Gingival epithelial cells heterozygous for Toll-like receptor 4 polymorphisms Asp299Gly and Thr399Ile are hypo-responsive to *Porphyromonas gingivalis*

Denis F. Kinane[1], Hideki Shiba[1,2], Panagiota G. Stathopoulou[1], Hongli Zhao[1], David F. Lappin[1,3], Amar Singh[1], Mehmet A. Eskan[1], Simone Beckers[4], Sabine Weigel[4], Brian Alpert[1], Thomas B. Knudsen[1].

[1]University of Louisville School of Dentistry, Louisville, KY; [2]Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan; [3]University of Glasgow Dental School, Glasgow, United Kingdom; [4]School of Medicine, University of Louisville, Louisville, KY

Corresponding author: Denis F. Kinane, BDS, PhD, FDS RCPS, FDS RCS
Oral Health and Systemic Disease
University of Louisville School of Dentistry
Louisville, KY 40292
PH: 502-852-3175
FX: 502-852-4052
Email: dfkina01@louisville.edu

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ABSTRACT

The Toll-like receptor (TLR4) is the major sensor for bacterial lipopolysaccharide and its two common co-segregating polymorphisms, Asp299Gly and Thr399Ile, which occur at a frequency of between 6 and 10%, have been associated with infectious diseases, LPS hypo-responsiveness and cardiovascular disease. *Porphyromonas gingivalis* is a Gram negative bacterium implicated in chronic periodontitis and is a known TLR4 and TLR2 agonist. We obtained two gingival epithelial cell primary cultures from subjects heterozygous for the TLR4 polymorphism Asp299Gly and compared response characteristics with similar cells from patients (four) with the wild type TLR4 genes. Cytokine responses and transcriptome profiles of gingival epithelial cell primary culture cells to TNFα challenge were similar for all primary epithelial cell cultures. *P. gingivalis* challenge however, gave markedly different responses for Asp299Gly heterozygous and wild-type epithelial cell cultures. The epithelial cells heterozygous for the TLR4 polymorphism Asp299Gly were functionally hypo-responsive, evidenced by differences in BD-2 mRNA expression, mRNA response profile by microarray analysis and by pro-inflammatory and chemokine cytokines at the protein and mRNA level. These findings emphasize variance in human epithelial cell TLRs, linked with Asp299Gly carriage, which results in a hypo-responsive epithelial cell phenotype less susceptible to Gram negative diseases and associated systemic conditions.

INTRODUCTION

The Toll-like receptors (TLRs) are a major class of eukaryotic receptors for microbial pathogen-associated molecular patterns (PAMPs). When TLRs recognize PAMPs, alone or in heterodimerization with other TLR or non-TLR molecules, they induce signals responsible for the activation of genes relevant to the host defense including the inflammatory and adaptive immune related cytokines[1]. These signaling pathways specify the release of cytokine profiles specific for particular PAMPs [1, 2] which implies that TLRs can confer a degree of specificity to the innate response. The released cytokine profile may influence the resultant inflammatory or adaptive immune response or some combination of these responses.

So far 13 members of the TLR family have been identified in mammals with 10 expressed at the protein level in humans. TLR2 recognizes lipoproteins of the outer membrane of Gram-positive and Gram-negative bacteria such as the lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, in cooperation with TLR1 or TLR6. TLR4 recognizes LPS from Gram-negative bacteria[1, 3]. TLRs are expressed in immune cells, as well as in epithelial cells. *In vitro* studies have shown TLR2 and TLR4 expression in dermal keratinocytes[4] and alveolar and bronchial epithelial cells[5, 6], as well as TLR expression in intestinal epithelial cells[7]. *In vivo* studies have shown TLR2 and TLR4 expression in renal epithelial cells[8] and TLR2 expression in alveolar epithelial cells[6]. Recently genes for TLR11 have been reported in mammals and it appears that carriage of TLR11 prevents infection by uropathogenic bacteria in mice[9]. These results suggest that the presence of TLRs is very common in epithelial barriers in the body, wherever the host is likely to encounter pathogenic microorganisms.

