# Variable Cell Responses to *P. gingivalis* Lipopolysaccharide

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## ABSTRACT

*Porphyromonas gingivalis* is a major etiological agent of chronic periodontal diseases, the virulence of which has been attributed to different factors, including lipopolysaccharide (LPS). We investigated the differential responses induced by *P. gingivalis* LPS stimulation of human umbilical vein endothelial cells and human oral epithelial cells. RT-PCR analysis showed that *P. gingivalis* LPS used Toll-like receptor 2 (TLR2) to activate epithelial cells and Toll-like receptor 4 (TLR4) to activate endothelial cells. Both cell types were stimulated by *P. gingivalis* LPS to produce pro-inflammatory cytokines. Cytokine Array assay showed that although patterns of cytokine expression were similar in both cell types, some cytokines were specifically secreted by the endothelial cells, and others were specific to epithelial cells. These results support the idea that the same LPS preparation can act as a TLR2 or TLR4 agonist, depending on TLR expression of the host cell, inducing cytokine profiles that differ according to the cell type.

**KEY WORDS:** *Porphyromonas gingivalis*, lipopolysaccharide, Toll-like receptors, cytokines.

## INTRODUCTION

*Porphyromonas gingivalis* (*P. gingivalis*) has been implicated as a major etiological agent of periodontal disease development and progression (Socransky and Haffajee, 1992). Inflammation following *P. gingivalis* infection leads to the destruction of periodontal tissues and resorption of alveolar bone, and can ultimately result in the loss of the tooth (Darveau *et al.,* 1997). A likely candidate for the initiation of a destructive inflammatory response is lipopolysaccharide (LPS), since it has been well-established that this bacterial cell wall component is a potent stimulator of the innate host defense system. Two members of the Toll-like receptor family, TLR2 and TLR4, have been identified as signaling receptors for bacterial cell wall components (Beutler, 2002).

A well-characterized innate host recognition pathway is that for *Escherichia coli* LPS, which starts with the initial binding of LPS to LPS-binding protein (LBP) and CD14 (Schumann *et al*., 1990; Wright *et al*., 1990). Transfer of *E. coli* LPS by either mCD14 or sCD14 to a cell-associated TLR4 and MD-2 protein complex (Da Silva Correia *et al*., 2001) initiates host cell activation pathways, leading to innate host defense mediator production.

*P. gingivalis* LPS receptor usage is very controversial. It has been shown to signal through TLR2 or TLR4 to activate different cell types to produce cytokines, such as macrophages (Hirschfeld *et al*., 2001), gingival fibroblasts (Wang and Ohura, 2002), gingival epithelial cells (Sandros *et al*., 2000), and endothelial cells (Nassar *et al*., 2002). These discrepancies were hypothesized to be due to differences in LPS preparations, since differently acylated LPS Lipid A moieties induce different cellular effects (Darveau *et al*., 2004; Reife *et al*., 2006), or to lipoproteic contaminants (Hashimoto *et al*., 2004).

In the present study, we investigated the differential inflammatory responses induced by *P. gingivalis* LPS when TLR4-expressing human umbilical vein endothelial cells and TLR2-expressing human oral epithelial cells were stimulated.

## MATERIALS & METHODS

All of the products and techniques are detailed in the Appendix.

### Cell Culture

Human umbilical vein endothelial cells were obtained from Lonza (Lonza Ltd, St Bauzire, France) and maintained in Endothelial Basal Medium supplemented with Single Quots (Lonza Ltd) according to the supplier's indications. Only cell passages 3 to 8 were used.

Human oral epithelial cells are a non-tumoral immortalized oral keratinocyte cell line, TERT-2 OKF-6 (BWH Cell Culture and Microscopy Core, Boston,

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MA, USA). The cells were cultivated in Defined Keratinocyte-SFM basal medium (KSFM) supplemented with a cocktail of growth supplements from Invitrogen (Carlsbad, CA, USA).

