J Clin Periodontol 2011; 38: 694–701 doi: 10.1111/j.1600-051X.2011.01741.x

Journal of Clinical Periodontology

Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity differentially modulates the expression of IL-6 and IL-8 in human gingival fibroblasts

Thanuja D. K. Herath[1], Yu Wang[2], Chaminda J. Seneviratne[1], Qian Lu[1], Richard P. Darveau[3], Cun-Yu Wang[4] and Lijian Jin[1]

[1]Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR, China; [2]Department of Pharmacology & Pharmacy, Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China; [3]School of Dentistry, University of Washington, Seattle, WA, USA; [4]School of Dentistry, University of California Los Angeles, Los Angeles, CA, USA

Herath TDK, Wang Y, Seneviratne CJ, Lu Q, Darveau RP, Wang CY, Jin L. Porphyromonas gingivalis lipopolysaccharide lipid A heterogeneity differentially modulates the expression of IL-6 and IL-8 in human gingival fibroblasts. J Clin Periodontol 2011; 38: 694–701. doi: 10.1111/j.1600-051X.2011.01741.x.

Abstract

Aim: *Porphyromonas gingivalis* lipopolysaccharide (LPS) displays a significant amount of structural heterogeneity, containing both tetra- ($LPS_{1435/1449}$) and penta-acylated (LPS_{1690}) lipid A structures. This study investigated the effects of the two isoforms of *P. gingivalis* LPS on the expression of IL-6, IL-8 and TNF-α in human gingival fibroblasts (HGFs).

Materials and methods: HGFs were stimulated with *P. gingivalis* $LPS_{1435/1449}$ and LPS_{1690} in both dose- (1 ng–10 μg/ml) and time-dependent (2–48 h) experiments. Total RNA and protein were extracted and used for analysis of the IL-6, IL-8 and TNF-α transcripts as well as IL-6 and IL-8 proteins, by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

Results: *P. gingivalis* LPS_{1690} significantly up-regulated the mRNA and protein expression of IL-6 and IL-8, whereas *P. gingivalis* $LPS_{1435/1449}$ did not induce significant host response. The expression levels of IL-6 and IL-8 up-regulated by *P. gingivalis* LPS_{1690} continuously increased with time course. In contrast, TNF-α transcript expression was up-regulated promptly by *P. gingivalis* LPS_{1690} after 2 h of stimulation and gradually declined afterwards.

Conclusions: This study suggests that *P. gingivalis* LPS heterogeneity may differentially modulate the pro-inflammatory cytokine expression in HGFs, which may contribute to periodontal pathogenesis.

Key words: cytokines; human gingival fibroblasts; lipopolysaccharide; periodontal disease; *Porphyromonas gingivalis*

Accepted for publication 20 April 2011

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.
This study was supported by the Hong Kong Research Grants Council (HKU766909M to LJJ).

Periodontitis is characterized by inflammatory destruction of periodontal tissues due to uncontrolled, detrimental bacteria–host interactions in susceptible individuals (Darveau 2010). *Porphyromonas gingivalis* is an important periodontopathogen and its effects differ significantly from those elicited by other pathogens (Kebschull & Papapanou 2011, Wade 2011). *P. gingivalis* lipopolysaccharide (LPS) is one of the key virulent attributes, which is significantly involved in periodontal pathogenesis (Bainbridge & Darveau 2001, Lu et al. 2009). In general, bacterial LPS contains three major components including

the outermost polysaccharide, core oligosaccharide regions and innermost lipid A structures (Dixon & Darveau 2005). Lipid A is biologically the most active part of LPS and it is a phosphorylated glucosamine disaccharide attached with multiple fatty acids. Its structure differs greatly among Gram-negative bacterial species depending on the differences in composition of attached fatty acids, number of phosphorylation sites and substituted groups attached to the phosphate residues (Dixon & Darveau 2005).

During periodontal disease process, *P. gingivalis* releases copious amount of outer membrane vesicles containing LPS that penetrate periodontal tissues and mediate an immuno-inflammatory response in the periodontal tissues (Darveau 2009). The exact nature and consequence of the host response to *P. gingivalis* LPS have been subjected to a debate, due to the conflicting results reported. Recently, it is revealed that *P. gingivalis* is able to generate heterogeneous LPS lipid A structures by changing the lipid A moiety through haemin-dependent modulation (Al-Qutub et al. 2006). Hence, *P. gingivalis* contains both tetra- ($LPS_{1435/1449}$) and penta-acylated (LPS_{1690}) lipid A structures and generates opposing effects on innate immune responses, thereby playing a key role in modulating host immuno-inflammatory response (Darveau et al. 2004, Al-Qutub et al. 2006, Reife et al. 2006).

Human gingival fibroblasts (HGFs) are the most abundant cell type in gingival tissues. They perpetually interact with microbes and their products, and the interplay between *P. gingivalis* and its LPS with HGFs plays a vital role in determining the healthy and diseased state of gingival tissues (Wang & Ohura 2002). Currently, it remains unknown how the heterogeneous lipid A structures of *P. gingivalis* LPS could affect host innate responses through modulation of the expression of pro-inflammatory cytokines in HGFs and the relevant clinical implications are largely unclear. This study investigated the modulatory effects of the two isoforms of *P. gingivalis* $LPS_{1435/1449}$ and LPS_{1690} on the expression of IL-6, IL-8 and TNF-α in HGFs.

Materials and Methods

Culture of HGFs

Primary HGFs were commercially obtained (ScienCell Research Laboratories, Carlsbad, CA, USA). The cells were continuously subcultured for over 10 passages and those in third to fourth passages were determined appropriate for the experiments without any signs of senescence. The cells suspended at 10^5 cell/ml were seeded on six-well-plates and grown until confluent at 37°C with 5% CO_2 in a culture medium for fibroblasts, consisting of basal medium with 2% foetal bovine serum containing penicillin/streptomycin (0.01% w/v) and fibroblast growth supplement (Di Domenico et al. 2003, Poggi et al. 2003).

