# Differential effects of LPS from Escherichia coli and Porphyromonas gingivalis on IL-6 production in human periodontal ligament cells.

Nebel, Daniel; Arvidsson, Joel; Lillqvist, Johan; Holm, Anders; Nilsson, Bengt-Olof

*Published in:*
Acta Odontologica Scandinavica

*DOI:*
10.3109/00016357.2012.734415

2013

# Differential effects of LPS from *Escherichia coli* and *Porphyromonas gingivalis* on IL-6 production in human periodontal ligament cells

**DANIEL NEBEL[1,2*], JOEL ARVIDSSON[1,2], JOHAN LILLQVIST[1,2], ANDERS HOLM[1] & BENGT-OLOF NILSSON[1]**

[1]Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden

[2]Department of Periodontology, Faculty of Odontology, Malmö University, SE-20506 Malmö, Sweden

**Short title:** Differential regulation of IL-6

Manuscript includes **6 figures**

***Correspondence:** Dr. Daniel Nebel

Department of Periodontology

Faculty of Odontology

Malmö University

SE-20506 Malmö

Sweden

Phone: +46-40-6658453

Fax: +46-40-925359

E-mail: Daniel.Nebel@mah.se

## Abstract

*Objective*. Periodontal ligament (PDL) cells produce IL-6 upon stimulation with inflammation promoters, but the signaling pathways involved have not been characterized. Here, we investigate underlying mechanisms behind regulation of PDL cell IL-6 production by *E. coli* and *P. gingivalis* LPS. *Material and methods.* Human PDL cells, endothelial cells and monocytes were stimulated with *E. coli* or *P. gingivalis* LPS in the presence or absence of pharmacological agents in order to disclose pathways involved in LPS signaling. Gene expression and cellular protein levels were assessed by quantitative real-time PCR and ELISA, respectively. *Results*. Stimulation with LPS from *E. coli* (1 µg/ml) for 24 h enhanced PDL cell IL-6 expression several fold demonstrated both on transcript and protein levels but *P. gingivalis* LPS (1-5 µg/ml) had no effect. TLR2 mRNA was more highly expressed than TLR4 transcript in PDL cells. Treatment with the non-selective nitric oxide synthase inhibitor L-NAME (100 µM) reduced *E. coli* LPS-induced PDL cell IL-6 by 30%, while neither aminoguanidine (10 µM), an inhibitor of inducible nitric oxide synthase, nor estrogen (17β-estradiol, 100 nM) influenced IL-6. Treatment with the glucocorticoid dexamethasone (1 µM) totally prevented the *E. coli* LPS-induced PDL cell IL-6. In endothelial cells, neither *E. coli* LPS nor *P. gingivalis* LPS promoted IL-6 production. In monocytes, serving as positive control, both *E. coli* and *P. gingivalis* LPS stimulated IL-6. *Conclusions*. *E. coli* LPS but not *P. gingivalis* LPS stimulates PDL cell IL-6 production through a glucocorticoid-sensitive mechanism involving nitric oxide formation probably via endothelial nitric oxide synthase.


**Key Words**: *cytokine, LPS, nitric oxide, periodontitis, PDL cells*

## Introduction

The periodontopathogen *Porphyromonas gingivalis* (*P. gingivalis*) is regarded as a major etiologic factor in periodontitis leading to production of cytokines/chemokines, such as interleukin-6 (IL-6), associated with the progression of the disease [1,2]. In the periodontium, not only classical inflammatory cells, such as white blood cells and macrophages, but also periodontal ligament (PDL) cells and gingival fibroblasts respond to bacterial lipopolysaccharide (LPS) by producing cytokines and chemokines, although the exact mechanisms have not been identified [3]. Endothelial cells and gingival epithelial cells have also been reported to produce cytokines in response to LPS stimulation, suggesting that these cell types may contribute to the cytokine/chemokine load in periodontal inflammation [4-6]. LPS is thought to promote cytokine/chemokine expression by binding to Toll-like receptors (TLRs), which through either a myeloid differentiation factor (MyD88)-dependent or a MyD88-independent pathway activates the transcription factor nuclear factor-κB (NF-κB) causing transcription of cytokine/chemokine genes, but still the mechanisms are not fully understood [7]. LPS primarily acts through TLR4 but LPS from some bacterial species also activates cells via TLR2 [7]. Interestingly, *P. gingivalis* LPS has been reported to act as an agonist for TLR2 but as an antagonist for TLR4 [8].

Estrogen has been shown to influence mechanisms involved in the inflammatory reaction, however its role in inflammation is complex involving both anti- and pro-inflammatory actions [9]. The biologically most active estrogen, 17β-estradiol ($E_2$), has been reported to modulate LPS-induced cytokine and chemokine expression in human PDL cells, suggesting that estrogen modulates periodontal inflammation by controlling PDL cell production of

cytokines and chemokines [10,11]. Estrogen-induced regulation of PDL cell cytokine/chemokine production is probably mediated via estrogen receptor β (ERβ), since this ER subtype is highly expressed in PDL cells, but we cannot completely rule out that also ERα is involved [12,13]. Nitric oxide (NO) is formed in an enzymatic reaction using the amino acid arginine as substrate, and NO regulates many important physiological and pathophysiological processes such as blood pressure and inflammation [14]. This reaction is catalyzed by three isoforms of nitric oxide synthase (NOS) named endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [14]. Interestingly, inhibition of NOS has been shown to prevent alveolar bone loss in experimental periodontitis, suggesting that NO modulates inflammation and bone metabolism/catabolism in periodontitis [15,16].

