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Differential Cytokine Patterns in Mouse Macrophages and Gingival Fibroblasts following Stimulation with *Porphyromonas gingivalis* or *Escherichia coli* Lipopolysaccharide

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Abstract

Background—A major cause of chronic inflammatory periodontal diseases is *Porphyromonas gingivalis* (*P. gingivalis*), a non-motile, gram-negative, rod-shaped, anaerobic bacterium. Within gingival tissue, both macrophages and fibroblasts participate in the immune response to foreign entities by releasing cytokines and expressing molecules to recruit and activate lymphocytes. However, the contribution of gingival macrophages and fibroblasts to the immune response to *P. gingivalis* infection is not fully known.

Methods—The AMJ2-C8 cell line (AM cells), a mouse alveolar macrophage cell line, and ESK-1 cells, a mouse gingival fibroblast cell line made in our laboratory, were treated with lipopolysaccharide (LPS) from either *P. gingivalis* or *Escherichia coli* (*E. coli*). The expression of immune response molecules was quantified by real-time PCR and enzyme-linked immunoassay.

Results—AM and ESK-1 cells responded differently to *P. gingivalis* and *E. coli* LPS stimulation. The ESK-1 gingival fibroblast cell line was more responsive to *E. coli* LPS stimulation as seen by elevated levels of interleukin (IL)-6, inducible nitric oxide (iNOS), and monocyte chemotactic protein-1 (MCP-1) expression relative to stimulation by *P. gingivalis* LPS. Conversely, the AM macrophage cell line was more responsive to *P. gingivalis* LPS stimulation, particularly for interleukin IL-1β, IL-6, and MCP-1, relative to stimulation by *E. coli* LPS.

Conclusion—These findings demonstrate that *E. coli* LPS induces a stronger cytokine/chemokine response in gingival fibroblasts, while *P. gingivalis* LPS induces a stronger response in macrophages.

Keywords

cytokines; fibroblasts; host response; immunology; inflammation and innate immunity

INTRODUCTION

Periodontal diseases are chronic inflammatory diseases of the oral cavity. Periodontal diseases are classified as either gingivitis or periodontitis; gingivitis is the milder form affecting only the gingiva, whereas periodontitis affects the periodontium, damaging the

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alveolar bone structure which can lead to tooth loss[1,2]. Early linear conceptual models of periodontal disease have advanced over the years to complex integrated hierarchical models of disease[3]. Current models recognize the importance of contributory factors affecting pathogenesis including host genetic variation, environmental factors, and host responses including activation of the inflammatory responses, and alteration of bone and connective tissue metabolism[3]. Even with the recognition of the interplay between multiple factors for periodontal disease pathogenesis, microbial challenge has remained the cornerstone of these models throughout their evolution.

One of the major contributing agents in the development of chronic inflammatory periodontal diseases is *Porphyromonas gingivalis* (*P. gingivalis*), a non-motile, gram-negative, rod-shaped, anaerobic bacterium. Previous reports have demonstrated an association between *P. gingivalis* and periodontal health[4-6]. In a study examining oral *P. gingivalis* infection, *P. gingivalis* was detected in 25% of healthy subjects compared to 79% of periodontitis patients; an odds ratio of 11.2 was reported for *P. gingivalis* infection in the periodontitis patients[4]. Several animal models including non-human primates have been shown to develop periodontal disease upon infection with *P. gingivalis*[7]. Furthermore, antibiotic treatment to clear the bacterial infection improves oral health[8-10].

Within gingival tissue, both macrophages and fibroblasts participate in the immune response to foreign entities. In healthy gingival tissue, macrophages are rare but increase in number during an inflammatory response. Conversely, fibroblasts are the most abundant cell type within the gingival tissue[11]. Both fibroblasts and macrophages release chemokines and cytokines to recruit and activate lymphocytes to sites of inflammation. Given the abundance of fibroblasts within the periodontium, one would expect fibroblasts to be the primary responder during *P. gingivalis* infection to recruit and activate macrophages and other immune cells. However, the interplay between gingival fibroblasts and macrophages during an immune response, particularly to *P. gingivalis* infection, is not fully known.