LPS preparation and stimulation

P. gingivalis LPS was provided by one of the co-authors (R. P. D.). It was isolated from *P. gingivalis* ATCC 33277 and prepared by the cold $MgCl_2$–ethanol procedure as described previously (Darveau et al. 2004, Al-Qutub et al. 2006). It was further purified with $<0.1\%$ of protein contamination. The fatty acids composition was analysed by gas chromatographic–mass spectroscopy. Separate extractions of *P. gingivalis* $LPS_{1435/1449}$ and LPS_{1690} were then produced and analysed by the matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Escherichia coli* LPS (JM 83-wild type strain) was provided by R. P. D. as well.

In the dose-dependent assay, HGFs were treated with various concentrations of *P. gingivalis* $LPS_{1435/1449}$ and LPS_{1690} as well as *E. coli* LPS as a positive control at the concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml for 24 h. Three wells containing only the culture medium served as the negative controls. Upon the results of dose-dependent assay, 1 μg/ml of *P. gingivalis* LPS or *E. coli* LPS was selected as the optimal dose for the subsequent time-dependent assay. HGFs were then incubated with 1 μg/ml of *P. gingivalis* LPS or *E. coli* LPS and harvested at 2, 12, 24 and 48 h. HGFs treated with culture medium alone served as the negative controls.

The culture media were collected and centrifuged at 4°C to remove any contaminated cell debris, and stored at –70°C for assay of protein levels. The attached cells were then washed with PBS and collected for extraction of total RNA and protein, respectively.

cDNA synthesis and quantitative real-time polymerase chain reaction analysis (Q-RT-PCR)

Total RNA was extracted from the homogenized HGFs using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNA was synthesized by reverse transcriptase-PCR at 43°C for 90 min. in a 20 μl of reaction mixture containing 1 μg of total RNA, 1 μl (200 U) of SuperScript™ First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA), 0.5 μg of oligo dT-primer, first-strand buffer, 10 mM DTT and 1 mM dNTPs. A control reaction was performed without reverse transcriptase for all isolates to verify the absence of genomic DNA contamination. Q-RT-PCR was performed by using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in three separate experiments. Amplification reactions were undertaken in 20 μl of reaction mixture containing 10 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems), 1 μl of cDNA template and 1 μl of each pair of primers for the targeting cytokine genes (Sigma, St. Louis, MO, USA). Real-time primer pairs were designed using ABI software to amplify a sequence containing two or more exons whenever possible, with the amplification efficiencies over 90%. The specific sequences for each pair of primers are listed in Table 1. β-actin was amplified as an internal control. The Q-RT-PCR reaction conditions were set at 95°C for 10 min. followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The results were analysed using the comparative cycle threshold (Ct) method as described previously (Lu et al. 2009). The expression level of each gene was normalized to a β-actin (ΔCt) and the fold changes for each gene were calculated by comparing the test and control samples from the $\Delta\Delta$Ct values.

Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Total protein from the homogenized HGFs was extracted using CelLytic™ MT-mammalian cell lysis extraction reagent (Sigma). The protein concentrations in both of the cell-bound fraction and culture media were measured, respectively, by a protein assay following the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA, USA). The levels of IL-6 and IL-8 were determined by ELISA with a minimal detectable concentration of 0.70 and 10 pg/ml, respectively. The absorbance values were determined by a microplate reader (Victor, Vienna, VA, USA) at an optical absorbance of 450 nm. The final con-

centration was determined with reference to a standard curve.

Statistical analysis

All experiments were repeated in three assays for Q-RT-PCR and two assays for ELISA. The results were presented as mean ± SD. The statistical significance of differences between the data sets from the dose-dependent assay was evaluated by student *t*-test, one-way analysis of variance (ANOVA) and post hoc testing using both the Bonferroni and LSD methods. Additionally, the repeated measures of ANOVA were used to determine the differences between the data sets from the time-dependent assay. A *p*-value with <0.05 was considered statistically significant. All statistical analysis was performed using a software program (SPSS 14.0, SPSS Inc, Chicago, IL, USA).

Results

Dose-dependent assay

The expression of IL-6 and IL-8 in HGFs was differentially regulated by *P. gingivalis* LPS_{1690} and $LPS_{1435/1449}$. *P. gingivalis* LPS_{1690} significantly induced the expression of IL-6 and IL-8 in a dose-dependent manner both at transcriptomic (Fig. 1a and b) and protein levels (Fig. 2a and c). In contrast, *P. gingivalis* $LPS_{1435/1449}$ did not induce the expression of these cytokines. Compared with *P. gingivalis* $LPS_{1435/1449}$, *P. gingivalis* LPS_{1690} significantly up-regulated IL-6 and IL-8 expression at both transcriptomic and protein levels, respectively, ($p<0.05$) (Fig. 1a and b, and Fig. 2). In addition, *P. gingivalis* LPS_{1690} at 1 μg/ml and/or 10 μg/ml induced higher levels of IL-6 and IL-8 expression than *E. coli* LPS (Fig. 1a and b, and Fig. 2). Moreover, secretory IL-6 and IL-8 protein levels were higher than those in the cellular compartment (Fig. 2). TNF-α level was only measured at transcriptomic level using Q-RT-PCR (Fig. 1c). *P. gingivalis* LPS_{1690} significantly up-regulated TNF-α mRNA expression as compared with *P. gingivalis* $LPS_{1435/1449}$. However, *E. coli* LPS induced the highest expression of TNF-α transcripts (Fig. 1c).