Although PDL cells are recognized producing cytokines in response to stimulation with inflammation promoters such as LPS, the mechanisms involved have not been fully explored. The objective of the present study was to characterize signaling mechanisms involved in LPS-induced stimulation of PDL cell IL-6 production. We show that LPS from *Escherichia coli* (*E. coli*) is a much more powerful stimulator of PDL cell IL-6 production than LPS from *P. gingivalis*. The involvement of estrogen, glucocorticoids and NO in LPS-induced IL-6 production is assessed using a pharmacological approach. We disclose that inhibition of NO production and treatment with glucocorticoids reduces *E. coli* LPS-induced IL-6 production. Furthermore, we show that both *P. gingivalis* and *E. coli* LPS stimulate monocyte IL-6 production, while they have no effect on IL-6 in endothelial cells, suggesting a cell type specific effect.

## Material and methods

*Cells and cell culture*

PDL cells were obtained from premolars extracted for orthodontic reasons. The patients and their parents were informed and the parents gave written consent. The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden. The periodontal ligament was gently scraped off from the middle third of the root surface and then washed in phosphate buffered saline (PBS) in order to avoid contamination from the gingival and apical tissues. Tissue explants from four premolars in two subjects (one boy and one girl 14 years of age) were seeded providing eight batches of PDL cells. These eight batches of cells responded identically to treatment with the different drugs specified below. Previous studies confirm that PDL cells obtained from teeth extracted for orthodontic reasons in young individuals show identical morphology and functional characteristics irrespective of donor [12,17]. The tissue explants were transferred to cell culture flasks containing Dulbecco's modified Eagle's medium supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), glutamine (1.16 g/l) and 10% fetal calf serum. The flasks were placed in a water-jacketed cell/tissue incubator with 5% $CO_2$ in air. The cells were allowed to migrate from the explants and after reaching confluence the cells were trypsinized (0.25% trypsin) and reseeded at a density of 80,000 cells/ml. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Lonza, Walkersville, MD, USA) and cultured in endothelial cell culture medium as recommended by the manufacturer. Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium as recommended. Experiments were performed in sub-confluent cells in passages 2–5.

*Experimental procedure*

Before experiments the normal culture medium was exchanged for phenol red-free culture medium containing dextran-coated charcoal stripped fetal calf serum to remove the estrogen-like activity of phenol red and estrogens derived from the serum. The phenol red-free culture medium with charcoal stripped fetal calf serum was used throughout the experiment. After 2 h pre-treatment with or without 17β-estradiol, $E_2$ (100 nM, Sigma Chemicals, St. Louis, MO, USA), dexamethasone (1 µM, Sigma), $N^G$-nitro-L-arginine methyl ester (L-NAME, 100 µM, Sigma) or aminoguanidine (10 µM, Sigma), the cells were treated with or without *E. coli* 0111:B4 LPS (1 µg/ml, Sigma) or *P. gingivalis* LPS (1-5 µg/ml, InvivoGen, San Diego, CA, USA) for 24-72 h in the continuous presence of $E_2$, L-NAME, dexamethasone or aminoguanidine. In order to detect any possible effect of estrogen we used a physiologically high concentration (100 nM) of $E_2$. The pre-ovulatory plasma concentration of $E_2$ is around 2 nM but increases in pregnancy several fold to about the same concentration as used by us in the present study [18]. L-NAME, at 100 µM, inhibits all three isoforms of nitric oxide synthase (NOS), i.e. eNOS, iNOS and nNOS, while aminoguanidine selectively reduces iNOS with an $IC_{50}$ of about 10 µM [19]. $E_2$ and dexamethasone were dissolved in dimethylsulfoxide (DMSO), while L-NAME and aminoguanidine were dissolved in PBS. Controls received vehicle as appropriate.

*Quantitative real-time PCR*

Cells were washed carefully in PBS and then total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Concentration and purity of RNA was measured at 260/280 nm in a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). One-step quantitative real-time PCR measurements were performed

using QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in duplicate. IL-6, monocyte chemoattractant protein-1 (MCP-1), TLR2 and TLR4 gene expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene as described by Pfaffl [20]. The PCR primers (QuantiTect Primer Assays) for IL-6 (Hs_IL6_1_SG), MCP-1 (Hs_CCL2_1_SG), TLR2 (Hs_TLR2_1_SG), TLR4 (Hs_TLR4_2_SG) and GAPDH (Hs_GAPDH_2_SG) were purchased from Qiagen.

*ELISA*

The cells were washed carefully in PBS and scraped off the culture dishes using cell scrapers (Sarstedt, Newton, NC, USA). Then the cells were sonicated 2 x 10 s on ice and centrifuged at 1700 x *g* at 4 °C for 5 min. The IL-6 protein was determined in the cell supernatant using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA). IL-6 protein level was determined according to instructions by the manufacturer. Each sample was analyzed in duplicate. The IL-6 level was normalized to the total protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

*Statistics*

Values are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for unpaired comparisons with Bonferroni correction for post hoc analysis as appropriate. P values less than 0.05 were regarded to denote statistical significance.

## Results

*Effects of E. coli and P. gingivalis LPS on IL-6 production in PDL cells*

Stimulation with *E. coli* LPS (1 µg/ml) for 24 h increased mRNA for IL-6 by 4 to 5 times, while, on the other hand, *P. gingivalis* LPS, administered at the same concentration (1 µg/ml), had no effect on PDL cell IL-6 mRNA expression (Fig. 1A). A similar pattern was observed for another important cytokine, the chemokine monocyte chemoattractant protein-1 (MCP-1) as well, i.e. stimulation with *E. coli* LPS (1 µg/ml) for 24 h increased MCP-1 mRNA by about 4 times, while *P. gingivalis* LPS (1 µg/ml) had no effect (Fig. 1B). Treatment with 1 µg/ml *E. coli* LPS for 24 h increased IL-6 protein concentration by about 30 times in PDL cells (Fig. 2), while, on the contrary, *P. gingivalis* LPS (1 µg/ml) had no effect on PDL cell IL-6 production confirming the mRNA data (Fig. 2). Treatment with *E. coli* LPS (1 µg/ml) for a longer time period (72 h) also increased IL-6 protein production, while treatment with *P. gingivalis* LPS (5 µg/ml) had no effect (Fig. 3).