Mutations exist in the TLRs. For example there is controversy on the disease association and potential biological function of the two common TLR4 polymorphisms Asp299Gly and Thr399Ile. These polymorphisms co-segregate and are present at a frequency of between 6-10%[10]. It appears that Asp299Gly is the functional polymorphism and Thr399Ile is in linkage disequilibrium. Reduced airway responsiveness to inhaled endotoxin[11] and a protective association against Legionnaires' disease[12] have been linked with heterozygous carriage of these TLR4 polymorphisms. Inflammatory responses in heterozygous individual are not uniformly impaired however and in some cell types such as peripheral monocytes, response differences are

not seen[13]. We considered that different cell types vary in their expression levels of TLRs and other receptors such as CD14 and that differences in TLR genotype may affect epithelial cells more than leukocytes, which have abundant TLRs on their surfaces and have abundant and multiple receptors for LPS such as CD14 and CD11b. Furthermore the innate immune protection afforded by epithelial barriers may be more pertinent in disease initiation and may a source of variation in susceptibility. Thus we investigated the biological function of epithelial cells heterozygous for the two TLR4 polymorphisms Asp299Gly and Thr399Ile and compared them to cells with normal TLR4 (wild-type) genotype in terms of their response characteristics at the protein and mRNA level.

RESULTS

Human Gingival Cell Culture and TLR polymorphisms: For these experiments we utilized multiple human primary gingival epithelial cultures (HGECs) and have focused in depth on six (HGEC-1, -2, -3, -5, -9 and -12) derived from healthy gingival tissues isolated from six healthy subjects. We focused our experiments predominantly on HGEC3, an epithelial primary culture heterozygous for the TLR4 polymorphisms of both Asp299Gly and Thr399Ile and the HGEC2 primary culture which was of the wild type genotype for the two TLR-4 polymorphisms. Three additional primary epithelial cultures were used representing further examples of heterozygous and wild type cells (HGEC-12 was heterozygous for Asp299Gly and HGEC-1 and -5 were of the wild type, HGEC-9 was also of the wild type but hypo-responsive suggesting that this phenotype is not solely restricted to Asp299Gly carriage). The two major TLR2 polymorphisms were not found in any of the cells studied (see Materials and Methods for details). We also noted from previous experiments that TNFα and *P. gingivalis* were useful representations of cytokine and bacterial challenges. TNFα was chosen for its crucial role as an inflammatory cytokine in periodontal disease and because it is a consistent representative of the other pro-inflammatory cytokine responses. *P. gingivalis* is one of the more researched periodontal microorganisms and displays interesting properties signaling through TLR-2 and 4 and producing a wide cytokine response[14].

Gene expression profiling

To document the feasibility of the approach, RNA was extracted from both Asp299Gly heterozygous and wild-type epithelial cultures cultures after 4 hours in normal growth medium

(control) or after challenge with TNFα and *P. gingivalis* exposure as described elsewhere. The assays were performed in triplicate (and later combined after RNA extraction to reduce variability in the technical preparation stages) for the two epithelial cell cultures and the two challenges and then done in triplicate again to total fifty four wells of epithelial cells from which we derived RNA for eighteen microarrays. Each microarray was performed in triplicate to improve modeling of procedural variation.

TLRs 1-10 mRNA expression in unstimulated epithelial cells

HGEC-1, -2 and -3 were examined for their expression of TLR 1-10 mRNA in the unstimulated state (Figure 1). All human TLRs mRNAs except TLR-8 mRNA were detected in the cell lines studied. Each epithelial cell had its own characteristic TLR mRNA expression pattern, in particular there were marked differences in TLR-1 and TLR-4 mRNA expressions among the three primary cultures. Figure 2 illustrates that the HGECs showed much less mRNA expressions of TLR-4 than the HGFs and OBA-9 and that HGEC-3, which carries the Asp299Gly polymorphism, has much reduced TLR4 mRNA expression.

Comparison of MIP-3α and BD-2 responses in epithelial cells.

The mRNA levels of MIP-3α and BD-2 were increased in all the cells tested following microbial challenge or cytokine treatment (Figure 4). The kinetics of the response for MIP-3α and BD-2 in general differed in that MIP3-α mRNA expression peaked at 3h whereas BD-2 mRNA peaked at 24h. The consistent MIP-3α mRNA levels support the fact that the different primary cultures are similar and relatively consistent despite potential variations due to the days of growth prior to reaching confluence. The halving of BD-2 mRNA levels in response to *P. gingivalis* challenge is informative in that it confirms other reports[22] suggesting the BD-2 production is governed by TLR-2 and TLR-4 and confirms the effectiveness of the TLR-4 SNP in reducing TLR-4 function (Figure 4).