Time-dependent assay

The expression of IL-6 and IL-8 in HGFs was up-regulated by *P. gingivalis* LPS_{1690} and *E. coli* LPS at different time points. IL-6 and IL-8 transcriptomic levels gradually increased and reached peak at 48 h (Fig. 3). Significantly increased expression of IL-6 and

Table 1. Nucleotide sequence of primers for real-time quantitative-polymerase chain reaction

Gene	Forward	Reverse
IL-6	AATCATCACTGGTCTTTTGGAG	GCATTTGTGGTTGGGTCA
IL-8	GAACCATCTCACTGTGTGTAAA	CACTCCTTGGCAAAACTG
TNF-α	TCTTCTCCTTCCTGATCGTG	GAAGATGATCTGACTGCCTG
β-actin	TTGGCAATGAGCGGTT	AGTTGAAGGTAGTTTCGTGGAT

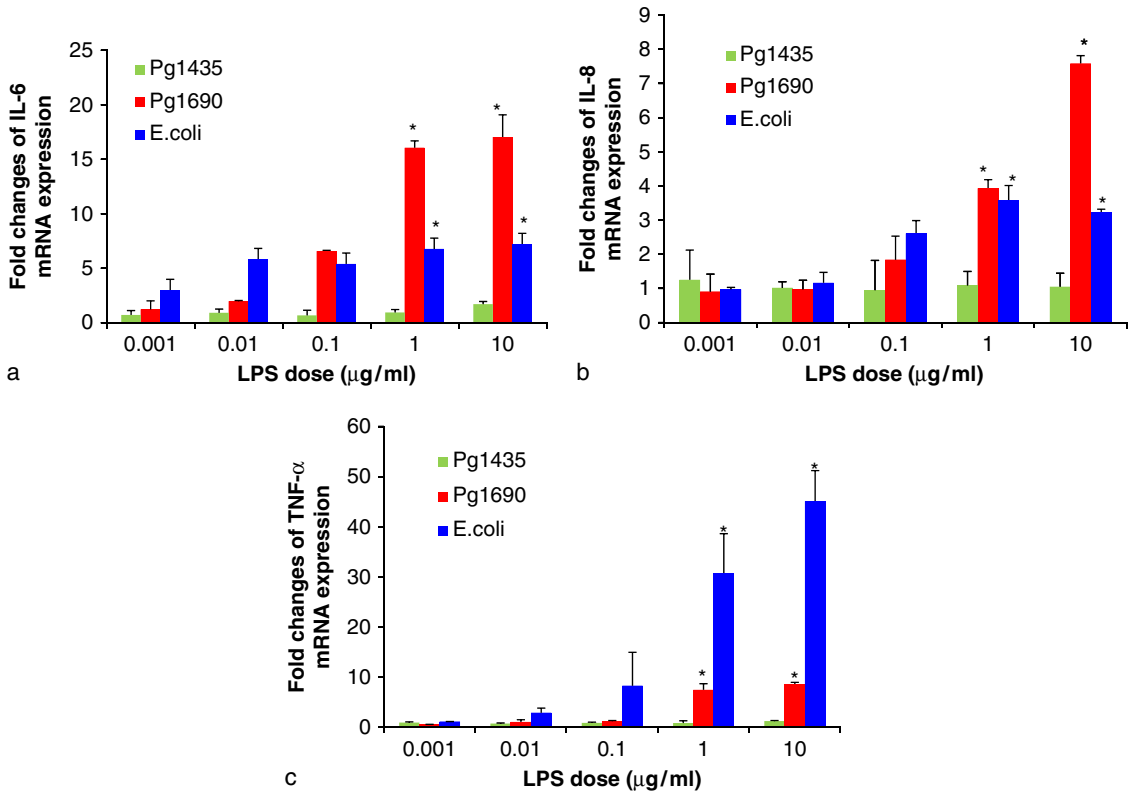

Fig. 1. Expression of IL-6 (a), IL-8 (b) and TNF-α (c) mRNAs in the cellular fractions of human gingival fibroblasts after the stimulation of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and *Escherichia coli* LPS in a dose-dependent assay (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml) for 24 h. The expression of mRNAs was measured by quantitative real-time polymerase chain reaction analysis. Each bar represents the mean ± SD of three independent experiments with three replicates. *Significant difference with a *p*-value <0.05 as compared with the controls without LPS treatment.