The *E. coli* LPS-induced (1 µg/ml) increase in PDL cell IL-6 protein production at 24 h was attenuated by about 30% by the non-selective NOS blocker L-NAME (100 µM), while, on the other hand, $E_2$, administered in a high but still physiological concentration (100 nM), had no effect (Fig. 2). The selective iNOS blocker aminoguanidine (10µM) had no effect on *E. coli* LPS-induced IL-6 (Fig. 2). The glucocorticoid dexamethasone (1 µM) completely abolished the PDL cell IL-6 production induced by 1 µg/ml *E. coli* LPS (Fig. 4).

*Expression of TLR2 and TLR4 in PDL cells*

*E. coli* LPS acts via TLR4, while *P. gingivalis* LPS is reported to act as an agonist for TLR2 and antagonist for TLR4 [7,8]. In order to assess if the effects of *E. coli* and *P. gingivalis* LPS on IL-6 correlate with differences in TLR2 and TLR4 expression, quantitative real-time PCR for TLR2 and TLR4 was performed in PDL cells using primers with identical efficiency. We show that PDL cells express about 12 times higher levels of TLR2 mRNA than TLR4 mRNA (Fig. 5). The expression levels of TLR2 and TLR4 were not affected by 24 h treatment with either *E. coli* or *P. gingivalis* LPS (1 μg/ml for both types of LPS, data not shown).

*Effects of E. coli and P. gingivalis LPS on IL-6 production in HUVEC endothelial cells*

Treatment with *E. coli* LPS (1 μg/ml) for 24 h had no effect on IL-6 concentration in human HUVEC umbilical vein endothelial cells (0.019±0.002 pg/μg protein in LPS-treated cells vs. 0.012±0.002 pg/μg protein in control cells, n=4 in each group). Stimulation with *P. gingivalis* LPS for 24 h had no effect on HUVEC IL-6 protein concentration neither at 1 (0.078±0.005 pg/μg protein in LPS-treated vs. 0.086±0.006 pg/μg protein in control cells, n=4 in each group) nor at 5 μg/ml (0.082±0.003 pg/μg protein in LPS-treated vs. 0.086±0.006 pg/μg protein in control cells, n=4 in each group). Treatment with *P. gingivalis* LPS (1 and 5 μg/ml) for a longer time period (72 h) had no effect on IL-6 (data not shown).

*Effects of E. coli and P. gingivalis LPS on IL-6 production in THP-1 monocytes*

Effects of *E. coli* and *P. gingivalis* LPS on IL-6 production in human THP-1 monocytes were determined for positive control. Treatment with 1 μg/ml *E. coli* LPS or 1 μg/ml *P. gingivalis* LPS for 24 h increased IL-6 protein by about 80 and 6 times, respectively (Fig. 6), showing

that monocytes produce IL-6 in response to stimulation with *E. coli* LPS as well as *P. gingivalis* LPS.

## Discussion

Here we show that *E. coli* LPS stimulates IL-6 production several fold in human PDL cells. On the other hand, LPS from *P. gingivalis* has no effect on PDL cell IL-6 production, suggesting that LPS derived from *E. coli* is a more powerful stimulator of PDL cell IL-6 production than LPS from *P. gingivalis*. Our data confirm those by Jones et al. [21] showing that *E. coli LPS* causes a much more powerful stimulation of IL-6 than *P. gingivalis* LPS in mouse gingival fibroblast cell line ESK-1 cells. Interestingly, Morandini et al. [22] show recently that stimulation with *P. gingivalis* LPS (the same LPS, from InvivoGen, as the one used by us in the present study), administered in the same concentration (1 μg/ml) and for the same time (1-24 h) as in our study, has no effect on IL-6 expression, neither on mRNA nor on protein levels, in PDL cells and gingival fibroblasts from the same donors. Indeed, Morandini et al. [22] demontrate that a high concentration (10 μg/ml) of *P. gingivalis* LPS promotes PDL cell IL-6 expression but only on the protein level and not on the mRNA level. Taken together these data strongly suggest that *P. gingivalis* LPS is a weak stimulator of IL-6 production both in PDL cells and gingival fibroblasts. *E. coli* LPS acts via TLR4, while *P. gingivalis* LPS is reported to act as an agonist for TLR2 and antagonist for TLR4 [7,8]. Here, we show that PDL cells express much higher levels of TLR2 than TLR4 mRNA, suggesting that the mechanism behind the differential effects of *E. coli* and *P. gingivalis* LPS is not associated with differences in TLR subtype expression but involves mechanisms down-stream of the TLRs. However, we cannot rule out the possibility that the differential response to *E. coli* and *P. gingivalis* LPS may involve differences in receptor-affinity for these two types of LPS.

Furthermore, our data show that *E. coli* LPS stimulates IL-6 production in PDL cells but not in HUVEC endothelial cells, suggesting that *E. coli* LPS acts in a cell type specific manner. HUVEC endothelial cells are widely used studying human endothelial cell physiology and pathophysiology. Indeed, Makó et al. [23] have recently shown that LPS is a poor stimulator of IL-6 in HUVECs probably due to the cellular localization of TLR4. These authors show that endothelial cell TLR4 is mainly localized to the Golgi apparatus. We used human THP-1 monocytes for positive control showing that both *E. coli* LPS and *P. gingivalis* LPS stimulate IL-6 production in these cells. Thus, the periodontopathogen *P. gingivalis* stimulates production of IL-6 by monocytes but not by PDL cells or endothelial cells.

