]. In HEK293 cells transfected with either *M. nemestrina* or human TLR4 and MD-2 encoding plasmids, NF- κ B expression was strikingly similar between the two species for all LPS variants tested (Figure 4). There were no significant differences in activation of the reporter assay between human and *M. nemestrina*. This suggests that the TLR4/MD-2 signaling complex in both species has a comparable capacity to respond to different LPS structures.

DISCUSSION

Many gram-negative bacteria, like *P. gingivalis*, can alter their LPS structures to modulate the host immune response, which may represent an important bacterial-specific virulence factor allowing invasion of the amniotic cavity and preterm labor. Our study objective was to determine whether LPS variants induced differential TLR4 signaling in macaque amniochorion and whether LPS sensitivity of human and *M. nemestrina* TLR4-MD-2 complex was similar. Overall, LPS induced a hierarchical inflammatory response that correlated with TLR4 activation. The strong TLR4 agonist elicited significantly higher levels of pro-inflammatory cytokines and prostaglandins than LPS variants known to be weak agonists or antagonists. These data suggest that LPS-induced inflammation in amniochorion requires a certain threshold of TLR4 signaling, which is determined by LPS structure. This requirement may play a role in determining whether an infection will elicit a robust response leading to rapid preterm birth or whether the host response can be evaded allowing for the development of a chronic infection leading to preterm birth after several weeks.

Comparable cytokine and prostaglandin responses for the weak TLR4 agonist and antagonists were expected, because a classic TLR4 antagonist is also a weak agonist. Prior comparison of gene activation profiles for the weak TLR4 agonist and TLR4 antagonists revealed few differences in human umbilical vein epithelial cells [30]. The modest elevations in IL-6 and IL-8 induced by the TLR4 antagonists and weak TLR4 agonist may have been due to TLR2 activation. Although synthetically made *P. gingivalis* lipid A does not activate TLR2, a complete LPS molecule may activate TLR2 in a region distinct from lipid A [31,32]. Alternatively, a contaminant from LPS extraction may activate TLR2.

The lack of transcriptional regulation of TLR4 in amniochorion is consistent with studies in other tissues [33,34]. Previous studies in an *ex vivo* model of human amniotic epithelium demonstrated TLR4 translocation from the apical surface to the basal membrane after LPS stimulation, which may represent one mechanism of posttranscriptional control [35]. TLR2 mRNA levels, however, were elevated in a pattern consistent with degree of TLR4 activation. *In vitro* studies using endothelial cells and murine alveolar macrophages also demonstrated TLR2 upregulation in response to LPS stimulation [36,37]. Whether this is due to TLR4 or TLR2 activation is unknown, but this finding suggests that activation of one TLR may alter amniochorion responsiveness of other TLR against future pathogens.

To our knowledge, this is the first time TLR4 and MD-2 from *M. nemestrina* have been cloned and LPS responsiveness characterized. The 95% similarity between human and *M. nemestrina* TLR4 proteins may be compared with 79% similarity with mouse and 64% with chicken TLR4

[38,39]. We have demonstrated that regions critical for LPS-TLR4 dimerization are essentially conserved; we would thus expect *M. nemestrina* TLR4/MD-2 to have a similar sensitivity to LPS as in the human homologues, which we have shown in a recombinant system. In this system, activation of NF- κ B by the weak agonist and antagonists was expected, because the system is designed to sensitively detect TLR4 activation; yet, in host cells, little cytokine transcription is observed accounting for the ability of antagonists and weak agonists to block stronger agonists. Cloning and functional characterization of a nonhuman primate TLR4/MD-2 with similar LPS sensitivity to human is important because species-specific differences in how the TLR4-MD-2 receptor complex recognizes variations in lipid A structures exist. For example, tetra- and penta-acylated LPS act as TLR4 antagonists or weak agonists in human cells, but as agonists in a murine system [40,41]. We found similar sensitivity of the human and *M. nemestrina* TLR4-MD-2 system to lipid A structures that are mostly hexa- (EcLPS), penta- (PgLPSweak) and tetra-acylated (PgLPSantag, LPSmsbB).

