Acta Odontologica Scandinavica. 2014; 72: 337–345

informa
healthcare

ORIGINAL ARTICLE

Different effects of *P. gingivalis* LPS and *E. coli* LPS on the expression of interleukin-6 in human gingival fibroblasts

OLEH ANDRUKHOV[1], SANDRA ERTLSCHWEIGER[1], ANDREAS MORITZ[2], HANS-PETER BANTLEON[3] & XIAOHUI RAUSCH-FAN[1,2]

[1]*Central Research Unit, Bernhard Gottlieb University Clinic of Dentistry, Medical University of Vienna ,* [2]*Division of Conservative Dentistry, Periodontology and Prophylaxis, Bernhard Gottlieb University Clinic of Dentistry, Medical University of Vienna, and* [3]*Division of Orthodontics, Bernhard Gottlieb University Clinic of Dentistry, Medical University of Vienna, Vienna, Austria*

Abstract

Objective. Gingival fibroblasts (GFs) produce pro-inflammatory cytokines in response to stimulation with lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, which is thought to be mediated by activation of toll-like receptors (TLR)2 and TLR4. The present study investigated the expression of interleukin (IL)-6, TLR2, and TLR4 in GFs of seven different donors upon stimulation with *P. gingivalis* LPS. The effects of *P. gingivalis* LPS were compared with those of TLR4 agonist *Escherichia coli* LPS and TLR2 agonist Pam3CSK4. ***Materials and methods.*** GFs were stimulated with *P. gingivalis* LPS, *E. coli* LPS or Pam3CSK4 and the expression of IL-6, TLR2 and TLR4 was measured by qPCR. The surface expression of TLR2 and TLR4 was measured by flow cytometry. ***Results***. In GFs from three donors, *P. gingivalis* LPS and Pam3CSK4 induced a markedly lower increase in IL-6 expression than *E. coli* LPS. This was accompanied by significant down-regulation of the TLR2 and TLR4 expression. In GFs from another four donors, an increase in IL-6 expression upon stimulation with *P. gingivalis* LPS and Pam3CSK4 was similar or even higher than that induced by *E. coli* LPS. In GFs of these donors, all stimuli induced an up-regulation of both mRNA and protein expression of TLR2 and did not influence that of TLR4. ***Conclusions.*** This study suggests that *P. gingivalis* LPS and *E. coli* LPS differently regulate cytokine production in human gingival fibroblasts. Regulation of the expression level of TLR2 and TLR4 by periodontal pathogens might be an important factor controlling the inflammatory response in GFs.

Key Words: *cytokines, innate immune response, pattern recognition receptors, periodontal disease*

Introduction

Periodontitis is an infection disease, induced by overgrow of some gram-negative bacteria in the dental plaque resulting in the chronic stimulation of innate immune system, excessive production of pro-inflammatory cytokines, destruction of periodontal tissue, and in worst cases tooth loss [1]. Immune response is initiated after recognition of bacteria-derived pathogen-associated molecular patterns by specific pattern recognizing receptors of the host cells. Lipopolysaccharide (LPS) is a component of gram-negative bacteria and is a potent inducer of immune response by various cell types [2]. Host cells recognize LPS through toll-like receptors (TLR), a family of trans-membrane protein, which are similar to the *toll* protein of *Drosophila* [3,4]. LPS from most gram-negative bacteria is recognized by TLR4 [4]. One of the few exceptions from this rule is LPS from *Porphyromonas gingivalis*, which is strongly associated with chronic and severe adult periodontitis [5]. In contrast to common LPS of enterobacteria, *P. gingivalis* LPS is a rather weak TLR4 agonist and activates mainly TLR2 [6–8]. More recent studies report that *P. gingivalis* LPS has heterogeneity in the structure and its different forms activate different signaling pathways [9,10].

Gingival fibroblasts (GFs) are the main constituent of the periodontium, they are responsible for the synthesis and degradation of connective tissue [11]. Numerous

Correspondence: Dr Oleh Andrukhov, Bernhard Gottlieb University Clinic of Dentistry, Medical University of Vienna, Sensengasse 2a, 1090 Vienna, Austria. Tel: +43 1 400702620. Fax: +43 1 400702609. E-mail: oleh.andrukhov@meduniwien.ac.at

(Received 26 April 2013; accepted 3 August 2013)

ISSN 0001-6357 print/ISSN 1502-3850 online © 2014 Informa Healthcare
DOI: 10.3109/00016357.2013.834535

studies within the last decades showed that GFs produce various pro-inflammatory cytokines in response stimulation with *P. gingivalis* LPS, which seems to play an important role in the progression of periodontal disease (for review, see [12,13]). Both TLR2 and TLR4 seem to mediate the cytokine production by GFs in response to *P. gingivalis* LPS [14–16]. However, large quantitative and qualitative differences in the production of pro-inflammatory cytokines were observed between cells isolated from different donors [17,18]. The exact reason for this heterogeneity is rather unclear, but plausibly these differences could be due to different expression of proteins involved in LPS-induced signaling. For example, one previous study on GFs showed that higher expression levels of the membrane CD14, which is required for interaction of LPS with TLRs, were associated with higher levels of cytokine production upon LPS stimulation [17]. Since TLR2 and TLR4 are primary receptors for *P. gingivalis* LPS, it could be assumed that their expression levels also correlate with the response of GFs. However, to date this question is poorly investigated.