We show in the present study that the NOS blocker L-NAME, blocking all three isoforms of NOS, but not the selective iNOS blocker aminoguanidine, reduces partially *E. coli* LPS-induced IL-6 production in PDL cells, suggesting that the signaling pathway involves NO formed via eNOS and/or nNOS rather than via iNOS. L-NAME was used in a concentration (100 µM) that inhibits all NOS isoforms and aminoguanidine in a concentration (10 µM) that is equivalent to its $IC_{50}$ concentration for iNOS [19]. Our results suggesting that eNOS but not iNOS may be involved in *E. coli* LPS-induced PDL cell IL-6 production are supported by findings by Kikuiri et al. [24] showing that human PDL cells express eNOS but not iNOS as demonstrated both on mRNA and protein levels. The nNOS isoform is primarily expressed in neuronal tissues making it less likely that this NOS isoform is involved [14]. Here, we show that the attenuation of PDL cell IL-6 production by L-NAME is small compared to that of the glucocorticoid dexamethasone which totally prevents *E. coli* LPS-induced IL-6. Indeed, Jönsson et al. [25] have previously observed that *E. coli* LPS-induced GROα chemokine production is abolished by dexamethasone in PDL cells. Taken together, these data suggest that both IL- 6 and GROα chemokine expression in PDL cells is sensitive to glucocorticoid

treatment. Glucocorticoids are supposed to exert their anti-inflammatory effects by inhibiting NF-κB transcriptional effects [26]. Thus, it is reasonable to conclude that transcription of the IL-6 gene in PDL cells stimulated by *E. coli* LPS involves NF-κB since dexamethasone fully prevents *E. coli* LPS-induced IL-6.

Estrogen (17β-estradiol, $E_2$) has been reported to modulate inflammation and immune responses [9]. In the present study $E_2$ failed to prevent *E. coli* LPS-induced IL-6 in PDL cells, suggesting that estrogen modulates inflammation via another mechanism. In order to detect any possible effect of $E_2$ we used a high but still physiological concentration of $E_2$, i.e. 100 nM [18]. The lack of effect of $E_2$ on E. coli-induced IL-6 observed in the present study confirms data reported by Jönsson et al. [27] showing that both acute (24 h) and chronic (72 h) treatment with 100 nM $E_2$ has no effect on IL-6 production in human PDL cells stimulated by *E. coli* LPS.

In conclusion, we show that *E. coli* LPS, but not *P. gingivalis* LPS, is a powerful stimulator of PDL cell IL-6 production through a glucocorticoid-sensitive pathway, and that combined treatment with *E. coli* LPS and the non-selective NOS blocker L-NAME reduces *E. coli* LPS-induced IL-6, suggesting that *E. coli* LPS acts, at least partially, through stimulation of NO formation. Furthermore, we show that both *P. gingivalis* and *E. coli* LPS enhance IL-6 production several fold in monocytes, while they have no effect on IL-6 in endothelial cells, suggesting a cell type specific mechanism.

## Acknowledgements

We thank Dr. Gunilla Bratthall for her thoughtful advice, and Kristina Hamberg, Ina Nordström and Elisabeth Thornqvist for excellent technical assistance. This study was supported by grants from the Swedish Research Council, the Swedish Dental Society, the Greta and Johan Kocks Foundation, the Thuréus Foundation, the Odontological Faculty at Malmö University and the Medical Faculty at Lund University.

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

***Ethical approval:*** The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden.

## References

[1] Feng Z, Weinberg A. Role of bacteria in health and disease of periodontal tissues. Periodontol 2000 2006;40:50-76.

[2] Trevilatto PC, Scarel-Caminaga RM, de Brito RB Jr., de Souza AP, Line SR. Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. J Clin Periodontol 2003;30:438-42.

[3] Jönsson D, Nebel D, Bratthall G, Nilsson BO. The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell. J Periodontal Res 2011;46:153-7.

[4] Sawa Y, Ueki T, Hata M, Iwasawa K, Tsuruga E, Kojima H, et al. LPS-induced IL-6, IL-8, VCAM-1 and ICAM-1 expression in human lymphatic endothelium. J Histochem Cytochem 2008;56:97-109.

[5] Annand AR, Bradley R, Ganju RK. LPS-induced MCP-1 expression in human microvascular endothelial cells is mediated by the tyrosine kinase Pyk2 via the p38 MAPK/NF-κB-dependent pathway. Mol Immunol 2009;46:962-8.

[6] Sandros J, Karlsson C, Lappin DF, Madianos PN, Kinane DF, Papapanou PN. Cytokine responses of oral epithelial cells to Porhpyromonas gingivalis infection. J Dent Res 2000;79:1808-14.

[7] Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. Lab Invest 2006;86:9-22.

[8] Yoshimura A, Kaneko T, Kato Y, Golenbock DT, Hara Y. Lipopolysaccharides from periodontopathic bacteria Porphyromonas gingivalis and Capnocytophaga ochracea are antagonists for human toll-like receptor 4. Infect Immun 2002;70:218-25.

[9] Nilsson BO. Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. *Inflamm Res* 2007;56:269-73.

[10] Shu L, Guan SM, Fu SM, Guo T, Cao M, Ding Y. Estrogen modulates cytokine expression in human periodontal ligament cells. J Dent Res 2008;87:142-7.

[11] Nebel D, Jönsson D, Norderyd O, Bratthall G, Nilsson BO. Differential regulation of chemokine expression by estrogen in human periodontal ligament cells. J Periodont Res 2010;45:796-802.