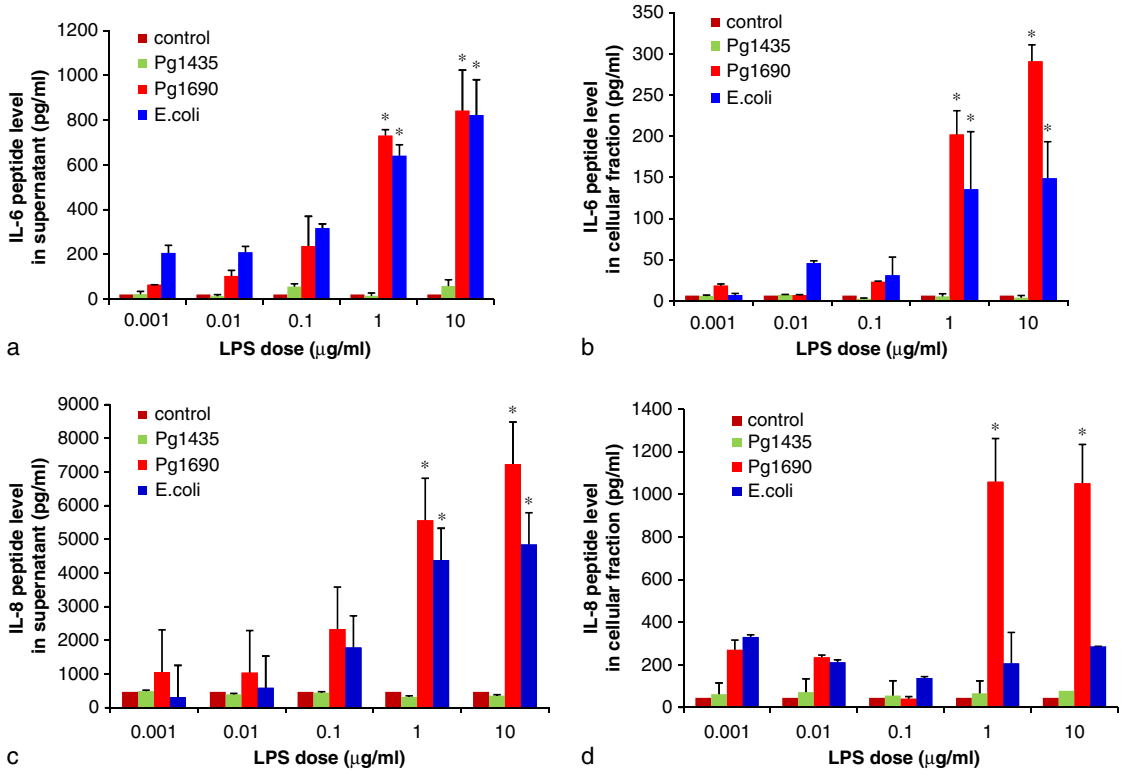

Fig. 2. Expression of IL-6 (a and b) and IL-8 (c and d) proteins in the culture supernatants (a and c) and cellular fractions (b and d) of human gingival fibroblasts after the stimulation of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and *Escherichia coli* LPS in a dose-dependent assay (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml) for 24 h. The protein expression levels were measured by enzyme-linked immunoadsorbent assay. Each bar represents the mean ± SD of three independent experiments with two replicates. *Significant difference with a p-value < 0.05 as compared with the controls without LPS treatment.

IL-8 levels were observed with reference to the controls at 24 h and 48 h (Figs. 3a and b, and 4a–c). In contrast, the up-regulation of IL-6 by *E. coli* LPS was more prompt and the expression reached the peak level within 2 h (Fig. 3a and 4b). Prolonged incubation with *P. gingivalis* LPS_{1690}, induced secretion of comparable levels of IL-6 in the culture supernatant and reached even higher level (but not significant) than that induced by *E. coli* LPS. In contrast, *P. gingivalis* $LPS_{1435/1449}$ did not show inducement in IL-8 mRNA or protein (Figs. 3b, 4c and d). *P. gingivalis* LPS_{1690} seemed to be a more potent stimulator in inducing IL-8 than *E .coli* LPS (Fig. 3b, and 4c and d).

TNF-α transcript level was rapidly induced at 2 h when incubated with *P. gingivalis* LPS_{1690} (Fig. 3c). However, this induction gradually decreased with incubation time.

Discussion

P. gingivalis LPS is a pathogen-associated molecular pattern (PAMP) and it has been a subject of continuous debate over the controversial data on its immuno-modulatory activity (Bainbridge & Darveau 2001, Dixon & Darveau 2005). Hence, some argue *P. gingivalis* LPS as a PAMP to induce the innate immune response, whilst others state that it facilitates the bacteria evading innate immune system (Yamazaki et al. 1992, Agarwal et al. 1995, Reife et al. 1995, Ogawa et al. 1996, Ogawa & Uchida 1996, Yoshimura et al. 1997). It is only recently that the significant role played by lipid A heterogeneity of Gram-negative bacterial LPS in modulating host innate response came into light (Darveau et al. 2004, Al-Qutub et al. 2006, Lu et al. 2009).

The present study examined for the first time the functional relevance of this structural heterogeneity of *P. gingivalis* LPS isoforms in HGFs as a modulator of the cytokine network. Penta-acylated *P. gingivalis* LPS_{1690} induced a significant host innate response in HGFs as evident from the higher levels of IL-6 and IL-8 expressions at both transcriptomic and protein levels. In contrast, when *P. gingivalis* $LPS_{1435/1449}$ interacted with HGFs, none of the aforementioned pro-inflammatory cytokines was expressed significantly. Hence, it appears that lipid A heterogeneity imparts a different immuno-modulatory activity on *P. gingivalis* LPS isoforms. Although no in vitro studies have been performed in HGFs, similar observations have been made when other cell types are induced with heterogeneous lipid A structure (Reife et al. 2006, Lu et al. 2009). For instance, *P. gingivalis* LPS with penta-acylated lipid A induces E-selection expression in human endothelial cells, whereas *P. gingivalis* LPS with tetra-acylated lipid A structures does not (Reife et al. 2006). Furthermore, *P. gingivalis* LPS with tetra- and penta-acylated lipid A structures interacts differently with TLR4, where *P. gingivalis* $LPS_{1435/1449}$ acts as a TLR4 antogonist for E-selection expression in human endothelial cells. We recently showed that the human β-defensins (hBD)-1, -2 and -3 levels in the reconstituted human gingival epithelia are significantly up-regulated by *P. gingivalis* LPS_{1690}. Conversely, hBD expression is down-regulated by *P. gingivalis* $LPS_{1435/1449}$