The aim of the present study was to investigate the effect of *P. gingivalis* LPS on the expression levels of interleukin (IL)-6, TLR2 and TLR4 in human GFs isolated from seven different individuals. Since *P. gingivalis* might activate both TLR2 and TLR4, we compared its effect with those of TLR4 agonist *E. coli* LPS and TLR2 agonist synthetic lipopeptide Pam3CSK4.

Materials and methods

Cell culture and reagents

Gingival fibroblasts were isolated from the gingival tissue of seven periodontally healthy donors undergoing routine extraction of their third molar teeth. Donors were informed in details and signed agreement before the surgical procedures. All donors had no systemic disease, donor's age ranged from 26–60 years. Donors 1, 2 and 7 were males, donors 3, 4, 5 and 6 were females. Donors 1, 3, 6 and 7 were non-smokers, donors 2, 4 and 5 were smokers. Human GFs were isolated by cutting the gingival tissue into smaller pieces with a sterile scalpel blade and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), streptomycin (50 µg/ml) and penicillin (100 U/ml) under humidified air atmosphere containing 5% CO_2 at 37°C. The medium was changed every 3 days and cells were cultured for 10–15 days until confluent monolayers were formed. Cells were further passaged by trypsinization and then again grown to confluence in 75 cm^2 cell culture flasks. Cell lines from passage levels 5–8 were used in this study.

Commercially available preparation of *P. gingivalis* LPS, *E. coli* 0111:B4 LPS, and synthetic lipopeptide Pam3CSK4 (all Invivogen, San Diego, CA) were used. Preparations of bacterial LPS were purified by the supplier according to the method of Hirschfeld et al. [19]. As reported by another study, these LPS preparations are free from contaminating lipoproteins [20].

GFs stimulation

GFs were seeded in a 6-well plate (10^6 cell in 3 ml medium per well). After 24 h, the culture medium was replaced with DMEM medium containing 2% FCS and cells were stimulated with one of the following substances: *P. gingivalis* LPS, 1 µg/ml; TLR2 agonist Pam3CSK4, 1 µg/ml; TLR4 agonist *E. coli* LPS, 0.1 µg/ml. Non-stimulated cells cultured in DMEM medium with 2% FCS were taken as a negative control. After 24 h stimulation, cells were collected and the expression of TLR2, TLR4, andIL-6 was analyzed. The stimulation conditions were similar to that used in our previous study, in which maximal response of GFs to *P. gingivalis* LPS was observed after 24 h stimulation [21].

Real time PCR

Isolation of total cellular mRNA and its following transcription into cDNA was performed using TaqMan® Gene Expression Cells-to-CT™ kit (Ambion/Applied Biosystems, Grand Island, NY) according to the manufacturer's instruction. For each group, 10^5 cells were taken for mRNA isolation. Real-time PCR was performed on an ABI Prism SDS 7000 (Applied Biosystems) in paired reactions using the Taqman® gene expression assays with following ID numbers (all from Applied Biosystems): IL-6, Hs00985639_m1; TLR2, Hs00610101_m1; TLR4, Hs00152939_m1; β-actin, Hs99999903_m1. Triplicate real time PCR reactions were performed in 96-well plates, each well contained 10 µl of the TaqMan® Gene Expression Master Mix, 1 µl TaqMan® Gene Expression Assay (both from Applied Biosystems), 5 µl of nuclease free water and 4 µl of template DNA. Thermocycling conditions were as follows: initiation at 95°C for 10 min, then 40 cycles each of them consisting of denaturation at 95°C for 15 s and hybridization-elongation at 60°C for 2 min. The point at which the PCR product was first detected above a fixed threshold (termed cycle threshold, C_t) was determined for each sample. Changes in the expression of target genes were calculated using $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = (C_t^{target} - C_t^{\beta\text{-actin}})_{sample} - (C_t^{target} - C_t^{\beta\text{-actin}})_{control}$, taking the sample without any treatment as a control.

Flow cytometry analysis

Cells were washed in FACS buffer (3% FCS, 0.9% NaN_3 in phosphate buffered saline), and used for

staining. 5×10^5 cells were re-suspended in 50 µl of FACS buffer and incubated with one of the following monoclonal antibodies conjugated with phycoerythrin (all eBioscience, San Diego, CA): mouse anti-human toll-like receptor 2 antibody (clone TL 2.1), mouse anti-human toll-like receptor 4 (clone HTA125) antibody, mouse IgG2a isotype control antibody. The incubation was performed in a dark place on ice. After incubation, the cells were washed twice with ice-cold FACS buffer, re-suspended in 500 µl of FACS buffer and analyzed for TLRs expression. Analysis was performed with a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) equipped with an argon laser tuned at 488 nm. Cell counting was limited by 10 000 events. The protein expression level was quantified based on the relationship of the mean fluorescence intensities of cells treated with one of TLRs antibodies to those treated with isotype control antibody.

Statistical analysis

The statistical differences compared to the control group were analyzed by *t*-test or ANOVA's statistic. All statistical analysis was performed using statistic program SPSS 17.0. Data are expressed as mean ± SD. Differences were considered to be statistically significant at $p < 0.05$.