[12] Jönsson D, Andersson G, Ekblad E, Liang M, Bratthall G, Nilsson BO. Immunocytochemical demonstration of estrogen receptor β in human periodontal ligament cells. Arch Oral Biol 2004;49:85-8.

[13] Tang X, Meng H, Han J, Zhang L, Hou J, Zhang F. Up-regulation of estrogen receptor-beta expression during osteogenic differentiation of human periodontal ligament cells. J Periodont Res 2008;43:311-21.

[14] Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-42.

[15] Leitão RF, Ribeiro RA, Chaves HV, Rocha FA, Lima V, Brito GA. Nitric oxide synthase inhibition prevents alveolar bone resorption in experimental periodontitis in rats. J Periodontol 2005;76:956-63.

[16] Herrera BS, Martins-Porto R, Maia-Dantas A, Campi P, Spolidorio LC, Costa SK, et al. iNOS-derived nitric oxide stimulates osteoclast activity and alveolar bone loss in ligature-induced periodontitis in rats. J Periodontol 2011;82:1608-15.

[17] Jönsson D, Wahlin Å, Idvall I, Johnsson I, Bratthall G, Nilsson BO. Differential effects of estrogen on DNA synthesis in human periodontal ligament and breast cancer cells. J Periodont Res 2005;40:401-406.

[18] Cornil CA, Ball GF, Balthazart J. Functional significance of the rapid regulation of the rapid regulation of brain estrogen action: where do the estrogens come from? Brain Res 2006;1126:2-26.

[19] Nilsson BO. Biological effects of aminoguanidine: An update. Inflamm Res 1999;48:509-15.

[20] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

[21] Jones KJ, Ekhlassi S, Montufar-Solis D, Klein JR, Schaefer JS. Differential cytokine patterns in mouse macrophages and gingival fibroblasts after stimulation with Porphyromonas gingivalis or Escherichia coli lipopolysaccharide. J Periodontol 2010;81:1850-7.

[22] Morandini ACF, Sipert CR, Gasparoto TH, Greghi SL, Passanezi E, Rezende M, et al. Differential production of macrophage inflammatory protein-1α, stromal-derived factor-1, and IL-6 by human cultured periodontal ligament and gingival fibroblasts challenged with lipopolysaccharide from P. gingivalis. J Periodontol 2010;81:310-7.

[23] Makó V, Czúcz J, Weiszhár Z, Herczenik E, Matkó J, Prohászka Z, et al. Proinflammatory activation pattern of human umbilical vein endothelial cells induced by IL-1β, TNF-α, and LPS. Cytometry A 2010;77:962-70.

[24] Kikuiri T, Hasegawa T, Yoshimura Y, Shirakawa T, Oguchi H. Cyclic tension force activates nitric oxide production in cultured human periodontal ligament cells. J Periodontol 2000;71:533-9.

[25] Jönsson D, Amisten S, Bratthall G, Holm A, Nilsson BO. LPS induces GROα chemokine production via NF-κB in oral fibroblasts. Inflamm Res 2009;58:791-6.

[26] Clark AR. Anti-inflammatory functions of glucocorticoid-induced genes. Mol Cell Endocrinol 2007;275:79-97.

[27] Jönsson D, Nebel D, Bratthall G, Nilsson BO. LPS-induced MCP-1 and IL-6 production is not reversed by oestrogen in human periodontal ligament cells. Arch Oral Biol 2008;53:896-902.

## Figure legends

**Fig. 1.** Stimulation with *E. coli* LPS (1 µg/ml) but not *P. gingivalis* (PG) LPS (1 µg/ml) for 24 h increases mRNA for (**A**) IL-6 and (**B**) MCP-1 in human PDL cells. Values are means ± S.E.M. of 6 observations in each group. * and *** represent $P<0.05$ and $P<0.001$. N.S. = not significant.

**Fig. 2.** *E. coli* LPS-induced PDL cell IL-6 protein production is reduced by the non-selective NO synthase blocker L-NAME. Human PDL cells were stimulated for 24 h with *E. coli* or *P. gingivalis* (PG) LPS (1 µg/ml) in the absence or presence of L-NAME (100µM), 17β-estradiol ($E_2$, 100 nM) or the inducible NO synthase blocker aminoguanidine (AG, 10µM). IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 4-12 observations in each group. * and *** represent $P<0.05$ and 0.001, respectively. N.S. = not significant.

**Fig. 3.** Treatment with 1 µg/ml *E. coli* LPS but not 5 µg/ml *P. gingivalis* (PG) LPS for 72 h increases IL-6 protein production in human PDL cells. IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 4 observations in each group. ** represents $P<0.01$. N.S. = not significant.

**Fig. 4.** The glucocorticoid dexamethasone (Dexa, 1 µM) abolishes completely PDL cell IL-6 protein production induced by 1 µg/ml *E. coli* LPS at 24 h. IL-6 content was normalized to

total protein concentration in each sample. Values are means ± S.E.M. of 5 observations in each group. ** and *** represent P<0.01 and P<0.001, respectively. N.S. = not significant.

**Fig. 5.** Human PDL cells express about 12 times higher levels of TLR2 mRNA than TLR4 mRNA. Values are means ± S.E.M. of 6 observations in each group. *** represents P<0.001.

**Fig. 6.** Stimulation with *E. coli* or *P. gingivalis* (PG) LPS (1 µg/ml for both *E. coli* and *P. gingivalis* LPS) for 24 h enhances IL-6 protein production by about 80 and 6 times, respectively in human THP-1 monocytes used for positive control**.** IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 5 observations in each group. * and *** represent P<0.05 and P<0.001, respectively.