### Cell Stimulations with *P. gingivalis* LPS

Endothelial cells and epithelial cells were stimulated by LPS at a final concentration of 1 μg/mL in complete cell culture medium supplemented with 10% heat-inactivated serum. Stimulations proceeded for 0, 0.5, 2, and 6 hrs, and were stopped when the cells were rinsed with PBS. In a second set of experiments, neutralizing monoclonal antibodies (mAbs) were used to block TLR2 and TLR4 agonist-induced cellular activation. Both cell types were pre-treated for 1 hr with the neutralizing mAbs at a final concentration of 1 μg/mL in the cell culture medium. The medium was then removed, the cells were washed with PBS, and the medium containing 1 μg/mL of LPS was added. Then, stimulations proceeded for 0, 0.5, 2, and 6 hrs.

### Enzyme-linked Immunosorbent Assay (ELISA)

Pro-inflammatory cytokine levels were measured in the supernatants of cells stimulated for 24 hrs. Interleukin (IL)-8 was measured in endothelial cells and tumor necrosis factor (TNF)-α in epithelial cell supernatants. ELISA assays were performed as instructed by the manufacturer (Strathmann Biotec AG, Hamburg, Germany). Each experiment was repeated three times, and the values are given as mean values ± standard deviations.

### Cytokine Antibody Array

Endothelial cells and epithelial cells were stimulated with 1 μg/mL LPS as described. Untreated cells were used as negative controls for cytokine secretion. Cell culture supernatants were collected after 24 hrs and analyzed with cytokine antibody array membranes (RayBiotech Inc., Norcross, GA, USA) according to the manufacturer's instructions. The protocol and the method for analysis of cytokine array membranes are detailed in the Appendix.

### Reverse-transcription-PCR (RT-PCR)

The levels of mRNA encoding for TLR2, TLR4, IL-8, TNF-α, and GAPDH were quantified by RT-PCR. Specific primer pairs and methods for quantification of RT-PCR end-products are presented in the Appendix. Each experiment was repeated at least four times, and the values are given as mean values ± standard deviations.

### Statistical Analysis

The non-parametric Kruskal-Wallis ANOVA on ranks and the non-parametric Mann-Whitney rank sum test were used in this study (see Appendix for details). Differences between two numbers were considered significant when the confidence interval exceeded 95% ($P < 0.05$).

## RESULTS

### *P. gingivalis* LPS Can Use Either TLR4 or TLR2 to Stimulate Cells

We checked the expression levels of TLR2 and TLR4 mRNA in endothelial cells and epithelial cells after a 1 μg/mL LPS challenge. RT-PCR experiments revealed that the endothelial cells expressed only TLR4 mRNA, the transcription of which was increased about 2.5-fold upon LPS stimulation (Fig. 1A). The use of a neutralizing TLR4 mAb led to a significant inhibition of TLR4 gene transcription. Conversely, the use of an anti-TLR2 antibody induced no significant change in TLR4 mRNA up-regulation, and the neutralization of both receptors led to the same inhibition as anti-TLR4 used alone (Fig. 1A). Epithelial cells were negative for the TLR4 mRNA, whereas TLR2 mRNA was detected with a 2.5-fold up-regulation upon LPS stimulation. Anti-TLR2 antibody antagonized the LPS stimulation, whereas anti-TLR4 had no effect, and pre-treatment with both mAbs showed no additional effect (Fig. 1B).

### Role of TLR2 and TLR4 in Cytokine Induction by *P. gingivalis* LPS

It has already been shown that *P. gingivalis* LPS triggers cytokine production from various cell types. To confirm receptor usage for cytokine synthesis, we investigated IL-8 and TNF-α expression and secretion in endothelial cells and in epithelial cells, respectively.

A 3-fold increase in the transcription of the IL-8 mRNA was observed as soon as 30 min post-stimulation in endothelial cells, and was sustained for 2 hrs before decreasing (Fig. 2A). Neutralizing anti-TLR4 mAb strongly inhibited the IL-8 mRNA up-regulation. Neutralization with anti-TLR2 mAb had no effect, and the use of both showed no additional inhibition. ELISA assays confirmed these observations at the protein level: A strong increase in IL-8 secretion, culminating at 1800 pg/mL upon LPS challenge, was inhibited by the neutralization of the TLR4 receptor, whereas the neutralization of TLR2 had no effect (Fig. 2B).