Results

Effect of P. gingivalis LPS, E. coli LPS and Pam3CSK4 on the mRNA expression level of IL-6 in GFs

The effect of *P. gingivalis* LPS, *E. coli* LP and Pam3CSK4 on the gene expression of IL-6 in human gingival fibroblasts isolated from different donors is shown in Figure 1. Quantitative inter-individual differences were observed in response to different stimuli. *P. gingivalis* LPS induce a significant increase in IL-6 expression levels in GFs from donors 3–7, whereas no changes in IL-6 expression were observed in donors 1 and 2. Pam3CSK4 induced a significant increase in IL-6 expression in GFs from all donors, excepting donor 3. *E. coli* LPS induced a significant increase in IL-6 mRNA expression levels in GFs isolated from all donors. The responses to *P. gingivalis* LPS and Pam3CSK4 were significantly lower ($p < 0.05$) than that to *E. coli* LPS in donors 1–3 and significantly higher than that to *E. coli* LPS in donors 4–7. In all donors, excepting donor 4, no

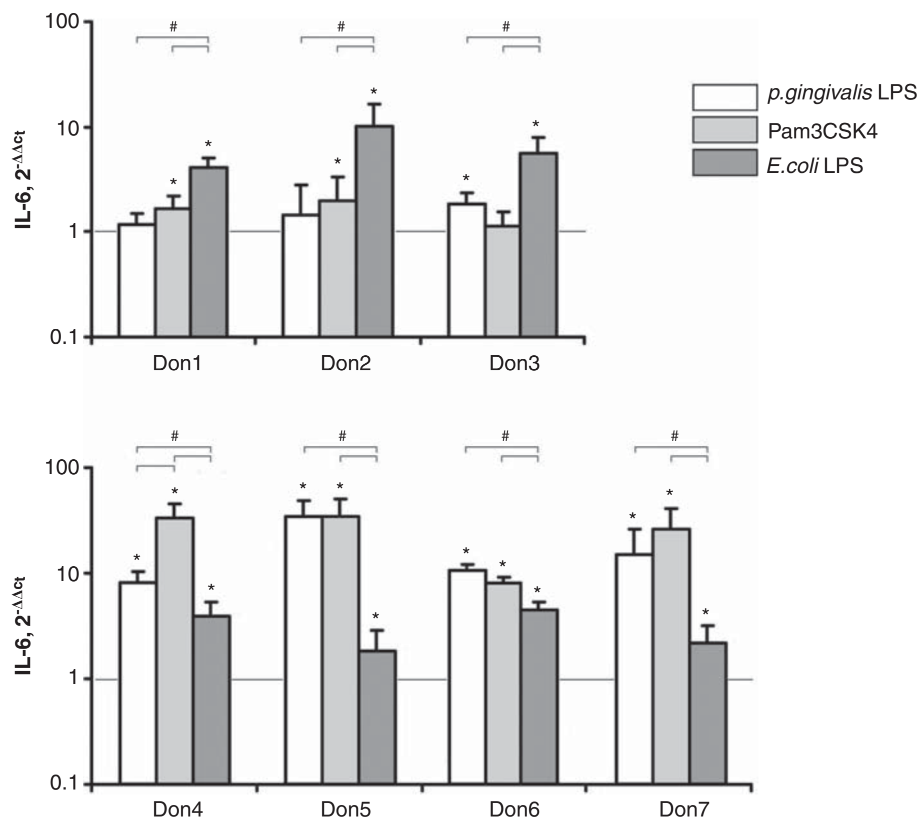

Figure 1. Effect of *P. gingivalis* LPS, Pam3CSK4 and *E. coli* LPS on the IL-6 expression levels in human GFs isolated from different donors. Human GFs were stimulated with different stimuli for 24 h and the changes in the expression levels of IL-6 were measured by qPCR. The *y*-axis indicates *n*-times changes in the mRNA expression levels ($2^{-\Delta\Delta Ct}$) of IL-6 compared to untreated control (= 1). *Significantly different from control, $p < 0.01$. # Significantly different between different stimuli, $p < 0.01$.