Currently, there are limited interventions to prevent preterm birth associated with infection. Microbial factors important in the pathogenesis of an ascending infection into the amniotic cavity are not well understood. Animal models have depended upon species in which the regulation of labor differs significantly from humans or upon expensive and relatively limited non-human primate models [42]. Altering LPS structure in order to antagonize TLR4 and downregulate the innate immune response has been proposed to account for the progression of periodontitis, bacterial survival and tissue invasion. Whether this mechanism could also dampen the placental immune response, promote bacterial survival and invasion of the amniotic cavity is unknown, but is supported by our prior work in a nonhuman primate model using a TLR4 antagonist to inhibit LPS-induced increases in pro-inflammatory cytokines and prostaglandins in amniotic fluid.[19] In addition to *P. gingivalis*, many other bacteria can modify LPS structures to evade host immunity [17,43,44]; key bacteria implicated in preterm birth have yet to be studied. Our results suggest that *M. nemestrina* has a TLR4-MD-2 receptor complex that differentially recognizes a variety of lipid A structures in a manner similar to humans. The monkey amniochorion explant model may allow investigation of TLR4 signaling and help identify potential intervention strategies to refine and limit whole animal experimentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Lawn JE, Wilczynska-Ketende K, Cousens SN. Estimating the causes of 4 million neonatal deaths in the year 2000. *Int J Epidemiol* 2006;35:706–718. [PubMed: 16556647]
2. Behrman, RE.; Stith Butler, A., editors. *Preterm Birth: Causes, Consequences, and Prevention*. Washington, D.C.: National Academies Press; 2006.
3. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* 2000;342:1500–1507. [PubMed: 10816189]
4. Vergnes JN, Sixou M. Preterm low birth weight and maternal periodontal status: a meta-analysis. *Am J Obstet Gynecol* 2007;196(135):e131–e137.

5. Han YW, Ikegami A, Bissada NF, Herbst M, Redline RW, Ashmead GG. Transmission of an uncultivated *Bergeyella* strain from the oral cavity to amniotic fluid in a case of preterm birth. *J Clin Microbiol* 2006;44:1475–1483. [PubMed: 16597879]
6. Han YW, Redline RW, Li M, Yin L, Hill GB, McCormick TS. *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. *Infect Immun* 2004;72:2272–2279. [PubMed: 15039352]
7. Michalowicz BS, Hodges JS, DiAngelis AJ, Lupo VR, Novak MJ, Ferguson JE, Buchanan W, Bofill J, Papapanou PN, Mitchell DA, Matseoane S, Tschida PA. Treatment of periodontal disease and the risk of preterm birth. *N Engl J Med* 2006;355:1885–1894. [PubMed: 17079762]
8. Offenbacher S, Beck JD, Jared HL, Mauriello SM, Mendoza LC, Couper DJ, Stewart DD, Murtha AP, Cochran DL, Dudley DJ, Reddy MS, Geurs NC, Hauth JC. Effects of periodontal therapy on rate of preterm delivery: a randomized controlled trial. *Obstet Gynecol* 2009;114:551–559. [PubMed: 19701034]
9. Polyzos NP, Polyzos IP, Mauri D, Tzioras S, Tsappi M, Cortinovis I, Casazza G. Effect of periodontal disease treatment during pregnancy on preterm birth incidence: a metaanalysis of randomized trials. *Am J Obstet Gynecol* 2009;200:225–232. [PubMed: 19254578]
10. Moore WE, Moore LV. The bacteria of periodontal diseases. *Periodontol* 2000 1994;5:66–77. [PubMed: 9673163]
11. Socransky SS, Haffajee AD, Dzink JL. Relationship of subgingival microbial complexes to clinical features at the sampled sites. *J Clin Periodontol* 1988;15:440–444. [PubMed: 3183069]
12. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. *Periodontol* 2000 1997;14:12–32. [PubMed: 9567964]
13. Reife RA, Shapiro RA, Bamber BA, Berry KK, Mick GE, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide is poorly recognized by molecular components of innate host defense in a mouse model of early inflammation. *Infect Immun* 1995;63:4686–4694. [PubMed: 7591124]
14. Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, Goodlett DR, Ernst RK, Darveau RP. Human Toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1- and 4'-phosphatase activities. *Cell Microbiol*. 2009
15. Al-Qutub MN, Braham PH, Karimi-Naser LM, Liu X, Genco CA, Darveau RP. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. *Infect Immun* 2006;74:4474–4485. [PubMed: 16861633]
16. Marsh PD, McDermid AS, McKee AS, Baskerville A. The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. *Microbiology* 1994;140(Pt 4): 861–865. [PubMed: 8012602]
17. Darveau RP, Charnetzky WT, Hurlbert RF, Hancock RE. Effects of growth temperature, 47-megadalton plasmid, and calcium deficiency on the outer membrane protein porin and lipopolysaccharide composition of *Yersinia pestis* EV76. *Infect Immun* 1983;42:1092–1101. [PubMed: 6315590]
18. Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* 1997;276:250–253. [PubMed: 9092473]
19. Adams Waldorf KM, Persing D, Novy MJ, Sadowsky DW, Gravett MG. Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. *Reprod Sci* 2008;15:121–127. [PubMed: 18187405]
20. Elovitz MA, Wang Z, Chien EK, Rychlik DF, Phillippe M. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. *Am J Pathol* 2003;163:2103–2111. [PubMed: 14578208]
21. Bainbridge BW, Coats SR, Pham TT, Reife RA, Darveau RP. Expression of a *Porphyromonas gingivalis* lipid A palmitylacyltransferase in *Escherichia coli* yields a chimeric lipid A with altered ability to stimulate interleukin-8 secretion. *Cell Microbiol* 2006;8:120–129. [PubMed: 16367871]
22. Kumada H, Haishima Y, Umemoto T, Tanamoto K. Structural study on the free lipid A isolated from lipopolysaccharide of *Porphyromonas gingivalis*. *J Bacteriol* 1995;177:2098–2106. [PubMed: 7721702]