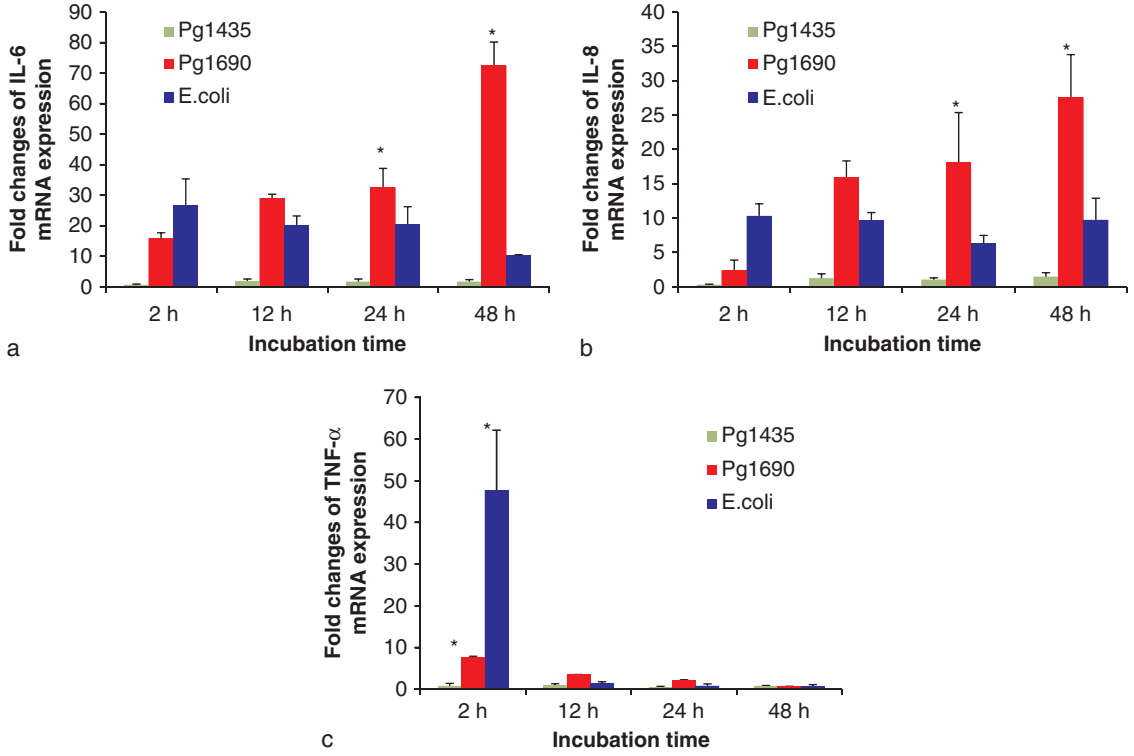

Fig. 3. Expression of IL-6 (a), IL-8 (b) and TNF-α (c) mRNAs in the cellular fractions of human gingival fibroblasts after the stimulation of *Porphyromonas gingivalis* lipopolysaccharide (LPS) (1 μg/ml) and *Escherichia coli* LPS (1 μg/ml) in a time-dependent assay (2–48 h). The expression of mRNAs was measured by quantitative real-time polymerase chain reaction analysis. Each bar represents the mean ± SD of three independent experiments with three replicates. *Significant difference with a p-value < 0.05 as compared with the controls without LPS treatment.

(Lu et al. 2009). It is also noteworthy that *P. gingivalis* LPS_{1690} elicits a higher and more persistent hBD-2 expression than *E. coli* LPS. Similarly, the present study shows that the HGFs exhibit a relatively prompt responsiveness to the hexa-acyl diphosphorylated lipid A of *E. coli* LPS, but a persistent responsiveness to the penta-acylated *P. gingivalis* LPS_{1690}. It could be hypothesized that the delayed response of IL-6 induction by *P. gingivalis* LPS_{1690} observed in the present study might induce a delayed host response which may facilitate *P. gingivalis* colonization and persistence. Further study is required to clarify this point.

Although lipid A is considered to be the most conserved moiety of the LPS molecule, recent studies have shown that relatively small changes in lipid A structure can greatly influence the host innate immune response (Dixon & Darveau 2005). In a previous study, Darveau and colleagues showed that the ability of *E. coli* LPS to orchestrate the innate immune response is considerably weak, when C12 fatty acid in the lipid A structure is replaced with C16 fatty acid as measured by the secretion of IL-8 levels (Bainbridge et al. 2006). Hence, it is conceivable that the lipid A structural changes in *P. gingivalis* LPS evoke a markedly different host innate response in HGFs as shown in the present study. Therefore, it is imperative to further discuss the implications of these findings along several lines.

Firstly, the present study provides additional evidence on the role of HGFs as a part of innate defense of gingival tissues. The primary function of HGFs is to maintain the tissue integrity and homeostasis by producing structural components such as collagen, elastin, glycoprotein and glycosaminoglycans. However, HGFs could also act as a regulator of the innate immune response by identifying the PAMP-like *P. gingivalis* LPS and respond by mounting an immuno-inflammatory response (Ara et al. 2009). Hence, HGFs are capable of expressing IL-6, IL-8 and TNF-α upon stimulation with LPS from oral bacteria (Tamura et al. 1992, Yamazaki et al. 1992, Kent et al. 1998, Tabeta et al. 2000, Ren et al. 2005, Mahanonda et al. 2007, Morandini et al. 2010). The present study demonstrates that *E. coli* LPS is also able to induce an immune response in HGFs, but the effect is more pronounced when *P. gingivalis* LPS_{1690} is used. Secondly, the present study for the first time reveals that lipid A heterogeneity in *P. gingivalis* LPS elicits a significantly different innate immune response in HGFs as measured by pro-inflammatory cytokines. IL-6 and IL-8 have been shown to be the active pro-inflammatory mediator of periodontal diseases (Ozmeric et al. 1998, Trevilatto et al. 2003). IL-6 is a multifunctional interleukin which activates the down-stream cascades related to immuno-inflammatory pathways. IL-8 induces the microbicidal activity by chemoattracting PMNs through the CXCR1 receptors, and it helps migration of neutrophils through the wall of blood vessels. It has been shown that Gram-negative bacterial LPS such as of *E. coli* and *P. gingivalis* could stimulate the IL-6 and IL-8 production in HGFs, although no attempts have been made to determine how the structural heterogeneity of *P. gingivalis* LPS can affect the expression of these cytokines (Kent et al. 1999, Ren et al. 2005, Ara et al. 2009, Lu et al. 2009, Morandini et al. 2010). Further, it