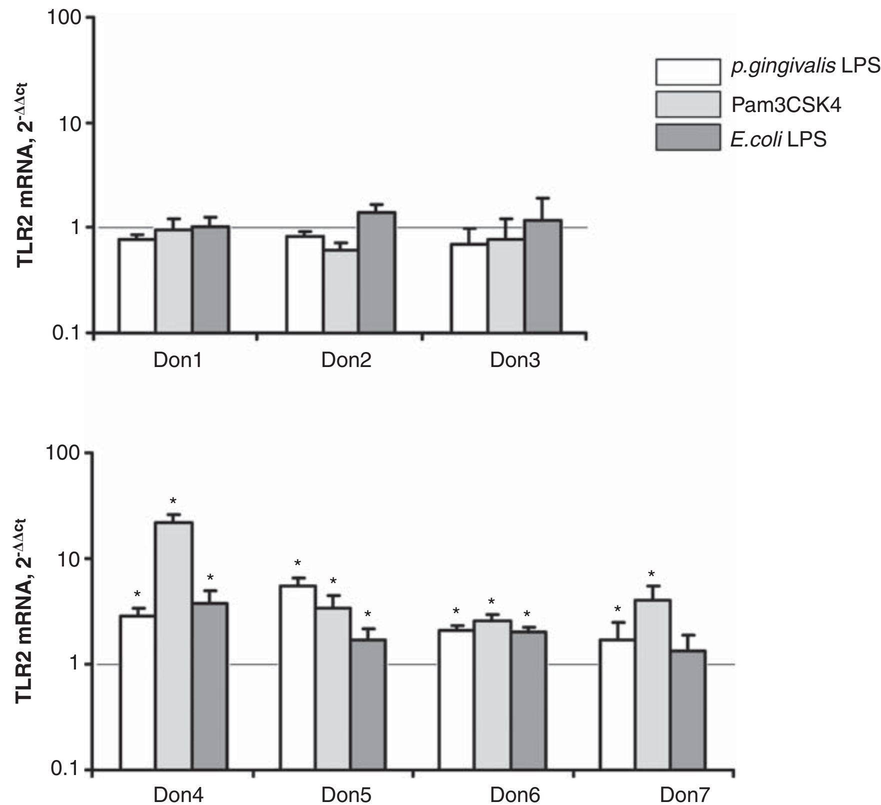

Figure 2. Effect of *P. gingivalis* LPS, Pam3CSK4 and *E. coli* LPS on the mRNA expression levels of TLR2 in human GFs isolated from different donors. Human GFs were stimulated with different stimuli for 24 h and the changes in the expression levels of TLR2 were measured by qPCR. The *y*-axis indicates *n*-times changes in the mRNA expression levels ($2^{-\Delta\Delta Ct}$) of TLR2 compared to untreated control (= 1). *Significantly higher than control, $p < 0.01$.

significant difference in IL-6 expression in response to *P. gingivalis* LPS and Pam3CSK4 was observed. In donor 4, *P. gingivalis* LPS induced significantly lower IL-6 expression levels than Pam3CSK4 ($p < 0.01$).

Effect of P. gingivalis LPS, E. coli LPS and Pam3CSK4 on the expression of TLR2 in GFs

The effect of *P. gingivalis* LPS, *E. coli* LPS and Pam3CSK4 on the mRNA and protein expression TLR2 expression levels in human gingival fibroblasts is shown in Figures 2 and 3, respectively. The inter-individual differences in changes of TLR2 expression in GFs isolated from different donors were observed. In donors 1–3 stimulation with *P. gingivalis* LPS induced a significant decrease in surface expression of TLR2, whereas no changes in the TLR2 mRNA expression levels were found. Qualitatively similar responses were observed in GFs of these donors also after stimulation with *E. coli* LPS and Pam3CSK4. In donors 4–7 stimulation with *P. gingivalis* LPS resulted in a significant increase of TLR2 expression on both protein and mRNA levels. A qualitatively similar response was observed after stimulation with *E. coli* LPS in GFs from donors 4–6 and after stimulation with Pam3CSK4 in GFs from donors 4–7. No significant changes in TLR2 expression levels were observed after stimulation with *E. coli* LPS in GFs from donor 7.

Effect of P. gingivalis LPS, E. coli LPS and Pam3CSK4 on the expression of TLR4 in GFs

The effect of *P. gingivalis* LPS, *E. coli* LPS and Pam3CSK4 on the mRNA and protein expression TLR4 expression levels in human gingival fibroblasts is shown in Figures 4 and 5, respectively. The inter-individual differences in changes of TLR4 expression in GFs isolated from different donors were observed. In some donors (donors 1–3) stimulation with *P. gingivalis* LPS induced significant decrease in surface expression of TLR4, whereas no changes in the TLR4 mRNA expression levels were found. A qualitatively similar response was observed after stimulation of GFs from these donors with *E. coli* LPS and Pam3CSK4 LPS. In other donors (donors 4–7) stimulation with *P. gingivalis* LPS did not induce any changes of TLR4 expression on both protein and mRNA levels. Similarly, no changes in TLR4 expression levels were observed in GFs from these donors after stimulation with *E. coli* LPS and Pam3CSK4.

Discussion

Gingival fibroblasts actively participate in homeostasis of periodontium and play an important role in immune response in periodontal disease [12,13]. In the present