In this study, we sought to quantify the response of a mouse AMJ2-C8 alveolar macrophage cell line (AM cells) and a mouse gingival fibroblast cell line produced in our laboratory (designated ESK-1) that were exposed to *P. gingivalis* or *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS). The underlying hypothesis for these studies was that *P. gingivalis* LPS would induce different cytokine and chemokine responses in AM and ESK-1 cells compared to stimulation with *E. coli* LPS, and that the effect of *P. gingivalis* LPS stimulation would be to activate stronger proinflammatory cytokine responses in macrophages than in gingival fibroblasts. Here we show that the AM macrophage cell line indeed responds to *P. gingivalis* LPS by producing a stronger proinflammatory response, whereas *E. coli* LPS was a stronger inductive signal for cytokine/chemokine responses from gingival fibroblasts.

MATERIALS AND METHODS

Preparation of Retrovirus containing the SV40 Large T Antigen

The PT67 viral-packaging cell line† was grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS and 1% penicillin/streptomycin (DMEM-10). Fugene6‡ was used to transfect the PT67 cells with the pBABE-puro SV40 large T (LT) retroviral plasmid§, which encodes the SV-40 LT antigen, a molecule with a known ability to transform cells. Stable transfectants were selected with 2 μg/ml puromycin. PT67 pBABE-puro SV40 LT

†American Type Culture Collection; Manassas, VA.
‡Roche; Basel, Switzerland.
§Addgene, Inc; Cambridge, MA (plasmid 13970)

cells were grown for 24 hours without puromycin prior to virus collection in supernatants, which were filtered through a 0.45-μm syringe filter.

AM Cells, Mice, and Generation of Stable Virus-Transformed Gingival Cell Lines

AMJ2-C8 cells[†], referred to as AM cells in this present study, were grown in DMEM-10 as a floating cell suspension and sub-cultured 3x weekly.

Adult female 6-10 week old C57BL/6 mice were purchased from Harlan (Indianapolis, IN). Animals were used according to protocols approved by the Institutional Animals Care and Use Committee of the University of Texas Health Science Center at Houston. After euthanization, gingival tissues were dissected from below the mandibular incisors, minced and placed in a 24-well Costar tissue culture plates[‖] in DMEM-10 in a 37°C incubator in a 10% CO_2 environment as previously reported[12]. After 1-2 weeks, several wells had cell growth consisting of 40-60% confluent monolayer. The pBABE-puro SV40 LT retrovirus supernatant was mixed with 4 μg/ml polybrene, filtered through a 0.45-μm syringe filter, and added to the primary gingival cells for culture overnight. The infection was repeated the next day for higher infection efficiency. The viral media was replaced the following day with supplemented DMEM-10 without penicillin/streptomycin but containing 2 μg/ml puromycin. Gingival cells that proliferated in the presence of puromycin were grown for 2-3 weeks; 25% (v/v) supplemented DMEM-10 containing puromycin was replaced in well twice weekly. Wells with uniform cell growth consisting of 80-90% confluency were trypsinized and transferred to 60 mm tissue culture plates in supplemented DMEM. A cell culture displaying vigorous growth was split at a 1:4 ratio into 100 mm tissue culture plates. This line, designated ESK-1, could be maintained indefinitely by subculture twice weekly.

Cell Staining

ESK-1 was grown in Lab-Tek chambered cover glass slides[¶]. Cells were fixed in methanol for 5 min, air dried, and washed and rehydrated in PBS. A 1:50 dilution of anti-CD16/CD32 Fc blocking antibody[#] was added for 20 min at room temperature. 1:50 dilution of Alexa Fluor 488-labeled anti-vimentin antibody** reactive to mouse fibroblasts, or rat anti-mouse CD326 EP-Cam antibody (clone G8.8)[#] reactive to mouse epithelial cells, was reacted overnight at 4°C. After washing, biotin-labeled mouse anti-rat IgG[#] was added for 45 min at a dilution of 1:25 to cells stained with G8.8 antibody, washed with PBS, reacted with streptavidin-FITC[#] for 15 min followed by PBS washing. Cells were examined using a Nikon 80I microscope. Control staining was done using the mouse anti-rat IgG antibody followed by streptavidin-FITC[#] (for G8.8 staining) or streptavidin FITC-488[#] (for vimentin staining).