Cytokine protein assays

The cytokines produced by HGEC-2 and -3 cells following challenges with *P. gingivalis* and TNF-α indicated marked differences in the cell responses to *P. gingivalis* but consistency in the

responses to TNF-α. Significant changes in cytokine levels were declared when following challenge increases were twofold greater than baseline and the difference were also statistically significant. We categorized the cytokine responses generally into three response patterns. 'Discriminatory cytokines' were those that increased uniformly with TNFα challenge for both HGEC-2 (wild-type) and HGEC-3 (heterozygote) but showed marked differences between wild-type and heterozygote when challenged with *P. gingivalis.* These discriminatory cytokines were IL-6, IL-8 and GM-CSF. The second group comprised cytokines that increased with TNFa challenge only and were unchanged by *P. gingivalis* challenge, these were IL-1α, IL-1β, IFNγ, IL-3, IP-10, TNFα, RANTES. The third group included the cytokines that did not change significantly with either challenge and were: IL-2, -4, -5, -7, -10, IL-12p40, IL-12p70, IL-13, -15, MCP-1, MIP-1α and Eotaxin. Figure 3 depicts cytokine responses in group I for IL-6, and IL-8.

The most abundant cytokines produced were IL-8, IP-10, IL-6, IL-1α and GM-CSF, in that order. TNFα could only be measured in supernates of cells not challenged with TNFα for practical reasons. The cytokine responses were broadly comparable following TNFα challenge, but quite different following *P. gingivalis* challenge. HGEC-2 was more responsive to *P. gingivalis* than HGEC-3, markedly so for IL-6 and IL-8 and GM-CSF.

Two other hypo-responsive primary epithelial cell cultures were studied and these were HGEC-12 (a confirmed Asp299Gly heterozygote) and HGEC-9 (a wild-type which is also hypo-responsive) along with normal responsive HGEC-1 and HGEC-5 which are both confirmed wild-type primary cultures. Analysis of the representative cytokine GM-CSF produced following the same challenges by these HGECs are shown in Figure 7 and reveal differences between hypo-responsive, heterozygote and wild-type were consistent in epithelial cells derived from other subjects (IL-6 and IL-8 gave similar trends but data is not shown).

Changes in gene expression profiles

Changes in the gene expression profile are depicted in Figure 5 for HGEC-2 (wild-type) and HGEC-3 (heterozygote) cells challenged with *P. gingivalis* and TNF-α. While TNF-α exposure gave similar changes in the gene expression profile for both primary epithelial cell cultures, *P. gingivalis* challenge resulted in quite different patterns in gene expression for the HGEC-2

(wild-type) and HGEC-3 (heterozygote). Differences in the expression profiles determined by Affymetrix microarray indicate that the wild-type appears more responsive to *P. gingivalis* than the Asp299Gly heterozygote.

Analysis of cell receptor, cytokine and signal transduction changes using high-throughput functional genomics procedures on the microarray data from the challenged heterozygote and wild type epithelial cells whilst informative, required further elucidation. Thus, informational pathways were built using PathwayAssist v3.0 (Stratagene) for predicting connections between genes that change following challenge (Figure 6). The orange colored areas denote statistically significant twofold increases and the red shading a highly significant greater than fourfold increase. Biocarta produced similar pathway maps (not shown) which support these findings which are remarkably consistent with the data from the cytokine protein assessments. Figure 6 shows the HGEC-2 (wild-type) *P. gingivalis* challenge data (lower diagram) is quite different from the HGEC-3 (Asp299Gly heterozygote) *P. gingivalis* challenge (upper diagram). Differences between TNF-α challenged wild-type and heterozygote cells were undetectable, that is they were almost identical in response, whereas they heterozygote and wild-type responses differed considerably with the *P. gingivalis* challenge (Figures 5, 6).