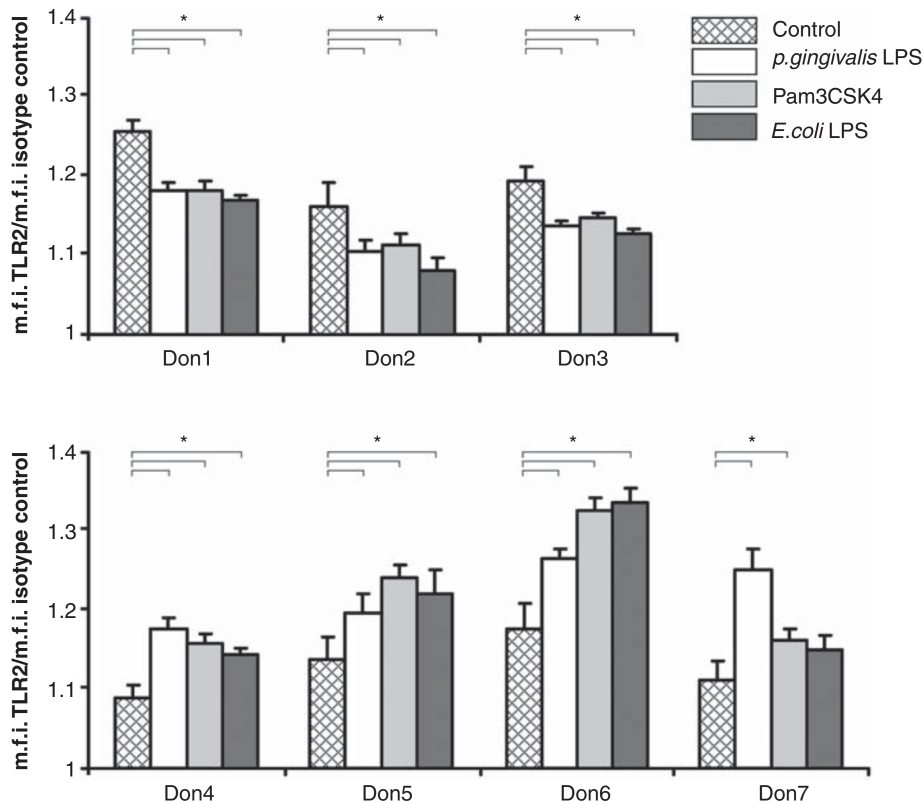

Figure 3. Effect of *P. gingivalis* LPS, Pam3CSK4 and *E. coli* LPS on the surface expression of TLR2 in human GFs isolated from different donors. Human GFs were stimulated with different stimuli for 24 h and the changes in the surface expression levels of TLR2 were measured by flow cytometry. The *y*-axis indicates the ratio of mean fluorescence intensities of GFs treated with anti-TLR2 antibodies to those treated with isotype control antibody. Data represent mean ± SD of three different acquisitions. *Significantly different from control, $p < 0.01$.

study, we found large inter-individual differences in the levels of IL-6 expression in GFs upon stimulation with periodontal pathogen *P. gingivalis* LPS, TLR4 agonist *E. coli* LPS and synthetic TLR2 agonist Pam3CSK4. Significantly different response of GFs to *P. gingivalis* LPS and *E. coli* LPS were observed. In cells isolated from donors 1–3, IL-6 expression in response to *P. gingivalis* LPS was significantly lower than that in response to *E. coli* LPS, whereas in other donors (4–7) the response to *P. gingivalis* LPS was significantly higher than that to *E. coli* LPS. Some previous studies also describe different responses of host cells to these two LPS. Particularly, *P. gingivalis* LPS is shown to induce significantly higher production of IL-1β in the human monocytic U937 cell line than *E. coli* LPS [22]. A former study showed that *P. gingivalis* LPS was not able to induce IL-6 expression in mouse human periodontal ligament cells (PDLs), whereas *E. coli* LPS induced IL-6 production by these cells [23]. A study on human monocyte cell line THP-1 showed stimulation with *P. gingivalis* LPS and *E. coli* LPS results in activation of distinct intracellular signaling pathways [24].

Substantial similarities between responses of GFs to *P. gingivalis* LPS and synthetic TLR2 agonist Pam3CSK4 were observed. There were no significant difference in responses to *P. gingivalis* LPS and Pam3CSK4 in GF of all donors, excepting donor 4. The changes in the IL-6 expression upon stimulation with *P. gingivalis* LPS and Pam3CSK4 seemed to correlate with the changes in the TLR2 expression levels. Thus, in GFs from one group of donors (donors 1–3), *P. gingivalis* LPS and Pam3CSK4 induced significantly lower IL-6 expression compared to *E. coli* LPS. At the same time, the surface expression levels of TLR2 was significantly decreased in these cells upon stimulation with all substances. In GFs from another group of donors (donors 4–7) an increase of IL-6 expression upon stimulation with *P. gingivalis* LPS and Pam3CSK4 was similar or even higher than that induced by *E. coli* LPS. In this group, *P. gingivalis* LPS and Pam3CSK4 induced an increase of TLR2 expression on both mRNA and protein levels. Thus, it seems that increases in TLR2 expression were associated with higher IL-6 expression levels and, thus, with a more intense response to *P. gingivalis* LPS and Pam3CSK4, which could be because these substances act through activation of the TLR2 signaling pathway. This conclusion is supported by a previous study, showing that knockdown of TLR2 in human GFs and PDLs decreased expression of IL-6 and IL-8 in response to stimulation with both *P. gingivalis* LPS and Pam3CSK4 [25].