For flow cytometric analyses, cells were recovered from tissue culture plates and reacted with a 1:50 dilution of anti-CD16/CD32 Fc blocking antibody[#] for 20 min at room temperature. Cells were washed and reacted with either: PE-labeled anti-mouse EpCam (clone G8.8)[#], Alexa Fluor-labeled anti-vimentin antibody**, PE-labeled rat anti-mouse CD45 leukocyte common antigen (LCA)[#], PE-rat anti-mouse CD14[#], or biotin-labeled anti-mouse toll-like receptor (TLR)2[#], for 20 min at 4°C. The latter group was washed and reacted with streptavidin-PE[#] for 15 min at 4°C. Cells were fixed and analyzed on a FACSCalibur flow cytometer with Cell Quest software[††].

[‖]Fisher Scientific; Pittsburgh, PA.
[¶]Nalge; Rochester, NY.
[#]BD Bioscience Pharmingen; San Diego, CA.
[**]Santa Cruz Biologicals; Santa Cruz, CA.
[††]BD Bioscience; San Jose, CA.

Cell Stimulation

AM and ESK-1 cells were seeded at concentrations of 1×10^6 cells/ml in 5% FBS, RPMI-1640 medium and exposed to 1 μg/ml of *P. gingivalis* LPS (97-99% pure)[‡‡], or *E. coli* LPS (99% pure)[§§] for 24 hr in a 37°C incubator with 5% CO_2. Cells were collected and processed for either flow cytometry or quantitative real-time PCR. Cell-free supernatants were collected for enzyme-linked assay.

Quantitative Real-time PCR (qRT-PCR)

Following the incubation, total RNA was isolated from the AM and ESK-1 cells[‖ ‖] according to the manufacturer's instructions. SYBR Green qRT-PCR was used to analyze mRNA expression of the indicated genes[¶¶] according to the manufacturer's instructions. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method[13] and normalized to GAPDH. Gene specific primers [B7 related protein-1 (B7RP-1), IL-1β, IL-6, inducible nitric oxide synthase (iNOS), MCP-1, macrophage inflammatory protein (MIP-1α), MIP-1β, and thymus epithelial chemokine-1 (TECK-1) [##] or (IL-23p19 and GAPDH)[***] were used (Table 1).

Enzyme-Linked Immunoassay (EIA)

Cell-free supernatants were collected after 24 hr of stimulation and analyzed for synthesis of IL-6 and MCP-1[†††] according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were done using Student's *t*-test determinations for comparison of mean values of replicate samples. A *p*-value of ≤ 0.05 was regarded as statistically significant.

RESULTS

Characterization of Transformed Gingival Cell Lines

The ESK-1 cell line was derived as described in the Materials and Methods. After several months of continuous in vitro culture, ESK-1 cells were stained with G8.8 antibody, which is reactive with an adhesion molecule found on epithelial cells or with an anti-vimentin antibody, which is reactive to intermediate filaments found in fibroblasts. ESK-1 cells were completely non-reactive with the G8.8 antibody (Fig. 1A), and were strongly reactive with the anti-vimentin antibody (Fig. 1B-C), indicating a fibroblast origin of those cells.

Based on flow cytometric analyses, ESK-1 cells did not express the CD45 leukocyte common antigen (CD45-LCA). Because CD45 LCA is expressed on all hematopoietic cells except erythrocytes, this confirmed that ESK-1 were not of hematopoietic origin. Similar to the fluorescence staining shown in panels Fig. 1A-C, ESK-1 cells did not express the epithelial cell marker, G8.8 (Fig. 1E), although they expressed vimentin (Fig. 1F), a marker commonly associated with fibroblasts. About two-thirds of the ESK-1 cells expressed CD14 (Fig. 1G) and most expressed TLR2 (Fig. 1H).