The stimulation of epithelial cells induced a 2.5-fold up-regulation of the TNF-α mRNA transcription, which was strongly inhibited by the use of the anti-TLR2 mAb only (Fig. 2C). ELISA assays confirmed the increased secretion of TNF-α, which culminated at 140 pg/mL at 24 hrs and was almost completely abolished when the epithelial cells were pre-treated with TLR2 mAb, whereas the use of TLR4 mAb had no effect (Fig. 2D).

### Differential Profiles of Cytokines Secreted in Endothelial Cells and Epithelial Cells upon LPS Stimulation

Having established that endothelial cells are activated through TLR4 and epithelial cells through TLR2, we decided to compare the overall pro-inflammatory signals induced by *P. gingivalis* LPS in these cells stimulated for 24 hrs with 1 μg/mL LPS. Cytokine profiles were examined with a cytokine antibody array that was able to detect 40 different cytokines. Supernatants of unstimulated

cells contained basal levels of some cytokines, in particular IL-8 and IP-10. Cytokines secreted upon LPS stimulation were divided into three categories: those secreted by both cell types, those secreted only or more strongly in endothelial cells, and those secreted only or more strongly in epithelial cells (Table). Although they exhibited some differences, the cytokine profiles of stimulated endothelial and epithelial cells were somewhat similar. Both cell types secreted interleukins involved in the innate immune response, including: IL-1α, IL-6, IL-6R, and IL-10; immunoregulatory cytokines, including IL-4, IL-12p40, and IL-12p70; chemokines, such as IP-10, MIP-1α, MIP-1β, and MIP-1δ; and growth and differentiation factors, such as ICAM-1, M-CSF, G-CSF, TIMP-2, and PDGF-BB. Stimulated endothelial cells specifically secreted soluble TNF receptors I and II, IL-13, IL-15, TGF-β, IL-8, MCP-1, and RANTES. TNF-α, IFN-γ, eotaxin, and eotaxin 2 were more strongly secreted by epithelial cells.

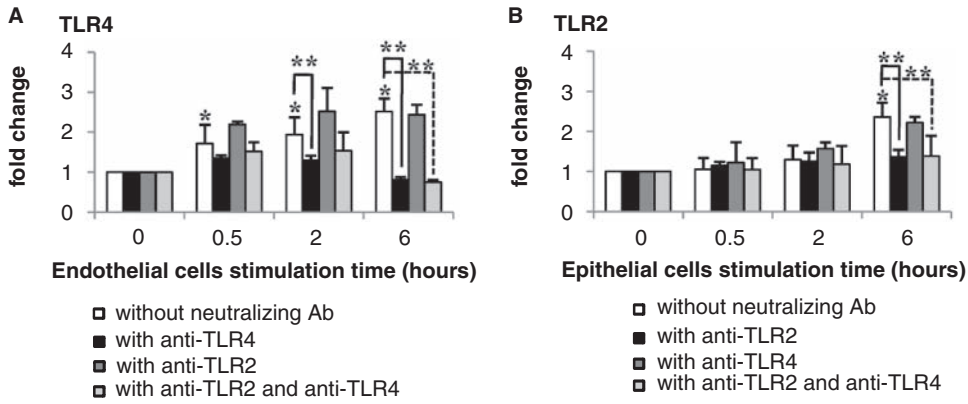


**Figure 1.** Effect of *Porphyromonas gingivalis* LPS on selected mRNA levels. *P. gingivalis* LPS stimulated TLR4 gene expression in human umbilical vein endothelial cells (HUVEC) **(A)** and TLR2 gene expression in human oral epithelial cells (HOEC) **(B)** according to the stimulation time. mRNA levels were evaluated by semi-quantitative RT-PCR in HUVEC and HOEC stimulated for 6 hrs by 1 μg/mL of LPS only, or were pre-treated with a neutralizing mAb directed against TLR2, TLR4, or both for 1 hr, and then stimulated by 1 μg/mL of LPS. Each experiment was repeated at least four times, and data were reported as the mean fold-change value ± standard deviation. *Statistical significance between *P. gingivalis* LPS-treated cells and time 0 ($P < 0.05$, ANOVA on ranks test). **Statistical significance between samples treated by LPS only and samples pre-treated with neutralizing antibodies before LPS stimulation ($P < 0.05$, Mann-Whitney rank sum test).