23. Reife RA, Coats SR, Al-Qutub M, Dixon DM, Braham PA, Billharz RJ, Howald WN, Darveau RP. Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity: differential activities of tetra- and penta-acylated lipid A structures on E-selectin expression and TLR4 recognition. *Cell Microbiol* 2006;8:857–868. [PubMed: 16611234]
24. Somerville JE Jr, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP. A novel Escherichia coli lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J Clin Invest* 1996;97:359–365. [PubMed: 8567955]
25. Coats SR, Pham TT, Bainbridge BW, Reife RA, Darveau RP. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. *J Immunol* 2005;175:4490–4498. [PubMed: 16177092]
26. Fortunato SJ, Menon R, Swan KF, Lyden TW. Organ culture of amniochorionic membrane in vitro. *Am J Reprod Immunol* 1994;32:184–187. [PubMed: 7880402]
27. Menon R, Peltier MR, Eckardt J, Fortunato SJ. Diversity in cytokine response to bacteria associated with preterm birth by fetal membranes. *Am J Obstet Gynecol* 2009;201(306):e301–e306.
28. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;458:1191–1195. [PubMed: 19252480]
29. Matsushima N, Tanaka T, Enkhbayar P, Mikami T, Taga M, Yamada K, Kuroki Y. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics* 2007;8:124. [PubMed: 17517123]
30. Chen C, Coats SR, Bumgarner RE, Darveau RP. Hierarchical gene expression profiles of HUVEC stimulated by different lipid A structures obtained from Porphyromonas gingivalis and Escherichia coli. *Cell Microbiol* 2007;9:1028–1038. [PubMed: 17166236]
31. Kumada H, Haishima Y, Watanabe K, Hasegawa C, Tsuchiya T, Tanamoto K, Umemoto T. Biological properties of the native and synthetic lipid A of Porphyromonas gingivalis lipopolysaccharide. *Oral Microbiol Immunol* 2008;23:60–69. [PubMed: 18173800]
32. Sawada N, Ogawa T, Asai Y, Makimura Y, Sugiyama A. Toll-like receptor 4- dependent recognition of structurally different forms of chemically synthesized lipid As of Porphyromonas gingivalis. *Clin Exp Immunol* 2007;148:529–536. [PubMed: 17335558]
33. Nhu QM, Cuesta N, Vogel SN. Transcriptional regulation of lipopolysaccharide (LPS)-induced Toll-like receptor (TLR) expression in murine macrophages: role of interferon regulatory factors 1 (IRF-1) and 2 (IRF-2). *J Endotoxin Res* 2006;12:285–295. [PubMed: 17059692]
34. Fan J, Kapus A, Marsden PA, Li YH, Oreopoulos G, Marshall JC, Frantz S, Kelly RA, Medzhitov R, Rotstein OD. Regulation of Toll-like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. *J Immunol* 2002;168:5252–5259. [PubMed: 11994482]
35. Adams KM, Lucas J, Kapur RP, Stevens AM. LPS induces translocation of TLR4 in amniotic epithelium. *Placenta* 2007;28:477–481. [PubMed: 17055575]
36. Satta N, Kruihof EK, Reber G, de Moerloose P. Induction of TLR2 expression by inflammatory stimuli is required for endothelial cell responses to lipopeptides. *Mol Immunol* 2008;46:145–157. [PubMed: 18722665]
37. Oshikawa K, Sugiyama Y. Regulation of toll-like receptor 2 and 4 gene expression in murine alveolar macrophages. *Exp Lung Res* 2003;29:401–412. [PubMed: 12888452]
38. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999;189:615–625. [PubMed: 9989976]
39. Leveque G, Forgetta V, Morroll S, Smith AL, Bumstead N, Barrow P, Loreda-Osti JC, Morgan K, Malo D. Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica serovar Typhimurium infection in chickens. *Infect Immun* 2003;71:1116–1124. [PubMed: 12595422]
40. Rallabhandi P, Awomoyi A, Thomas KE, Phalipon A, Fujimoto Y, Fukase K, Kusumoto S, Qureshi N, Szein MB, Vogel SN. Differential activation of human TLR4 by Escherichia coli and Shigella flexneri 2a lipopolysaccharide: combined effects of lipid A acylation state and TLR4 polymorphisms on signaling. *J Immunol* 2008;180:1139–1147. [PubMed: 18178854]
41. Saitoh S, Akashi S, Yamada T, Tanimura N, Kobayashi M, Konno K, Matsumoto F, Fukase K, Kusumoto S, Nagai Y, Kusumoto Y, Kosugi A, Miyake K. Lipid A antagonist, lipid IVa, is distinct