DISCUSSION

Porphyromonas gingivalis, a known TLR4 and TLR2 agonist gives differential responses for epithelial cells heterozygous for the two TLR4 polymorphisms Asp299Gly and Thr399Ile. Responses to TNFα were broadly similar for both cell types. Changes in mRNA levels analyzed by Affymetrix microarray techniques suggest that protein and mRNA analyses were consistent in that TNF induced similar changes in the cytokines produced and the gene expression profile for both primary cell cultures but *P. gingivalis* gave markedly different effects. Furthermore real time PCR analyses revealed that the expression of TLR2 and TLR4 mRNA varied between the epithelial cell types basally and when exposed to TNFα or killed *P. gingivalis*. The primary cultures showed marked differences in TLR2 and TLR4 responses but identical responses for macrophage inflammatory protein (MIP)-3a indicating that the cells were otherwise normal. HGEC-2, at gene and protein level, is more responsive to *P. gingivalis* than HGEC-3.

Mutations exist in the TLRs and there is controversy on the disease association and potential biological function of the two common TLR4 polymorphisms Asp299Gly and Thr399Ile. The polymorphisms in question co-segregate and are present at a frequency of between 6 and 10%[10]. Reduced airway responsiveness to inhaled endotoxin[11] and a protective association against Legionnaires' disease[12] have been associated with heterozygous carriage of these TLR4 polymorphisms. Inflammatory responses in heterozygous individual are not uniformly impaired however and in some cell types such as peripheral monocytes and leucocytes, response differences are not seen[13, 15]. When fibroblasts from the same individual were analyzed, the data emphasized that different cell types vary in the range and intensity of the TLR mRNA expressed. Additional examples of wild-type and heterozygous primary epithelial cell cultures show similar differences in challenge induced cytokine production (Figure 7). Although we did not study monocytes from the TLR4 heterozygous subject we propose that there may be little difference in TLR4 response due to the TLR4 excess on these cells and the multiple additional LPS responsive receptors such as CD14, CD11b, CXCR4, HSPs on the surface of these professional phagocytes[16]. Figure 8 supports this argument and demonstrates that inhibition of TLRs on a challenged monocyte cell line does not reduce its cytokine production. This may be the reason Erridge et al.[13] in their activity assays with human monocytes were unable to demonstrate a functional effect for the Asp299Gly polymorphism.

In contrast to the challenged TLR responses, the MIP3a responses of cells were identical emphasizing that the variability seen in TLR responses is not an artifact but may be relevant to susceptible phenotypes for human diseases including periodontal disease. The mRNA levels of MIP-3α and BD-2 were increased in all the cells tested following microbial challenge or cytokine treatment (Figure 4). The consistent MIP-3α mRNA levels suggest that the primary epithelial cultures comprise a relatively robust model. The halving of BD-2 mRNA levels in response to *P. gingivalis* challenge supports a recent report suggesting the BD-2 production is triggered by TLR-2 and TLR-4 ligation and confirms the effectiveness of the TLR-4 SNP in reducing TLR-4 function[17] (Figure 4).

Thus we conclude that the epithelial cells heterozygous for the TLR4 polymorphisms Asp299Gly and Thr399Ile are functionally hypo-responsive, evidenced by differences in TLR expression by real time PCR, mRNA response profile by microarray analysis and by pro-inflammatory and chemokine cytokines by protein analyses. This finding underlines that differences do exist in epithelial cells of patients and may relate to the TLRs expressed on their surface and associated genes. Furthermore there are clearly differences between fibroblasts and epithelial cells within the same subject and this may contribute to the explanation for the lack of effect of these polymorphisms on the responsiveness of other cells such as leukocytes previously reported[13] and may relate to the abundant LPS related receptors present on leucocytes (TLRs, CD14, CD11b). The consistent protein and gene differences of epithelial cells heterozygous for the two common TLR4 SNPs suggests they have a biological function even if only one impaired allele is present. This hypo-responsive phenotype is possibly limited to 'front line' epithelial cells, these cells having low basal expression of TLRs prior to microbial or cytokine perturbation. The literature indicates that these polymorphisms confer resistance to developing Gram negative infections such as Legionnaires' disease[12] and are associated with a blunted response to inhaled LPS in humans[11] but the impact on diseases such as gingivitis and periodontitis remains to be tested. In the future, it may be possible to identify patients with greater susceptibility (mediated through TLR expression differences) and this information could be utilized diagnostically or therapeutically as well as to elucidate the etiology and pathogenesis of these diseases.