[‡‡]IvivoGen; San Diego, CA.
[§§]Sigma-Aldrich; St. Louis, MO.
[‖ ‖]RNeasy Minikit, Qiagen; Valencia, CA.
[¶¶]iScript One-Step RT-PCR Kit with SYBR Green; Biorad; Hercules, CA.
[##]DNA Technologies; Coralville, IA.
[***]SA Bioscience; Frederick, MD.
[†††]ELISA Ready-SET-Go Kits; eBioscience; San Diego, CA.

AM cells expressed CD45-LCA (Fig. 1I), indicating hematopoietic origins of those cells. They also were positive for vimentin (Fig. 1K), CD14 (Fig. 1L), and TLR2 (Fig. 1M), but did not express the epithelial marker, G8.8 (Fig. 1J). The findings for CD14 expression are consistent with reports of variably expression on both myeloid cells and fibroblasts[14], including gingival fibroblasts[15].

Differential Cytokine Patterns of Gingival Fibroblasts and Macrophages in Response to P. gingivalis and E. coli LPS

LPS from *P. gingivalis* and *E. coli* were used to evaluate the patterns of elicited cytokines of the ESK-1 gingival fibroblast cell line and the AM mouse macrophage cell line. Because both cell lines are homogeneous and thus uncontaminated by other cell types, this allowed us to accurately determine the effects of LPS stimulation. Gene expression for nine cytokines or their receptors were studied by qRT-PCR. Of those, gene expression for five cytokines, IL-6, iNOS, and MCP-1 ($p < 0.01$) and MIP-1α and MIP-1β ($p < 0.05$), were expressed at significantly higher levels in ESK-1 cells stimulated with *E. coli* LPS compared to stimulation with *P. gingivalis* LPS (Table 2). Only the B7RP-1, the ligand for the inducible costimulator (ICOS) molecule, and TECK-1, were expressed at higher levels ($p < 0.05$) in ESK-1 cells stimulated with *P. gingivalis* LPS compared to stimulation with *E. coli* LPS (Table 2).

The pattern of gene expression observed for AM cells differed from that of ESK-1 cells in that gene expression for seven cytokines, IL-1β, IL-6, and MCP-1 ($p < 0.01$) and IL-23, iNOS, MIP-1α, and MIP-1β ($p < 0.05$), were expressed at significantly higher levels following stimulation with *P. gingivalis* LPS compared to stimulation with *E. coli* LPS (Table 3). There was no significant difference between gene expression levels of *P. gingivalis* vs. *E. coli* LPS-stimulated AM cells for B7RP-1 or TECK-1 (Table 3).

To confirm that the findings observed at the transcriptional level held true with regard to secreted proteins, cytokine levels for IL-6 and MCP-1 were measured by EIA in cell-free supernatants 24 hrs post-stimulation with *P. gingivalis* or *E. coli* LPS. The patterns of cytokine synthesis at the protein levels closely reflected that of the transcriptional findings in that AM cells responded by producing more IL-6 and MCP-1 when stimulated with *P. gingivalis* LPS compared to stimulation with *E. coli* LPS (Fig. 2). Conversely, ESK-1 gingival fibroblasts produced more IL-6 and MCP-1 when stimulated with *E. coli* LPS compared to *P. gingivalis* LPS (Fig. 2).

DISCUSSION

In this study, the inflammatory responses elicited by exposure to *P. gingivalis* LPS or *E. coli* LPS were compared between the two primary cell types, macrophage and fibroblast, responsible for periodontal health and integrity. In accordance with previous results[12,16,17], exposure to either *P. gingivalis* LPS or *E. coli* LPS elicited a significant change in the expression of key proinflammatory mediators, particularly IL-6 and IL-1β, in the AM and ESK-1 cells. However, exposure to *P. gingivalis* LPS or *E. coli* LPS stimulation elicited very different responses in these homogenous cell lines. We anticipated that robust proinflammatory expression would be induced upon exposure to either LPS in both cell types with the presumption that, as the most dominant cell type in the gingival tissue and therefore the most likely cell to initiate an immune response, the ESK-1 fibroblasts may be more reactive to *P. gingivalis* LPS. However, our results indicate that the ESK-1 induction of inflammatory gene expression was significantly more robust in response to *E. coli* LPS as opposed to stimulation with *P. gingivalis* LPS. In contrast, the increase in inflammatory gene expression in the AM macrophages was more pronounced after stimulation with *P. gingivalis* LPS rather than *E. coli* LPS. Interestingly, gingival fibroblasts, the cell type most