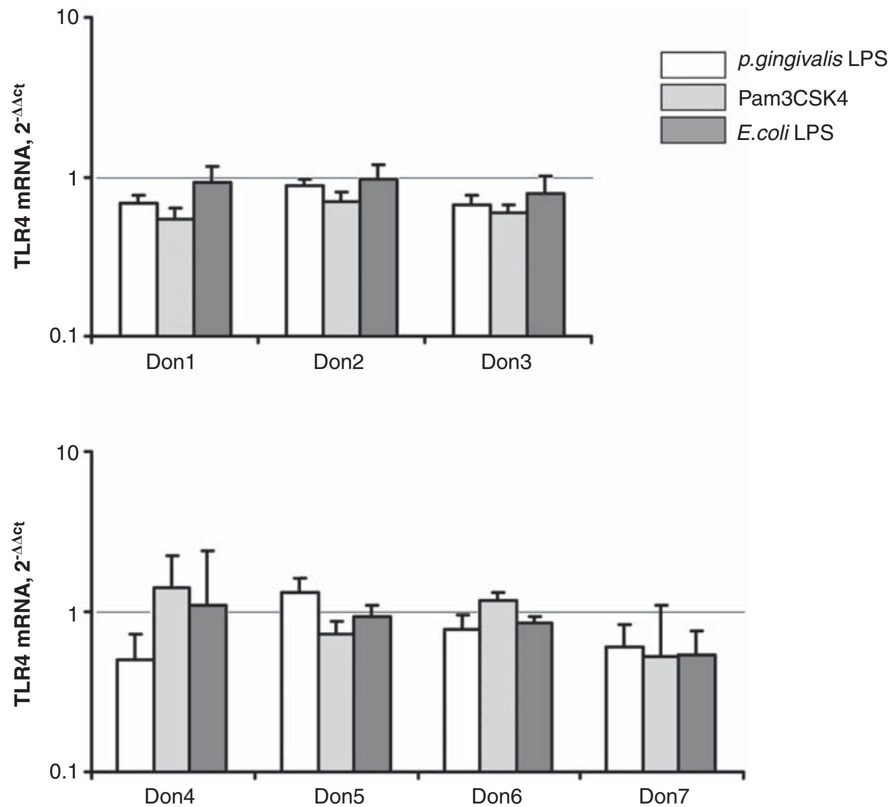

Figure 4. Effect of *P. gingivalis* LPS, Pam3CSK4 and *E. coli* LPS on the mRNA expression levels of TLR4 in human GFs isolated from different donors. Human GFs were stimulated with different stimuli for 24 h and the changes in the expression levels of TLR4 were measured by qPCR. The *y*-axis indicates *n*-times changes in the mRNA expression levels ($2^{-\Delta\Delta Ct}$) of TLR4 compared to untreated control (= 1).

It is well known that the structure of LPS bioactive moiety lipid A differs substantially between *P. gingivalis* and most other gram negative bacteria [26]. *P. gingivalis* LPS is thought to activate TLR2 rather than TLR4 [6,7,19,27,28]. However, some studies suggest that both TLR2 and TLR4 are involved in the *P. gingivalis* LPS signaling, which could be due to heterogeneity of its lipid A [10,29,30]. Our results suggest that TLR2-mediated signaling could play an important role in the response of gingival fibroblasts to *P. gingivalis* LPS. This conclusion is mainly based on two observations: first, high levels of IL-6 expression upon response to *P. gingivalis* LPS were observed only in GFs, where TLR2 expression was up-regulated. Second, the effects of *P. gingivalis* LPS were qualitatively similar to those of TLR2 agonist Pam3CSK4 and not to those of TLR4 agonist *E. coli* LPS.

We observed a large heterogeneity in the response of gingival fibroblasts to inflammatory stimuli, which was also shown by previous reports [17,18,31]. The reasons for this phenomenon are not entirely clear but it is rather possible that the heterogeneity could be accounted for by different genetic backgrounds of the host cells. Particularly, one previous report suggests that the ability of gingival fibroblasts to produce pro-inflammatory cytokines is determined by the expression levels of CD14, which is necessary for interaction of LPS with TLRs [17]. Our data suggest that differences in TLR expression and/or their regulation could also contribute to the heterogeneity in responses. One can assume the response of GF to LPS might be affected by such factors as age, gender, and smoking status. Previous studies showed that the cytokine production by GFs is affected by nicotine [32] and female sex hormones [33,34]. In our study, we did not find any relation between age, gender, and smoking status of donors and the response of GFs to different stimuli. However, the conclusion about the role of these factors in GFs response cannot be drawn from our results due to the low number of donors. The exact mechanisms underlying large inter-individuals heterogeneity of GFs responses remain to be elucidated.

In the present study the expression levels of both TLR2 and TLR4 was shown to be changed during inflammatory response. Regulation of the TLR expression in the host cells by bacterial LPS could be an important factor, regulating the intensity and duration of pro-inflammatory response [35–37]. This regulation is crucial because an excessive cytokine production due to inappropriate immune response is thought to be the main reason of periodontal tissue destruction [38]. The regulation of TLR expression level in GFs by bacterial lipopolysaccharides is also observed by some previous studies, but these results are rather controversial. Particularly, Wang et al. [14],