**Fig. 1**

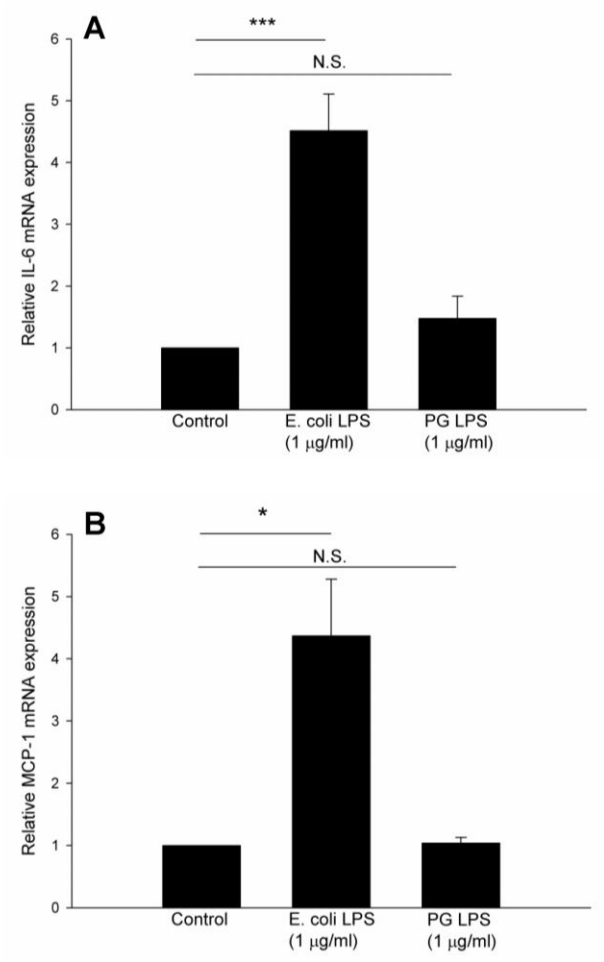

A
***
N.S.
Relative IL-6 mRNA expression
0
1
2
3
4
5
6
Control
E. coli LPS
(1 μg/ml)
PG LPS
(1 μg/ml)
B
*
N.S.
Relative MCP-1 mRNA expression
0
1
2
3
4
5
6
Control
E. coli LPS
(1 μg/ml)
PG LPS
(1 μg/ml)

**Fig. 2**

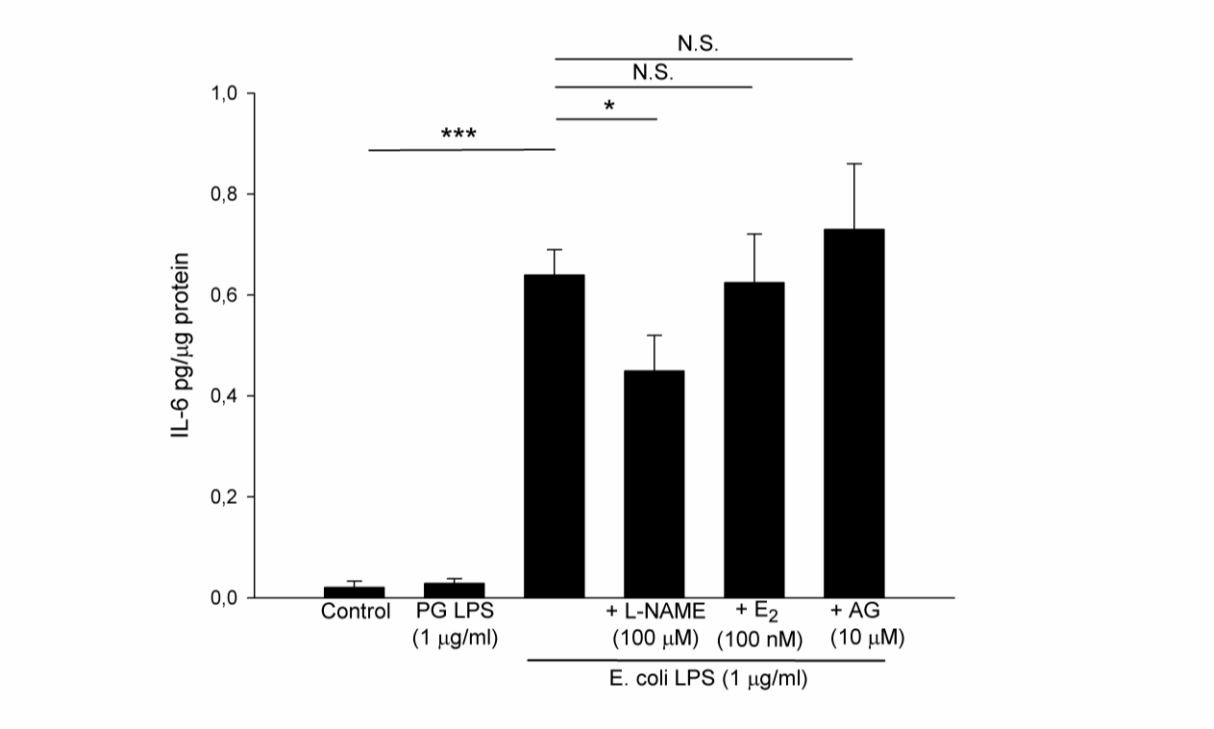

N.S.
N.S.
*
***
1,0
0,8
0,6
0,4
0,2
0,0
IL-6 pg/μg protein
Control
PG LPS
(1 μg/ml)
+ L-NAME
(100 μM)
+ E2
(100 nM)
+ AG
(10 μM)
E. coli LPS (1 μg/ml)