likely to first encounter and thereby initiate a response to *P. gingivalis* LPS within the oral cavity, actually has a comparatively muted response when considering the massive increase in inflammatory mediators generated in AM cells stimulated with *P. gingivalis* LPS .

Previous work has demonstrated the importance of CD14 and TLR expression in the host cell-LPS binding interaction[18,19]. CD14 is a co-receptor, expressed mainly by macrophages, that binds to a variety of microbial antigens to initiate host immune responses[20-23]. TLR2 and TLR4 are the primary TLRs responsible for signaling in response to bacterial cell wall componentswith TLR4 being the primary signaling receptor for LPS[19] while TLR2 is the primary signal transducer for lipoproteins or peptidoglycans[24,25]. Depending on the cell context, CD14, in the presence of lipopolysaccharide-binding protein (LBP)[20], and TLR2 and TLR4 contribute to LPS signaling events. *P. gingivalis* LPS signaling in particular has been demonstrated to be CD14 dependent[26]. The chemical structure of the major lipid A species comprising LPS differs markedly between the *E. coli* and *P. gingivalis* bacterial species[18]. As a result of these structural differences, *E. coli* and *P. gingivalis* LPS have varying affinities for the TLR and CD14 molecules. Published reports have produced conflicting results in terms of *P. gingivalis* LPS signaling. Hirschfeld et al. demonstrated that *P. gingivalis* LPS signaling occurred primarily through TLR2 but not TLR4[18,27], whereas others have shown that TLR4 in conjunction with CD14 play an important role in *P. gingivalis* LPS signaling[19,28-31].

Since ESK-1 cells are TLR2+, weakly TLR4+, and CD14-, the LPS signaling through these cells was primarily TLR2-dependent. Likewise, LPS signaling through the AM macrophage cell line should be primarily TLR2-dependent as AM cells are strongly TLR2+, weakly TLR4+, and weakly CD14+. However, TLR2 expression is greater for the AM cells in comparison to the ESK-1 cells, indicating that the AM cells may be more capable to respond to *P. gingivalis* LPS. These differences in surface receptor complex expression may account for the LPS preferences observed in the ESK-1 and AM cell lines in this study, although other signaling mechanisms or surface receptors cannot be ruled out. Regardless, these data suggest that local colonization of the oral cavity by *P. gingivalis* may proceed without detection by resident fibroblasts resulting in chronic disease as our data suggest a limited immune response would be initiated by gingival fibroblasts.

Others have shown similar results in comparisons of *E. coli* and *P. gingivalis* LPS[32]. Intramuscular injection of *E. coli* LPS into mice elicited a greater inflammatory response as measured by MCP-1, E-selectin, and P-selectin mRNA expression at the injection site in comparison to *P. gingivalis* LPS-treated mice[32]. Thus, *P. gingivalis* LPS appears to be less immunogenic than *E. coli* LPS under certain conditions. Our data indicate that secondary recruitment of macrophages to gingival tissue would be an important factor in gingival tissue destruction via inflammation as AM cells were more responsive to *P. gingivalis* LPS.

In summary, the findings reported here demonstrate that macrophage and gingival fibroblast cell lines differentially respond to LPS challenge depending on the bacterial source. ESK-1 fibroblasts preferentially responded to *E. coli* LPS whereas AM macrophages responded more robustly to *P. gingivalis* LPS. This indicates a model whereby upon *P. gingivalis* challenge, gingival fibroblasts may initiate a low level inflammatory response primarily through expression of MCP-1 and IL-6 to recruit and activate surrounding macrophages and other immune cells. The recruited macrophages then release massive proinflammatory cytokines to promote the immune response. Additional experiments targeting the gingival fibroblast/macrophage relationship in response to *P. gingivalis* challenge, such as conditioned media and co-culture experiments, may provide insight in that regard.