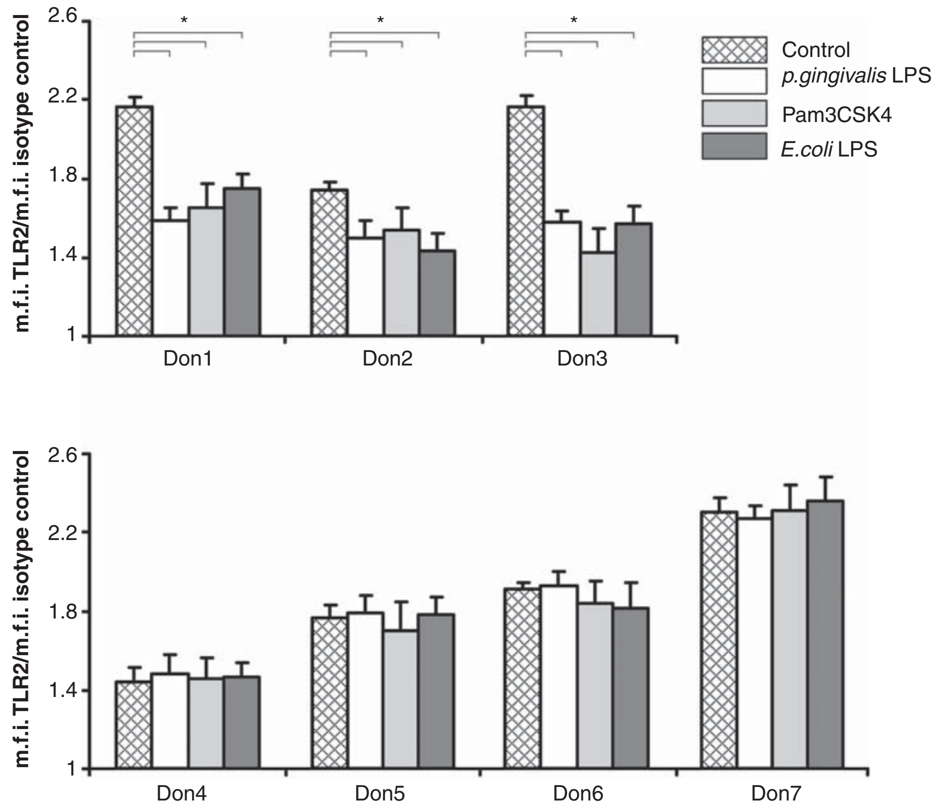

Figure 5. Effect of *P. gingivalis* LPS, Pam3CSK4 and *E. coli* LPS on the surface expression of TLR4 in human GFs isolated from different donors. Human GFs were stimulated with different stimuli for 24 h and the changes in the surface expression levels of TLR4 were measured by flow cytometry. The *y*-axis indicates the ratio of mean fluorescence intensities of GFs treated with anti-TLR4 antibodies to those treated with an isotype control antibody. Data represent mean ± SD of three different acquisitions. *Significantly different from control, $p < 0.01$.

using flow cytometry, showed that *P. gingivalis* LPS decreases the surface expression of TLR4 in GFs. Studies using real-time PCR show that the mRNA expression level of TLR2 and TLR4 is up-regulated by both *P. gingivalis* LPS [39,40] and *E. coli* LPS [41]. The controversy in the existing data could be explained by the heterogeneous structure of *P. gingivalis* LPS. A recent study shows that the expression of TLR4 in human GFs is stimulated by *P. gingivalis* LPS with penta-acetylated lipid A, whereas the expression of TLR2 is strongly stimulated by *P. gingivalis* LPS with tetra-acetylated lipid A [9]. The regulation of TLRs expression could also play a role in the progression of periodontal disease. For example, recent studies show that *P. gingivalis* induces stronger mRNA expression of TLR2 in GFs isolated from periodontitis patients than in GFs isolated from control subjects [40,42]. Our data showed that the changes in the TLR2 expression upon stimulation with *P. gingivalis* LPS are heterogeneous depending on the cells donor. This heterogeneity might also contribute to different individual susceptibility to periodontal infection.

The data on surface expression of TLR2 in GFs measured by flow cytometry were in agreement with the measurements of corresponding mRNA expression by real time PCR. In contrast, changes in the surface expression of TLR4 were not always in line with the expression of corresponding mRNA. Particularly, an increase in the TLR4 mRNA in GFs from donors 4–7 upon stimulation with Pg-LPS and Pam3CSK4 was not accompanied with an increase in the surface expression of TLR4 protein. Similarly, stimulation of GFs from donors 1–3 did not influence the mRNA level of TLR4 but resulted in significantly lower surface expression of this protein. These observations suggest that the expression level of TLR4 in GFs is regulated on a post-transcriptional level. For example, in human biliary epithelial cells microRNA *let-7i* down-regulates TLR4 expression due to degradation of TLR4 mRNA [43]. However, the existence of a similar mechanism in human GFs needs to be further investigated.

Acknowledgement

We thank Mrs Nguyen Phuong Quynh and Mrs Susanne Günzer for their help in preparing and performing the experiments.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Jenkinson HF, Dymock D. The microbiology of periodontal disease. Dent Update 1999;26:191–7.

[2] Triantafilou M, Triantafilou K. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol 2002;23:301–4.

[3] Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394–7.

[4] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev 2004;4:499–511.

[5] Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994;5: 78–111.

[6] Hirschfeld M, Weis JJ, Toshchakov V, Salkowski CA, Cody MJ, Ward DC, et al. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun 2001;69:1477–82.

[7] Martin M, Katz J, Vogel SN, Michalek SM. Differential induction of endotoxin tolerance by lipopolysaccharides derived from Porphyromonas gingivalis and Escherichia coli. J Immunol 2001;167:5278–85.

[8] Coats SR, Reife RA, Bainbridge BW, Pham TT, Darveau RP. Porphyromonas gingivalis lipopolysaccharide antagonizes Escherichia coli lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infect Immun 2003;71:6799–807.

[9] Herath TD, Darveau RP, Seneviratne CJ, Wang CY, Wang Y, Jin L. Tetra- and penta-acylated lipid A structures of Porphyromonas gingivalis LPS differentially activate TLR4-mediated NF-kappaB signal transduction cascade and immune-inflammatory response in human gingival fibroblasts. PLoS ONE 2013;8:e58496.

[10] Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, et al. Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. Infect Immun 2004;72:5041–51.

[11] Hassell TM. Tissues and cells of the periodontium. Periodontol 2000 1993;3:9–38.

[12] Wang PL, Ohura K. Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. Crit Rev Oral Biol Med 2002;13:132–42.

[13] Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. Periodontol 2000 2007; 43:41–55.

[14] Wang PL, Oido-Mori M, Fujii T, Kowashi Y, Kikuchi M, Suetsugu Y, et al. Heterogeneous expression of Toll-like receptor 4 and downregulation of Toll-like receptor 4 expression on human gingival fibroblasts by Porphyromonas gingivalis lipopolysaccharide. Biochem Biophys Res Commun 2001;288:863–7.