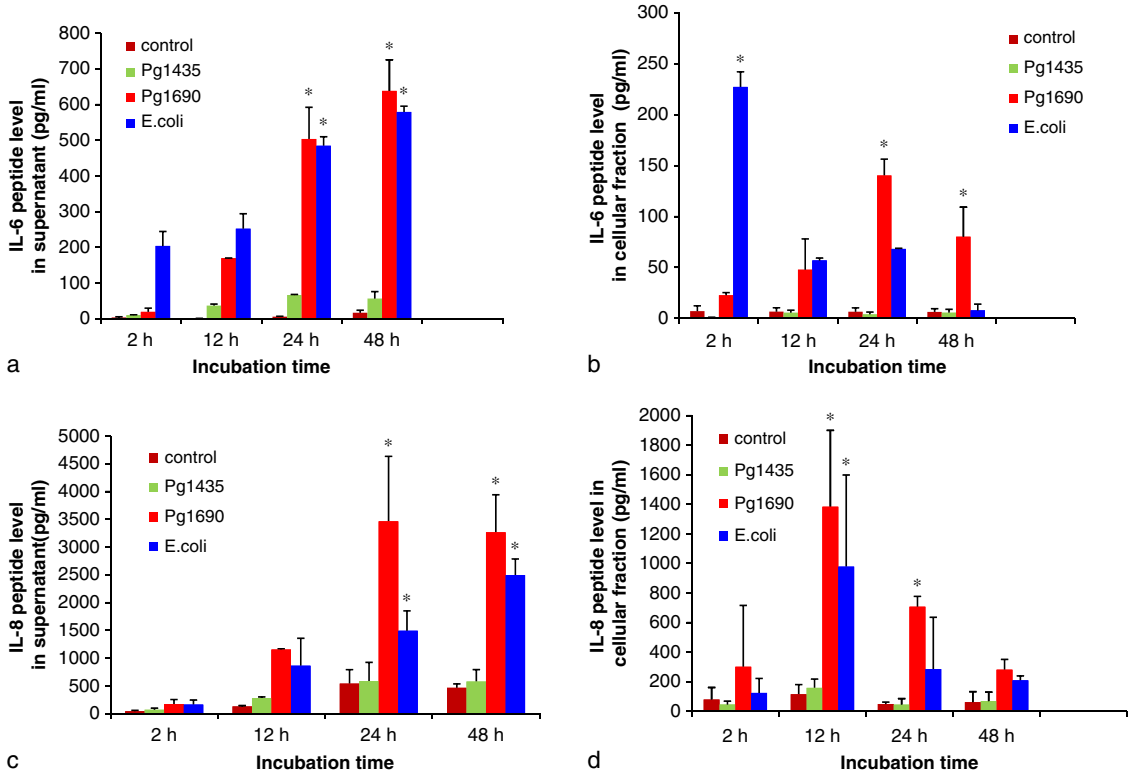

Fig. 4. Expression of IL-6 (a and b) and IL-8 (c and d) proteins in the culture supernatants (a and c) and cellular fractions (b and d) of human gingival fibroblasts after the stimulation of *Porphyromonas gingivalis* lipopolysaccharide (LPS) (1 μg/ml) and *Escherichia coli* LPS (1 μg/ml) in a time-dependent assay (2–48 h). The protein expression levels were measured by enzyme-linked immunoadsorbent assay. Each bar represents the mean ± SD of three independent experiments with two replicates. *Significant difference with a p-value <0.05 as compared with the controls without LPS treatment.

is observed that HGFs do not develop LPS tolerance during repetitive exposure (Ara et al. 2009). Therefore, as shown in the present study it is imperative to determine LPS structure, before embarking on studies of LPS, as the structural difference may elicit a markedly different host response. Thirdly, this study shows that TNF-α level is rapidly induced by *P. gingivalis* LPS_{1690} and gradually declined with time. TNF-α is an acute phase cytokine and induces the production of IL-6. But when IL-6 level is increased it acts as a negative feedback to inhibit TNF-α production (Leal-Berumen et al. 1996, Starkie et al. 2003). TNF-α mediates its biological activity via two receptors known as TNFR55 and TNFR75, which can be found in both soluble and membrane-bound forms (Martel-Pelletier et al. 1995). Interestingly TNF-α binding to the HGFs markedly reduces with the presence of TNF-α mediated by shedding the TNFR75 as sTNFR75 (Ohe et al. 2000). Therefore, it could be speculated that the gradual decline in TNF-α activity observed in the present study may be due to the increased shedding of sTNFR, inhibitory effect of IL-6 or other mechanisms to be defined in further studies.