**Fig. 3**

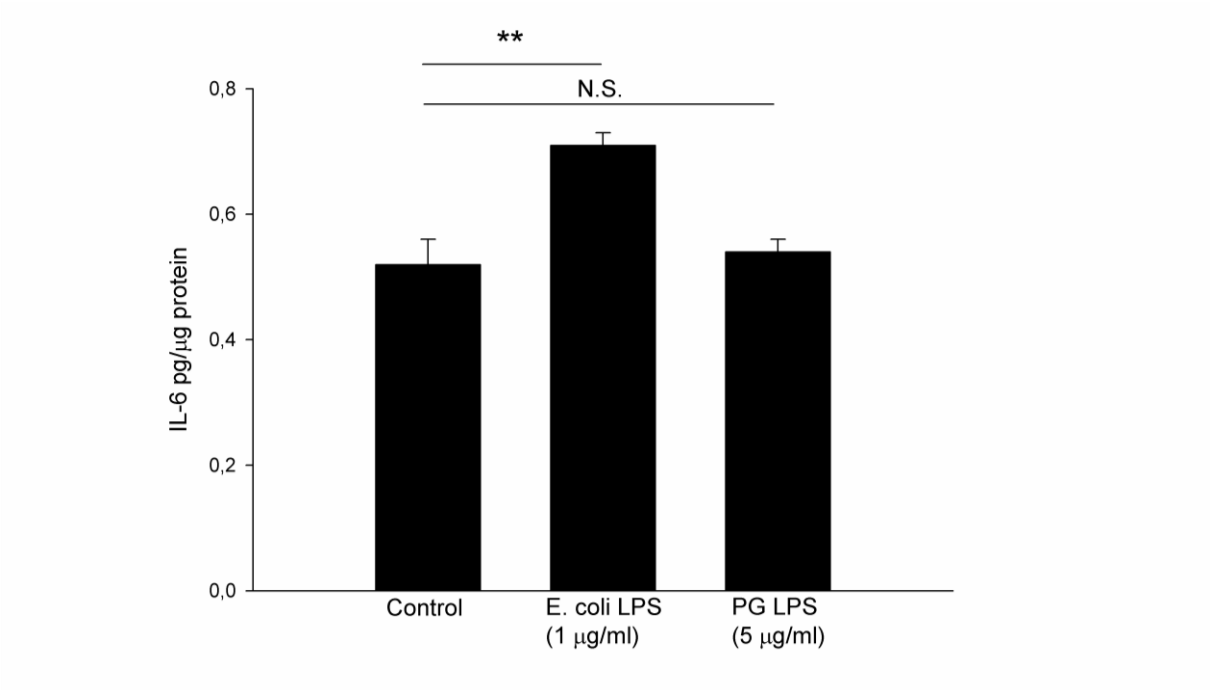

**
N.S.
0,8
0,6
0,4
0,2
0,0
IL-6 pg/μg protein
Control
E. coli LPS
(1 μg/ml)
PG LPS
(5 μg/ml)

**Fig. 4**

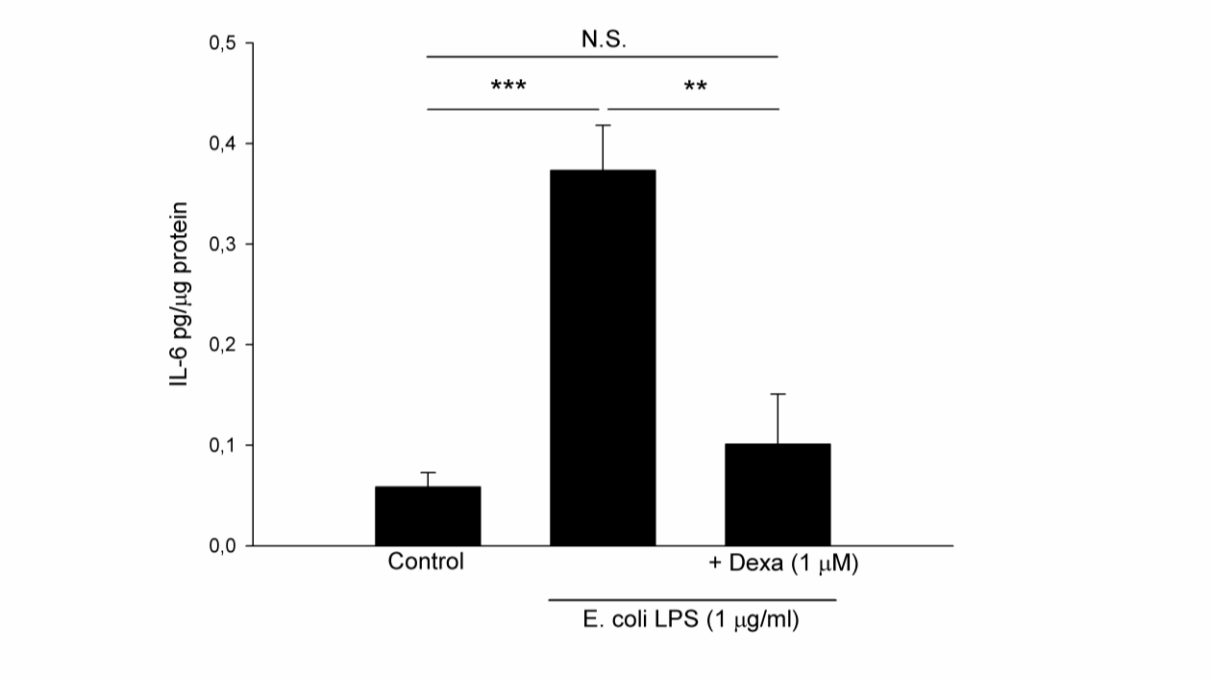

N.S.
***
**
0,5
0,4
0,3
0,2
0,1
0,0
IL-6 pg/μg protein
Control
+ Dexa (1 μM)
E. coli LPS (1 μg/ml)