METHODS

In order to investigate the biological function of epithelial cells with normal TLR4 (wild-type) and heterozygous for the two TLR4 polymorphisms Asp299Gly and Thr399Ile we set up human primary gingival epithelial cell (HGEC) cultures and challenged them with various cytokines and *P. gingivalis* then determined their response characteristics at the protein and mRNA level.

Genotyping of TLR2 and 4 genes

Determination of the TLR2 and 4 gene mutations was accomplished with PCR and restriction fragment length polymorphism (RFLP) as previously described[18, 19] and confirmed by an allele discrimination assay utilizing the ABI 7500 system (Applied Biosystems) for an end-point assay of allele carriage[20]. In the RFLP procedure, detection of two TLR2 gene single nucleotide polymorphism (Arg677Trp and Arg753Gln) was performed with the following primers: forward 5'-GCCTACTGGGTGGAGAACCT-3' and reverse 5'-GGCCACTCCAGGTAGGTCTT-3' [18]. The PCR product size is 340bp. Since both TLR2 polymorphisms eliminate an Aci I restriction site[18,19], the results of ACI digestion of the PCR products for these allotypes are: 227, 75 and 38bp (wild-type), 265 and 75 bp (Arg753Gln) and 302 and 38 bp (Arg677Trp). For detection of the TLR4 gene two single nucleotide polymorphism (Asp299Gly and Thr399Ile), the following two pairs of primers were used: Asp299Gly: forward 5'-AGCATACTTAGACTACTACCTCCATG-3' and reverse 5'-GAGAGATTTGAGTTTCAATGTGGG-3', Thr399Ile: forward 5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3' and reverse 5'-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3' [19]. Digestion of the PCR products was performed with Nco I for detection of Asp299Gly, yielding 188 bp (wild-type), and 168 and 20 bp (Asp299Gly). Digestion of the PCR products with Hinf I for detection of Thr399Ile yielded 124 bp (wild-type), and 98 and 26 bp (Asp299Gly). DNA (100ng) from HGEC-1, -2 -3 and OBA-9 was used as a template for the subsequent PCR of TLR2 and TLR4 using a High Fidelity Expand system in a total volume of 25 μl (Roch). PCR was performed under the following conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 25 s followed by one elongation step at 72°C for 5 min for TLR2 polymorphism, 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30s followed by one elongation step at 72°C for 10min for TLR4 Asp299Gly polymorphism and 95°C for 15 min, followed by 35 cycles of 94°C

for 30 s, 60°C for 30 s and 72°C for 30 s followed by one elongation step at 72°C for 10min for TLR4 Thr399Ile polymorphism. 4 µl of each PCR product was incubated at 37°C for 2 h with 10 units Aci I, 10 units Nco I or 10 units Hinf I in a total volume of 10 µl. Samples were subjected to electrophoresis on a 2.5% agarose gel, run for 1.5 h at 100V and stained with ethidium bromide.

Cell Culture

For these experiments we utilized multiple human primary gingival epithelial cultures (HGECs) but have focused in depth on three (HGEC-1, -2 and -3) derived from three healthy gingival tissues isolated and maintained as previously described[21, 22]. The cell suspension was centrifuged at 120 x *g* for 5 min, and the pellet was suspended in MCDB153 medium (pH 7.4) (Sigma, St. Louis, MO) containing 10 µg/ml insulin, 5 µg/ml transferrin, 10 µM 2-mercaptoethanol, 10 µM 2-aminoethanol, 10 nM sodium selenite, 50 µg/ml bovine pituitary extract, 100 units/ml penicillin, 100 µg/ml streptomycin and 50 ng/ml amphotericin B (medium A) [18, 19]. The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO_2 and 95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured. HGF-1 and -2 was obtained from explants of gingival connective tissues separated from epithelium. The explants were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO, Buffalo, NY), 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml amphotericin B (medium B). When the HGFs reached confluence, they were harvested and subcultured. HGEC-1 and HGF-1 or HGEC-2 and HGF-2 were derived form the same gingival biopsy. The immortalized human gingival epithelial cell line, OBA-9 was kindly provided by Dr. Shinya Murakami (Osaka University, Japan).