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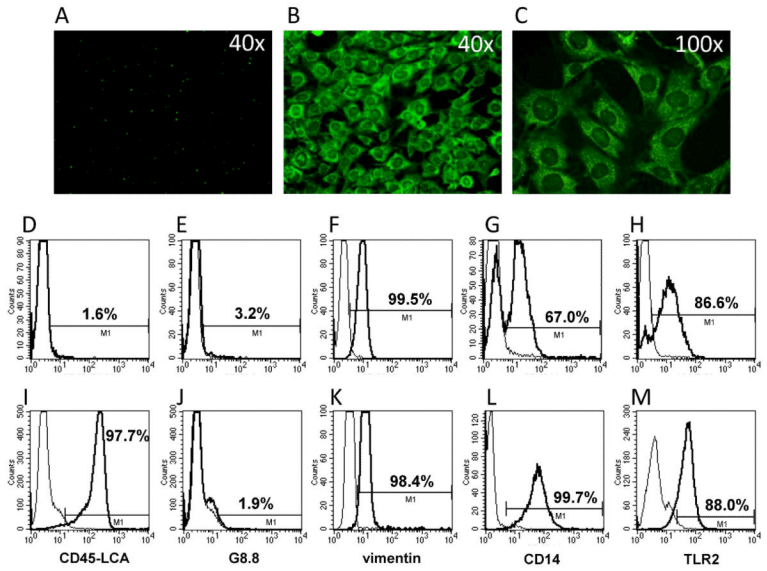

Figure 1.

Phenotypic characterization of ESK-1 cells. ESK-1 cells were non-reactive with (**A**) G8.8 mAb but were highly-reactive with (**B-C**) anti-vimentin antibody, indicating fibroblast origin of those cells. The lack of expression of the (**D**) CD45-LCA antigen indicates that ESK-1 cells were not of hematopoietic origin. ESK-1 cells were further confirmed by flow cytometry to be fibroblasts based (**E**) a lack of G8.8 staining, and (**F**) reactivity with anti-vimentin antibody. (**G**) Two-thirds of the ESK-1 cells expressed the CD14 co-receptor of TLR4, and also expressed the (**H**) TLR2 marker. AM cells expressed (**I**) CD45-LCA, (**K**) vimentin, (**L**) CD14, and (**M**) TLR2, but did not express (**J**) G8.8.

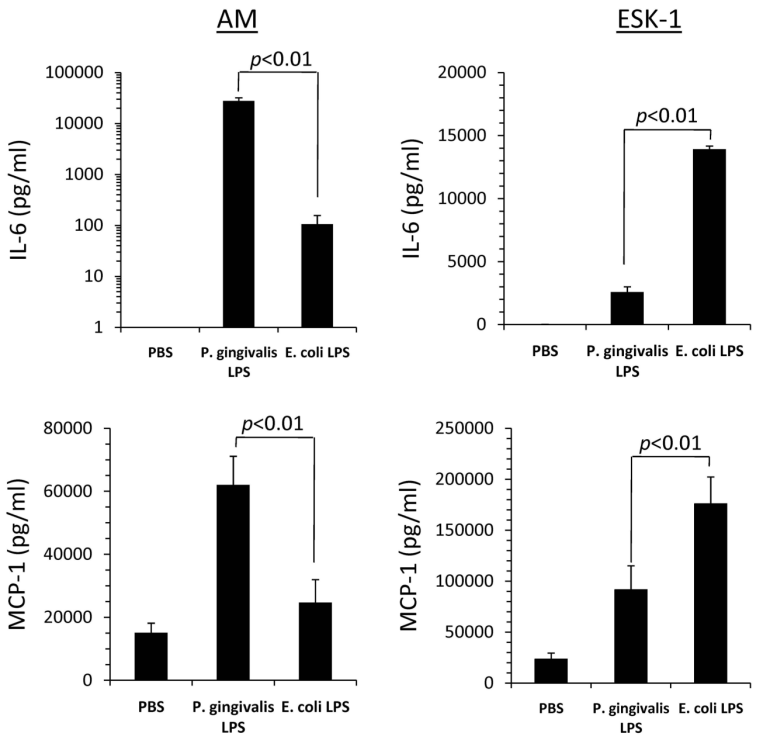

Figure 2.