**Fig. 5**

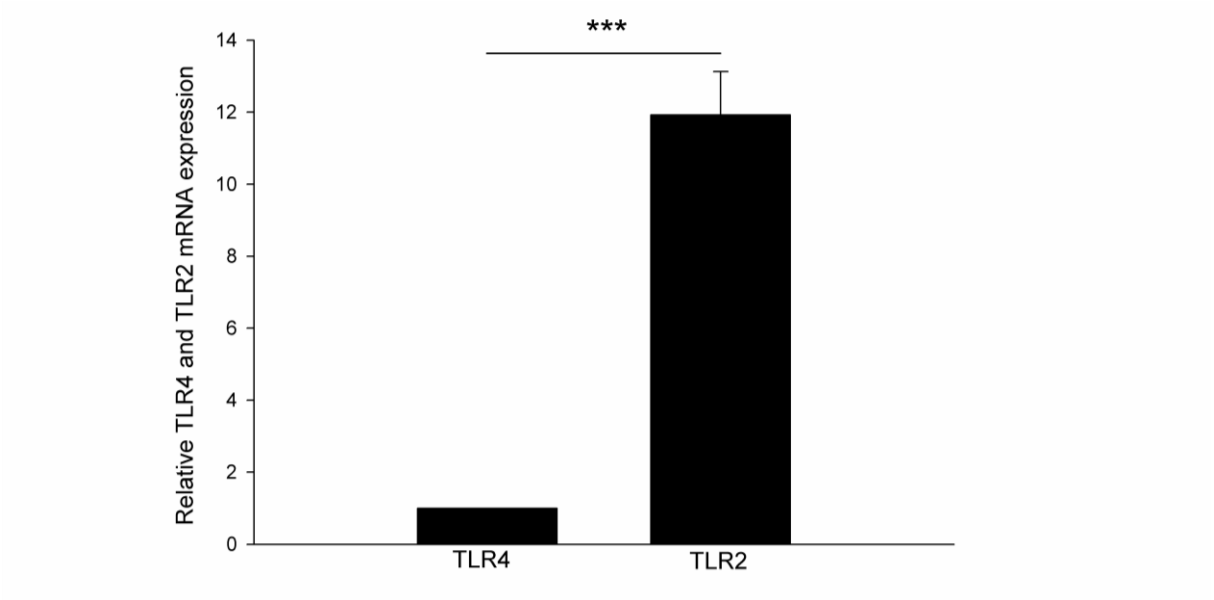
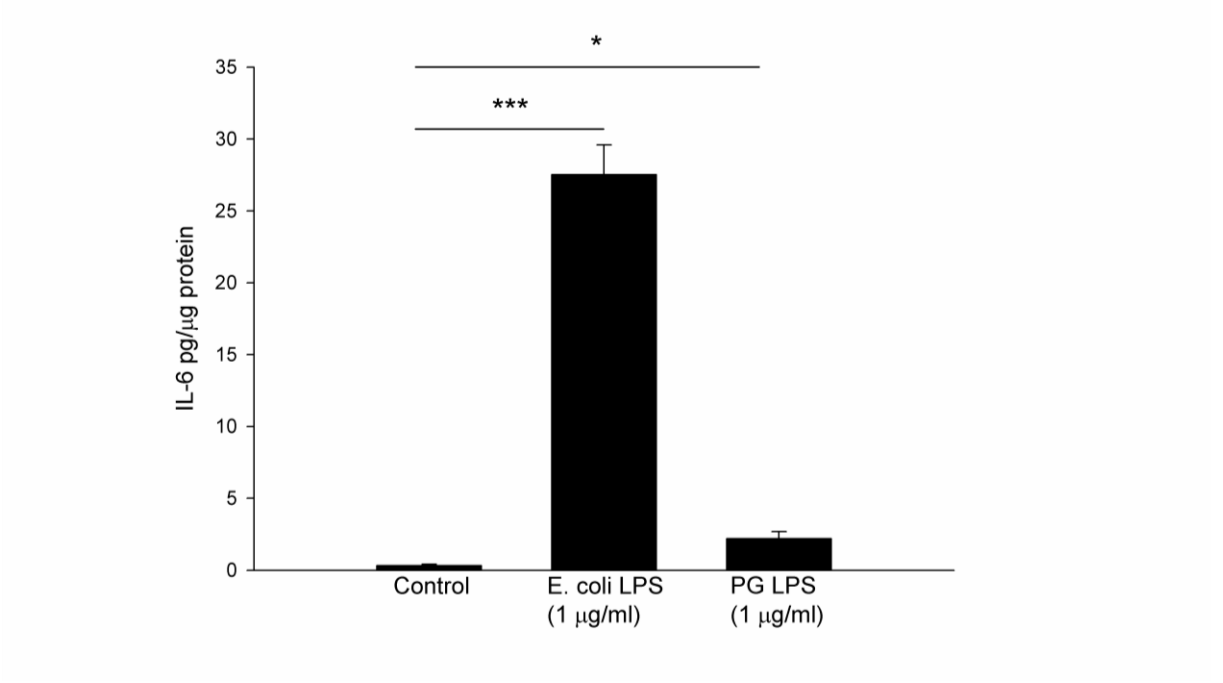

***
Relative TLR4 and TLR2 mRNA expression
14
12
10
8
6
4
2
0
TLR4
TLR2

**Fig. 6**


*
***
35
30
25
20
15
10
5
0
IL-6 pg/μg protein
Control
E. coli LPS
(1 μg/ml)
PG LPS
(1 μg/ml)