P. gingivalis residing in the dental plaque biofilms perpetually interacts with gingival tissues. *P. gingivalis* or its LPS is sensed by the HGFs and subsequently they activate innate response by releasing pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α. It has currently been concerned that the significant changes in bacterial composition may occur as a result of inflammation rather than being its cause (Wade 2011). Darveau and colleagues have recently showed that haemin acts as a crucial environmental regulator for *P. gingivalis* to change its TLR4 agonist penta-acylated LPS_{1690} structure to TLR4 antagonist tetra-acylated $LPS_{1435/1449}$ structure, where LPS_{1690} and $LPS_{1435/1449}$ are produced predominantly under low and high haemin concentrations, respectively (Al-Qutub et al. 2006). As the haemin concentrations increase markedly in uncontrolled periodontal inflammation, this structural modification of *P. gingivalis* LPS may disguise the host innate mechanisms and facilitate invasion of *P. gingivalis* into the gingival tissues. Hence, local cytokine paralysis could lead to devastating effects on host defense in the periodontium, where bacterial exposure is constant and the host may no longer be able to detect the presence of pathogenic bacteria like *P. gingivalis* and direct leucocytes for their removal, thereby contributing to periodontal destruction.

The present study suggests that *P. gingivalis* LPS heterogeneity may differentially modulate the pro-inflammatory cytokine expression in HGFs. It is reasonable to speculate that *P. gingivalis* may utilize its ability of transformation of LPS lipid A structure, and hence modulate innate host response in a favourable way to gain access and multiply inside the gingival tissues under certain conditions, subsequently leading to periodontal destruction.

Acknowledgements

The authors are grateful to Ivy Law and Joanne Yip for technical assistance. This work received the International Association for Dental Research

(IADR)/Lion Dental Research Award in July 2010.

References

Agarwal, S., Piesco, N. P., Johns, L. P. & Riccelli, A. E. (1995) Differential expression of IL-1 beta, TNF-alpha, IL-6, and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. *Journal of Dental Research* **74**, 1057–1065.

Al-Qutub, M. N., Braham, P. H., Karimi-Naser, L. M., Liu, X., Genco, C. A. & Darveau, R. P. (2006) Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. *Infection & Immunity* **74**, 4474–4485.

Ara, T., Kurata, K., Hirai, K., Uchihashi, T., Uematsu, T. & Imamura, Y. et al. (2009) Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. *Journal of Periodontal Research* **44**, 21–27.

Bainbridge, B. W., Coats, S. R., Pham, T. T., Reife, R. A. & Darveau, R. P. (2006) Expression of a Porphyromonas gingivalis lipid A palmitylacyltransferase in *Escherichia coli* yields a chimeric lipid A with altered ability to stimulate interleukin-8 secretion. *Cellular Microbiology* **8**, 120–129.

Bainbridge, B. W. & Darveau, R. P. (2001) Porphyromonas gingivalis lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. *Acta Odontologica Scandinavica* **59**, 131–138.

Darveau, R. P. (2009) The oral microbial consortium's interaction with the periodontal innate defense system. *DNA & Cell Biology* **28**, 389–395.

Darveau, R. P. (2010) Periodontitis: a polymicrobial disruption of host homeostasis. *Nature Review Microbiology* **8**, 481–490.

Darveau, R. P., Pham, T. T., Lemley, K., Reife, R. A., Bainbridge, B. W. & Coats, S. R. et al. (2004) Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infection & Immunity* **72**, 5041–5051.

Di Domenico, G., Del Vecchio, L., Postiglione, L. & Ramaglia, L. (2003) Immunophenotypic analysis of human gingival fibroblasts and its regulation by granulocyte-macrophage colony-stimulating factor (GM-CSF). *Minerva Stomatologica* **52**, 81–87.

Dixon, D. R. & Darveau, R. P. (2005) Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. *Journal of Dental Research* **84**, 584–595.

Kebschull, M. & Papapanou, P. N. (2011) Periodontal microbial complexes associated with specific cell and tissue responses. *Journal of Clinical Periodontology* **38** (Suppl. 11), 17–27.

Kent, L. W., Rahemtulla, F., Hockett, R. D. Jr., Gilleland, R. C. & Michalek, S. M. (1998) Effect of lipopolysaccharide and inflammatory cytokines on interleukin-6 production by healthy human gingival fibroblasts. *Infection & Immunity* **66**, 608–614.

Kent, L. W., Rahemtulla, F. & Michalek, S. M. (1999) Interleukin (IL)-1 and Porphyromonas gingivalis lipopolysaccharide stimulation of IL-6 production by fibroblasts derived from healthy or periodontally diseased human gingival tissue. *Journal of Periodontology* **70**, 274–282.

Leal-Berumen, I., Snider, D. P., Barajas-Lopez, C. & Marshall, J. S. (1996) Cholera toxin increases IL-6 synthesis and decreases TNF-alpha production by rat peritoneal mast cells. *Journal of Immunology* **156**, 316–321.

Lu, Q., Darveau, R. P., Samaranayake, L. P., Wang, C. Y. & Jin, L. J. (2009) Differential modulation of human β-defensins expression in human gingival epithelia by Porphyromonas gingivalis lipopolysaccharide with tetra- and penta-acylated lipid A structures. *Innate Immunity* **15**, 325–335.

Mahanonda, R., Sa-Ard-Iam, N., Montreekachon, P., Pimkhaokham, A., Yongvanichit, K., Fukuda, M. M. & Pichyangkul, S. (2007) IL-8 and IDO expression by human gingival fibroblasts via TLRs. *Journal of Immunology* **178**, 1151–1157.

Martel-Pelletier, J., Mineau, F., Jolicoeur, F. C. & Pelletier, J. P. (1995) Modulation of TNFSR55 and TNFSR75 by cytokines and growth factors in human synovial fibroblasts. *The Journal of Rheumatology* **43**, 115–119.

Morandini, A. C., Sipert, C. R., Gasparoto, T. H., Greghi, S. L., Passanezi, E., Rezende, M. L., & Pichyangkul, S. (2010) Differential production of macrophage inflammatory protein-1alpha, stromal-derived factor-1, and IL-6 by human cultured periodontal ligament and gingival fibroblasts challenged with lipopolysaccharide from *P. gingivalis*. *Journal of Periodontology* **81**, 310–317.