- from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *Int Immunol* 2004;16:961–969. [PubMed: 15184344]
42. Elovitz MA, Mrinalini C. Animal models of preterm birth. *Trends Endocrinol Metab* 2004;15:479–487. [PubMed: 15541647]
43. Lepper PM, Triantafilou M, Schumann C, Schneider EM, Triantafilou K. Lipopolysaccharides from *Helicobacter pylori* can act as antagonists for Toll-like receptor 4. *Cell Microbiol* 2005;7:519–528. [PubMed: 15760452]
44. Triantafilou M, Gamper FG, Lepper PM, Mouratis MA, Schumann C, Harokopakis E, Schifferle RE, Hajishengallis G, Triantafilou K. Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells. *Cell Microbiol* 2007;9:2030–2039. [PubMed: 17419716]

TLR4 Agonists

TLR4 Antagonists

Figure 1.

Structures of LPS variants. PgLPSweak and PgLPSantag were isolated from *P. gingivalis* grown under low and high haemin conditions, respectively. The structures have been elucidated using MALDI-TOF and mass spectrometry.

Figure 2.

Luminex and ELISA analysis of cytokines and prostaglandins in amniochorion explants supernatant 24-hours after LPS stimulation tested were TNF- α , IL-1 β , IL-6, IL-8, PGE $_2$, and PGF $_{2\alpha}$. Values are means $\pm$ SEMs of N=6 independent experiments. Values were log-transformed and analyzed using ANOVA with a Bonferroni correction applied to pairwise comparisons.

Figure 3. Quantitative RT-PCR analysis of gene expression in amniochorion explants 24-hours after LPS stimulation for TLR1, TLR2, TLR4, TLR6, MyD88, and NF-κB. Values are means ± SEMs of N=6 independent experiments. Analysis by ANOVA with a Bonferroni correction yielded no significant differences.

Figure 4.

Recombinant TLR4 signaling system to compare human and *M. nemestrina* TLR4 activation in HEK293 cells. Data are expressed as the fold increase in relative light units (which represents the ratio of NF-κB-luciferase to β-actin *Renilla*-luciferase expression) relative to that of a no-stimulation control. There were no significant differences between human and *M. nemestrina* activation of the reporter assay by Wilcoxon rank sum with Bonferroni correction.

Table 1

Primers used to amplify *M. nemestrina md-2* and *tlr4* genes. Restriction sites are shown in lower case.

Primer name	Sequence	Restriction site
SJ110	TCAgctagcCTGCTTACTGAAAAGGAAGAGTCTG	NheI
SJ112	GGGATTGTGCAATTTGACCATTTGAAGaattcCGA	EcoRI
SJ113	TCGgaattcTTCAATGGTCAAATTGCACAATCCC	EcoRI
SJ114	CAGCATTTGACACACTCAACaagcttCAGGTAC	HinDIII
SJ115	GTACCTGaagcttGTTGAGTGTGTCAAATGCTG	HinDIII
SJ116	AAgctagcGGATGATGCCAGGATGACGTCTGC	NheI
SJ117	ATgtttaaaCTGGGCAAGAAATGCCTCAGGAGT	PmeI
SJ123	ATcacgtgCTCAATTTATTCTAATTTGAATTAGGTTGGTGTATGATG	PmlI