**Table.** Major Differences in Cytokine Secretion by Human Umbilical Vein Endothelial Cells and Human Oral Epithelial Cells Treated for 24 hrs with 1 μg/mL *P. gingivalis* LPS

| | Endothelial and Epithelial Cells[a] | Endothelial Cells[b] | Epithelial Cells[c] |
|---|---|---|---|
| IL of innate immunity | IL-6 | IL-15 | IL-1α |
| | IL-6R | TNFRI | TNF-α |
| | IL-10 | TNFRII | |
| IL of specific immunity | IL-12p40 | IL-13 | IFN-γ |
| | IL-12p70 | TGF-β | |
| | IL-4 | | |
| Chemokines | IP-10 | IL-8 | Eotaxin |
| | MIP-1α | MCP-1 | Eotaxin 2 |
| | MIP-1β | RANTES | |
| | MIP-1δ | | |
| Differentiation and growth factors | PDGF-BB | | |
| | ICAM-1 | | |
| | TIMP-2 | | |
| | G-CSF | | |
| | M-CSF | | |

Abbreviations: Interleukins (IL) of innate immunity: TNF-α, tumor necrosis factor α; IL-6R, interleukin-6 receptor; sTNFRI and sTNFRII, soluble TNF receptors I and II. Interleukins of specific immunity and immunoregulation: TGF-β1, Transforming Growth Factor β1; IFN-γ, interferon-γ. Chemokines: MCP-1, monocyte chemoattractant protein-1; MIP-1α, β, and δ, macrophage inflammatory protein 1α, β, and δ; RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted. Growth and differentiation factors: PDGF-BB, platelet-derived growth factor; ICAM-1, intercellular cell adhesion molecule 1; TIMP2, tissue inhibitor of matrix metalloproteinase 2; M-CSF macrophage-colony-stimulating factor; G-CSF, granulocyte-colony-stimulating factor.

[a] Cytokines strongly secreted by both cell types.

[b] Cytokines secreted more strongly or only by human umbilical vein endothelial cells.

[c] Cytokines secreted more strongly or only by human oral epithelial cells.

## DISCUSSION

*Porphyromonas gingivalis* is a pathogen that produces numerous virulence factors, including fimbriae and LPS that are detected by Toll-Like receptors of the innate immune system, resulting in cell activation and induction of pro-inflammatory responses from the host (Zhou *et al*., 2005).

TLR4 is the major signal transducer for most types of LPS, while TLR2 was suggested to be a signal transducer for other bacterial components, such as peptidoglycans and lipoproteins (Takeuchi *et al*., 1999). However, *P. gingivalis* LPS has been shown, in previous studies, to activate cells through either TLR4 (Wang and Ohura, 2002; Kumada *et al*., 2008) or TLR2 (Hirschfeld *et al*., 2001; Hajishengallis *et al*., 2006). These discrepancies were explained by trace amounts of lipoprotein contamination in native *P. gingivalis* LPS preparations (Hashimoto *et al*., 2004).

However, in this study, we used a commercial *P. gingivalis* LPS preparation purified from lipoprotein contaminants that non-specifically activate TLR2 (Hirschfeld *et al*., 2000). When