[15] Hatakeyama J, Tamai R, Sugiyama A, Akashi S, Sugawara S, Takada H. Contrasting responses of human gingival and periodontal ligament fibroblasts to bacterial cell-surface components through the CD14/Toll-like receptor system. Oral Microbiol Immunol 2003;18:14–23.

[16] Asai Y, Hashimoto M, Fletcher HM, Miyake K, Akira S, Ogawa T. Lipopolysaccharide preparation extracted from Porphyromonas gingivalis lipoprotein-deficient mutant shows a marked decrease in toll-like receptor 2-mediated signaling. Infect Immun 2005;73:2157–63.

[17] Sugawara S, Sugiyama A, Nemoto E, Rikiishi H, Takada H. Heterogeneous expression and release of CD14 by human gingival fibroblasts: characterization and CD14-mediated interleukin-8 secretion in response to lipopolysaccharide. Infect Immun 1998;66:3043–9.

[18] Scheres N, Laine ML, de Vries TJ, Everts V, van Winkelhoff AJ. Gingival and periodontal ligament fibroblasts differ in their inflammatory response to viable Porphyromonas gingivalis. J Periodontal Res 2010;45:262–70.

[19] Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000;165:618–22.

[20] Kocgozlu L, Elkaim R, Tenenbaum H, Werner S. Variable cell responses to P. gingivalis lipopolysaccharide. J Dent Res 2009;88:741–5.

[21] Sekot G, Posch G, Messner P, Matejka M, Rausch-Fan X, Andrukhov O, et al. Potential of the Tannerella forsythia S-layer to delay the immune response. J Dent Res 2011;90: 109–14.

[22] Roberts FA, Richardson GJ, Michalek SM. Effects of Porphyromonas gingivalis and Escherichia coli lipopolysaccharides on mononuclear phagocytes. Infect Immun 1997;65: 3248–54.

[23] Nebel D, Arvidsson J, Lillqvist J, Holm A, Nilsson BO. Differential effects of LPS from Escherichia coli and Porphyromonas gingivalis on IL-6 production in human periodontal ligament cells. Acta Odontol Scand 2013;71:892–8.

[24] Diya Z, Lili C, Shenglai L, Zhiyuan G, Jie Y. Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1beta, TNF-alpha and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. Innate Immun 2008;14:99–107.

[25] Morandini AC, Chaves Souza PP, Ramos-Junior ES, Brozoski DT, Sipert CR, Souza Costa CA, et al. Toll-like receptor 2 knockdown modulates interleukin (IL)-6 and IL-8- but not Stromal Derived Factor-1 (SDF–1/CXCL12) in human periodontal ligament and gingival fibroblasts. J Periodontol 2012;84:535–44.

[26] Ogawa T. Chemical structure of lipid A from Porphyromonas (Bacteroides) gingivalis lipopolysaccharide. FEBS Letts 1993; 332:197–201.

[27] Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses *in vivo*. J Immunol 2001;167:5067–76.

[28] Martin M, Schifferle RE, Cuesta N, Vogel SN, Katz J, Michalek SM. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. J Immunol 2003;171: 717–25.

[29] Reife RA, Coats SR, Al-Qutub M, Dixon DM, Braham PA, Billharz RJ, et al. 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;8:857–68.

[30] Herath TD, Wang Y, Seneviratne CJ, Lu Q, Darveau RP, Wang CY, et al. 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.

[31] Zhou J, Windsor LJ. Heterogeneity in the collagen-degrading ability of Porphyromonas gingivalis-stimulated human gingival fibroblasts. J Periodontal Res 2007;42:77–84.

[32] Almasri A, Wisithphrom K, Windsor LJ, Olson B. Nicotine and lipopolysaccharide affect cytokine expression from gingival fibroblasts. J Periodontol 2007;78:533–41.

[33] Yokoyama M, Hinode D, Masuda K, Yoshioka M, Grenier D. Effect of female sex hormones on Campylobacter rectus and human gingival fibroblasts. Oral Microbiol Immunol 2005;20:239–43.

[34] Lapp CA, Lapp DF. Analysis of interleukin-activated human gingival fibroblasts: modulation of chemokine responses by female hormones. J Periodontol 2005;76:803–12.

[35] Lorenz E. TLR2 and. TLR4 expression during bacterial infections. Curr Pharm Des 2006;12:4185–93.

[36] Kawai T, Akira S. TLR signaling. Sem Immunol 2007;19: 24–32.
[37] Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev 2005; 5:446–58.
[38] Van Dyke TE, Serhan CN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J Dent Res 2003;82:82–90.
[39] Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, et al. Toll-like receptors confer responsiveness to lipopolysaccharide from Porphyromonas gingivalis in human gingival fibroblasts. Infect Immun 2000;68: 3731–5.
[40] Wara-Aswapati N, Chayasadom A, Surarit R, Pitiphat W, Boch JA, Nagasawa T, et al. Induction of Toll-like receptor expression by porphyromonas gingivalis. J Periodontol 2012; 84:1010–18.
[41] Ren L, Leung WK, Loo TW, Jin L. Lipopolysaccharide--binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast. J Periodontal Res 2005;40:407–16.
[42] Scheres N, Laine ML, Sipos PM, Bosch-Tijhof CJ, Crielaard W, de Vries TJ, et al. Periodontal ligament and gingival fibroblasts from periodontitis patients are more active in interaction with Porphyromonas gingivalis. J Periodontal Res 2011;46:407–16.
[43] Chen XM, Splinter PL, O'Hara SP, LaRusso NF. A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem 2007;282:28929–38.