Bacterial strains and conditions

P. gingivalis 33277 were grown at 37°C in trypticase soy broth supplemented with 1g of yeast extract, 5 mg of hemin and 1 mg of menadione per liter under anaerobic conditions of 85% N_2, 10% H_2, and 5% CO_2 for 2 days. After cultivation, the bacteria were harvested by centrifugation, washed three times in phosphate-buffered saline (PBS), heat-inactivated for 1 hour at 60°C and suspended in MCDB153 medium containing 10 µg/ml insulin, 5 µg/ml transferrin, 10 µM 2-mercaptoethanol, 10 µM 2-aminoethanol and 10 nM sodium selenite (medium C).

Challenge assay

Primary epithelial cultures and cell lines at the fourth or fifth passage were harvested, seeded at a density of $1x10^5$ cells/6-well culture plate coated with type I collagen, and maintained in 2 ml of medium A. After six days, the confluent cultures were washed twice with phenol red-free Hank's solution (pH 7.4) and heat-killed *P. gingivalis* ($5 x10^7$ cells/ml) suspended in 2 ml of medium C or TNF-α (10 ng/ml) (R&D Systems, Minneapolis, MI) were applied for the indicated periods. RNA and DNA (for SNP analysis) was extracted from each culture using TRIzol® (Invitrogen, Carlsbad, CA) and DNeasy® Tissue Kit (Qiagen, Valencia, CA), respectively, and quantified by spectrometry at 260 and 280 nm. Cell culture supernatant were then separated by centrifugation, removed and stored at -80ºC prior to cytokine protein assay by Luminex 100 technology using a multiplex for 22 cytokines (IL-1α, IL-1β, IL-2, -3, -4, -5, -6, -7, -8, -10, -12p40, -12p70, -13, -15, GM-CSF, IFNγ, TNFα, RANTES, MCP-1, MIP-1α and Eotaxin) (Upstate Laboratories).

Human monocyte experiments

The U-937 human monocyte cell line was kindly provided by Dr. Abu-Kwaik (University of Louisville, Medical School). The cells were cultured for three days in RPMI 1640 media (Invitrogen, with L-Glutamine) including 10 % FBS (heat inactivated), 100 U/ml of Penicillin/Streptomycin. The cells ($3x10^5$ /ml in 24 wells-plate) were seeded and differentiated toward macrophages by using 80 nM Phorbol 12-myristate 13-acetate (PMA, Sigma). After 48 hours, the cells were washed three times with 1x PBS. For the U937 human monocyte assays, anti-TLR4 HTA125 (20µg/ml, BioSciense), was added to cell cultures and incubated at 37 degree, 5% CO_2 for one hour before challenging with the bacteria. After one hour, the cells were stimulated with or without heat killed *P. gingivalis* (33277) for fours hours in the same conditions (cell to bacteria ratio was 1:100).

Real-time PCR for mRNA expression of TLR1-TLR10

Ten micrograms from each total RNA extract was used to perform 1st strand cDNA synthesis with High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) in a total volume of 100 µl. Real-time PCR with one-twentieth of each generating cDNA was performed with an ABI 7500

system (Applied Biosystems). TaqMan probes, sense primers and anti-sense primers for gene expression of human TLR1 toTLR10, human MIP-3α and BD-2 were purchased from Applied Biosystems along with probes and primers for human GAPDH. Using a Universal PCR Master Mix (Applied Biosystems), the reactions were carried out according to the manufacturer's protocol.