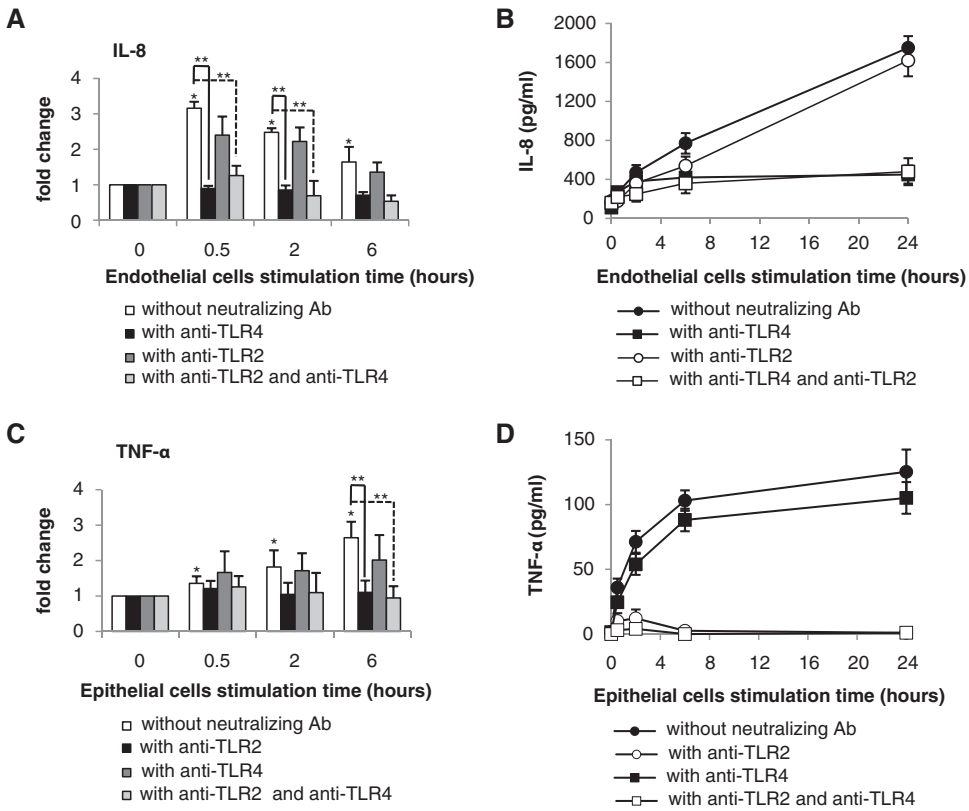


**Figure 2.** *P. gingivalis* LPS stimulated cytokine secretion by both endothelial and epithelial cells. IL-8 and TNF-α mRNA transcription was determined by semi-quantitative RT-PCR in human umbilical vein endothelial cells **(A)** and in human oral epithelial cells **(C),** respectively. IL-8 secretion by endothelial cells **(B)** and TNF-α secretion by epithelial cells **(D)** were quantified by ELISA. Cells were stimulated for various times by 1 μg/mL of *P. gingivalis* LPS or were pre-treated with a neutralizing mAb directed against TLR2, TLR4, or both for 1 hr, and then were stimulated by 1 μg/mL of LPS. Each experiment was repeated at least four times, and data were reported as the mean fold-change value ± standard deviation. *Statistical significance between LPS-treated cells and time 0 ($P < 0.05$, ANOVA on ranks test). **Statistical significance between samples treated by *P. gingivalis* LPS only and samples pre-treated with neutralizing antibodies before *P. gingivalis* LPS stimulation ($P < 0.05$, Mann-Whitney rank sum test).

endothelial and oral epithelial cells were challenged with this *P. gingivalis* LPS preparation, we observed that both TLR4-expressing endothelial cells and TLR2-expressing epithelial cells were able to respond to this LPS preparation. Therefore, our results support the idea that *P. gingivalis* LPS is a TLR2 and TLR4 agonist, depending on TLR expression of the host cell. This different receptor usage could be explained by the fact that even highly purified LPS preparations could contain different forms of the LPS lipid A moiety able to interact with both TLR2 and TLR4 (Darveau *et al*., 2004), thus inducing different cellular effects (Reife *et al.,* 2006; Kumada *et al.,* 2008). This suggests that *P. gingivalis* is able to modulate innate host responses by alterations in the relative amounts of these lipid A structures.

Analysis of our cytokine array data supports the hypothesis of a differential cellular effect by *P. gingivalis* LPS. TLR2 or TLR4 stimulation elicited a strong innate immune response in activated endothelial and epithelial cells, which resulted in the differential expression of a panel of inflammatory cytokines.