EIA analyses of IL-6 and MCP-1 production by AM and ESK-1 cells confirm, at the protein level, the differential effects of *P. gingivalis* vs. *E. coli* LPS stimulation as shown in the gene expression experiments described in Table 2 and 3.

Table 1

Primer sequences used in this study

Gene##	Forward Primer	Reverse Primer
B7RP-1	5′-GGACTCCATGAAGCAGGGTA-3′	5′-GCTCTGGGTAGCCATTCTTG-3′
IL-1β	5'-GCCCATCCTCTGTGACTCAT-3′	5'-AGGCCACAGGTATTTTGTCG-3′
IL-6	5′-GAGGATACCACTCCCAACAGACC-3′	5′-AAGTGCATCATCGTTGTTCATACA-3′
iNOS	5′-ACAACAGGAACCTACCAGCTCA-3′	5′-GATGTTGTAGCGCTGTGTGTCA-3′
MCP-1	5′-CTTCTGGGCCTGCTGTTCA-3′	5′-CCAGCCTACTCATTGGGATCA-3′
MIP-1α	5′-ACTGCCTGCTGCTTCTCCTACA-3′	5′-AGGAAAATGACACCTGGCTGG-3′
MIP-1β	5′-AAACCTAACCCCGAGCAACA-3′	5′-CCATTGGTGCTGAGAACCCT-3′
TECK-1	5′-GGAAAAGGCTAGTCCACTGGAA-3′	5′-CCTTGGACTTCATATGGTTTGACTT-3′
Gene***	Primer position location	
IL-23	Gene reference position 23-47 with amplicon sequence: gttagactcagaaccaaagga ggtg	
GAPDH	Gene reference position 309-328 with amplicon sequence: gaggccggtgctgagtatgt	

Table 2

ESK-1 Cell Relative Gene Expression

Gene	*P. gingivalis* LPS	*E. coli* LPS
B7RP-1	0.90 ± 0.05[a]	0.57 ± 0.02
IL-1β	1.43	12.43
IL-6	3.70 ± 0.21	43.82 ± 2.37[c]
IL-23	1.21 ± 0.33	0.97 ± 0.01
iNOS	0.93 ± 0.16	13.59 ± 1.54[c]
MCP-1	3.05 ± 0.20	12.40 ± 0.55[c]
MIP-1α	0.62 ± 0.06	1.06 ± 0.06[b]
MIP-1β	1.18 ± 0.37	3.94 ± 0.49[b]
TECK-1	1.01 ± 0.05[a]	0.68 ± 0.04

[a] Statistically-significant increase ($p<0.05$) compared to *E. coli* stimulation

[b] Statistically-significant increase ($p<0.05$) compared to *P. gingivalis* stimulation

[c] Statistically-significant increase ($p<0.01$) compared to *P. gingivalis* stimulation

Table 3

AM Cell Relative Gene Expression

Gene	*P. gingivalis* LPS	*E. coli* LPS
B7RP-1	1.43	1.53
IL-1β	210.74 ± 16.54[b]	2.67 ± 0.40
IL-6	42.77 ± 2.57[b]	1.56 ± 0.10
IL-23	6.96 ± 1.01[a]	1.24 ± 0.10
iNOS	5.33 ± 0.46[a]	1.12 ± 0.10
MCP-1	21.16 ± 1.18[b]	1.25 ± 0.11
MIP-1α	3.12 ± 0.60[a]	0.56 ± 0.06
MIP-1β	11.34 ± 1.35[a]	0.88 ± 0.03
TECK-1	1.20 ± 0.15	0.80 ± 0.01

[a]Statistically-significant increase ($p<0.05$) compared to *E. coli* stimulation

[b]Statistically-significant increase ($p<0.01$) compared to *E. coli* stimulation