Ogawa, T., Nakazawa, M. & Masui, K. (1996) Immunopharmacological activities of the nontoxic monophosphoryl lipid A of *Porphyromonas gingivalis*. *Vaccine* **14**, 70–76.

Ogawa, T. & Uchida, H. (1996) Differential induction of IL-1 beta and IL-6 production by the nontoxic lipid A from *Porphyromonas gingivalis* in comparison with synthetic Escherichia coli lipid A in human peripheral blood mononuclear cells. *FEMS Immunology and Medical Microbiology* **14**, 1–13.

Ohe, H., Takashiba, S., Naruishi, K., Chou, H. H., Yamada, H. & Nishimura, F. et al. (2000) Tumor necrosis factor-alpha (TNF-alpha)-induced and interleukin-1 beta (IL-1 beta)-induced shedding of TNF receptors from gingival fibroblasts. *Journal of Interferon & Cytokine Research* **20**, 1077–1082.

Ozmeric, N., Bal, B., Balos, K., Berker, E. & Bulut, S. (1998) The correlation of gingival crevicular fluid interleukin-8 levels and periodontal status in localized juvenile periodontitis. *Journal of Periodontology* **69**, 1299–1304.

Poggi, P., Rodriguez, Y., Baena, R., Rizzo, S. & Rota, M. T. (2003) Mouthrinses with alcohol: cytotoxic effects on human gingival fibroblasts in vitro. *Journal of Periodontology* **74**, 623–629.

Reife, R. A., Coats, S. R., Al-Qutub, M., Dixon, D. M., Braham, P. A., Billharz, R. J., Howald, W.N. & Darveau, R.P. (2006) *Porphyromonas gingivalis* lipopolysaccharide lipid A heterogeneity: differential activities of tetra- and penta-acylated lipid A structures on E-selectin expression and TLR4 recognition. *Cellular Microbiology* **8**, 857–868.

Reife, R. A., Shapiro, R. A., Bamber, B. A., Berry, K. K., Mick, G. E. & Darveau, R. P. (1995) Porphyromonas gingivalis lipopolysaccharide is poorly recognized by molecular components of innate host defense in a mouse model of early inflammation. *Infection & Immunity* **63**, 4686–4694.

Ren, L., Leung, W. K., Loo, T. W. & Jin, L. J. (2005) Lipopolysaccharide-binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast. *Journal of Periodontal Research* **40**, 407–416.

Starkie, R., Ostrowski, S. R., Jauffred, S., Febbraio, M. & Pedersen, B. K. (2003) Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. *The Journal of the Federation of the American Societies for Experimental Biology* **17**, 884–886.

Tabeta, K., Yamazaki, K., Akashi, S., Miyake, K., Kumada, H. & Umemoto, T. et al. (2000) Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. *Infection & Immunity* **68**, 3731–3735.

Tamura, M., Tokuda, M., Nagaoka, S. & Takada, H. (1992) Lipopolysaccharides of *Bacteroides intermedius* (*Prevotella intermedia*) and *Bacteroides* (*Porphyromonas*) *gingivalis* induce interleukin-8 gene expression in human gingival fibroblast cultures. *Infection & Immunity* **60**, 4932–4937.

Trevilatto, P. C., Scarel-Caminaga, R. M., de Brito, R. B. Jr., de Souza, A. P. & Line, S. R. (2003) Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. *Journal of Clinical Periodontology* **30**, 438–442.

Wade, W. G. (2011) Has the use of molecular methods for the characterization of the human oral microbiome changed our understanding of the role of bacteria in the pathogenesis of periodontal disease? *Journal of Clinical Periodontology* **38** (Suppl. 11), 7–16.

Wang, P. L. & Ohura, K. (2002) *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Critical Reviews in Oral Biology & Medicine* **13**, 132–142.

Yamazaki, K., Ikarashi, F., Aoyagi, T., Takahashi, K., Nakajima, T. & Hara, K. et al. (1992) Direct and indirect effects of *Porphyromonas gingivalis* lipopolysaccharide on interleukin-6 production by human gingival fibroblasts. *Oral Microbiology Immunology* **7**, 218–224.

Yoshimura, A., Hara, Y., Kaneko, T. & Kato, I. (1997) Secretion of IL-1 beta, TNF-alpha, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *Journal of Periodontal Research* **32**, 279–286.

Address:
Lijian Jin
Faculty of Dentistry,
The University of Hong Kong
34 Hospital Road
Hong Kong SAR
China
E-mail: ljjin@hkucc.hku.hk

Clinical Relevance

Scientific rationale for the study: P. gingivalis LPS is one of the major virulent attributes strongly involved in periodontal pathogenesis. It displays a significant amount of structural heterogeneity, containing both tetra- ($LPS_{1435/1449}$) and penta-acylated (LPS_{1690}) lipid A structures. The impacts of both isoforms of *P. gingivalis* LPS on innate host response remain elusive. This study investigated the modulatory effects of *P. gingivalis* LPS heterogeneity on the expression of IL-6, IL-8 and TNF-α in HGFs.

Principal findings: P. gingivalis LPS_{1690} significantly up-regulated the mRNA and protein expression of IL-6 and IL-8, whereas *P. gingivalis* $LPS_{1435/1449}$ did not induce significant host response. The expression levels of IL-6 and IL-8 up-regulated by *P. gingivalis* LPS_{1690} continuously increased with time course.

Practical implications: P. gingivalis LPS heterogeneity may differentially modulate the immuno-inflammatory response in HGFs and contribute to periodontal pathogenesis. This finding enhances the understanding on the molecular mechanisms of bacteria–host interactions that are crucial for development of novel treatment approaches to controlling periodontal diseases.