Several of the detected cytokines were produced by both cell types following *P. gingivalis* LPS stimulation. We detected, in particular, IL-1α and IL-6, which are involved in the acute-phase reaction and in periodontitis (Gabay and Kushner, 1999), and molecules involved in the recruitment and adhesion of leukocytes to inflammatory sites such as IP-10, MIP-1α, β, δ, ICAM-1, and M-CSF. However, only epithelial cells secreted TNF-α and Interferon-γ, which were detected at high levels in gingival crevicular fluids and periodontal tissues at diseased sites (Graves and Cochran, 2003), whereas IL-8, RANTES, and MCP-1 were secreted only by endothelial cells.

Why this differential response? In 2001, Hirschfeld *et al.* reported that murine macrophages stimulated by protein-free enterobacterial LPS and a similarly purified preparation of *P. gingivalis* LPS exhibited a differential expression of a panel of genes. They showed that *P. gingivalis* LPS exhibited potent Toll-like receptor 2 (TLR2), rather than TLR4, agonist activity, and they also showed that signaling through TLR2 and through TLR4 differed quantitatively and qualitatively. These findings were confirmed in a similar study on THP-1 cells (Zhang *et al*., 2008).

One hypothesis to explain this differential cytokine response could be a difference in the capacities of TLR2 and TLR4 to recruit additional signaling molecules to the LPS signaling complex. Human monocyte activation was found to be lipid-raft-dependent and to require the formation of heterotypic receptor complexes comprised of TLR2, TLR1, and CD11b/CD18 (Hajishengallis *et al*., 2006). Another hypothesis could be different affinities for CD14 of the different forms of *P. gingivalis* LPS lipid A moieties. Indeed, sCD14 is able to discriminate the slight structural differences between lipid A moiety from LPS preparations of *Fusobacterium nucleatum* and preparations of *Escherichia coli*-type synthetic lipid A (compound 506), which causes their distinct host cell activation abilities (Asai *et al.*, 2007). Finally, MD-2 enabled TLR2 to respond to non-activating protein-free LPS, LPS mutants, or lipid A by a physical association with TLR2, but this association appeared to be weaker than with TLR4, which could explain differential cellular effects (Dziarski *et al.*, 2001). Regardless of the mechanism, it appears that a divergence of upstream signaling complexes results in a differential distal gene expression.

The differential cytokine responses elicited by LPS stimulation of TLR2 or TLR4 could illustrate the course of infection

and pathology in *P. gingivalis* infection. In the early stage of infection, epithelial cells, which are the first line of immune defense, responded strongly to *P. gingivalis* LPS by producing IL-6, INF-γ, or TNF-α, leading to local tissue destruction. In the later stages of infection, bacteria and their virulence factors might move into the bloodstream through degraded gingival tissues and activate endothelial cells to express pro-inflammatory mediators such as IL-8 and MCP-1 that are involved in leukocyte chemotaxis and migration across the endothelium, as well as growth and differentiation factors, cell-adhesion molecules, and Toll-like receptors. Ultimately, *P. gingivalis* LPS stimulation could shift endothelial cells toward a pro-thrombotic state, which could be in line with recent studies demonstrating an association between periodontal disease and atherosclerosis (Scannapieco *et al.*, 2003). Indeed, atherogenesis is initiated by the binding and accumulation of leukocytes to the activated endothelium *via* the induced expression of adhesion molecules (Lusis, 2000), which could also be up-regulated by *P. gingivalis* LPS. Moreover, an increased expression of TLR2 and TLR4 in the aortas of hyperlipidemic mice was observed following oral challenge with *P. gingivalis* (Gibson *et al*., 2004). Finally, *P. gingivalis* has been identified in diseased atherosclerotic tissue (Haraszthy *et al*., 2000; Elkaim *et al*., 2008).

These findings could explain why a local persistent chronic infection may exert systemic effects by the interaction of periodontal pathogens or their virulence factors with the host immune system. However, further studies are needed to elucidate the precise molecular mechanisms by which periodontal pathogens could initiate or contribute to the progression of atherosclerosis.

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