Microarray analysis

TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) yielded good quality, intact RNA as revealed using RNA Nano Chips on the Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). All samples showed the desired bands for the 18S and 28S ribosomal subunits and no degradation was detected. Each RNA sample was converted to cDNA (10 μg) using Superscript cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, CA) and then into biotin-labeled cRNA with the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). Purification at each stage was performed by using the GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA). After confirming quality on the Agilent 2100 Bioanalyzer, 15 μg of cRNA were fragmented in 40 mmol/l Tris-acetate buffer, pH 8.1, containing 100mmol/l KOA_c and 30 mmol/l $MGOA_c$ by metal induced hydrolysis and cleanup. Samples were spiked with Affymetrix hybridization controls and 10 μg of fragmented cRNA was hybridized for 16 hours at 45ºC to the HG-U133A probe (Affymetrix, Santa Clara, CA) using the GeneChip Hybridization Oven at 60 rpm. The arrays were washed and stained in an Affymetrix GeneChip Fluidics Station 450 at the University of Louisville Core Facility using streptavidin-phycoerythrin (SAPE, Molecular Probes), biotinylated anti-streptavidin antibody (Vector Laboratories), and a second staining with streptavidin-phycoerythrin according to the Affymetrix protocol. Stained arrays were scanned at 488 nm with an Agilent GeneArray Scanner (Agilent, Palo Alto, CA) and computed with Microarray Suite 5.0 (MAS5) from Affymetrix. Signal intensities were quantified using one-step Tukey's Biweight method associated with the detection p-value ($p < 0.006$) to differentiate present vs absent cells. All samples were scaled to an average signal intensity of 150 after excluding the highest and lowest 2% of the data. The signals were imported into Partek Pro 6.0 and published into Data Mining Tool 3.0 (Affymetrix) for statistical reduction (Partek Incorporated, St. Charles, MO).

Data analysis: The Affymetrix GeneChip data was extracted and normalized to the median intensity using <models – PM-MM> (which subtracts the background mismatch probe intensity form the perfect match probe intensity) using Affymetrix GCOS software. Affymetrix uses a single-channel platform in which the final data output lists genes by probe ids, GenBank accession numbers, signal intensity for perfect match versus mismatch probe sets, and a call as to whether the expression is absent, marginal, or present based on technology-specific statistical metrics. In two-color platforms the primary comparison is defined as an expression ratio (T) for each gene, defined as T=Q/R where Q is query sample and R is reference sample. We used the Q and R measure in single channel platform by taking the respective controls (average of triplicate) as "Reference" denominator and treated samples as "Query". The ratio data was then transformed to logarithm base 2 (log2) and scaled each sample to median = 0 and S.D = 0.5. This produced a continuous spectrum of values treating up- and down-regulated genes in a similar fashion (e.g., +1.0 means 2-fold up-regulated and -1.0 means 2-fold down-regulated) [23]. The data was then imported into GeneSpring 7.0 (Silicon Genetics, Redwood City, CA) for further data management, statistics, analysis and representation. GeneSpring accounts for two different kinds of random variation to estimate the variability in gene expression measurements: measurement variation, corresponding to a single gene within each array; and replica sample variation specified by the user. Precision is based on variance components analysis (VCA) that calculates the standard error and *P*-value. VCA uses cross-replica and cross-gene error variations to give a mean expression. In this case ANOVA is run and multi-sample comparison uses t-test assuming equal variance in normalized data. Benjamini and Hochberg False Discovery Rate was applied as a beta correction to adjust the alpha level (P) such that the return is <1 gene predicted as a false positive[24].

Genes differentiating between unstimulated and stimulated states were determined by two-way ANOVA and FDR (false-discovery rate) protection (*p-value* < 0.005 for 1386Tu and *p-value* < 0.00039 for 1386Ln) overlap analysis was performed with GeneSpring v7.0 (Silicon Genetics, Redwood City, CA). Two-way ANOVA using a parametric test with variances assumed equal, p-value cutoff of 0.005 with no multiple testing corrections for Treatment and cell resulted in 144 genes.

We used several tools in GeneSpring for these purposes: (a) principal components analysis, a

decomposition tool for comparing a limited subset of samples; (b) hierarchical clustering, useful for a holistic view of the entire data set; and (c) K-means clustering to partition the genes groups of similar behaviors with respect to a computed group mean or centroid. Although clustering algorithms are good exploratory tools they are limited because we are necessarily forcing genes into expression clusters even though these genes may function in more than one biological pathway. Clustering was used in combination with meta-analysis (pathway maps, chromosome maps, computational gene network prediction, and so forth) to complete the picture[25]. Informational pathways were built using PathwayAssist v3.0 (Stratagene) and BioRag (Bioresource for array genes) at www.biorag.org for predicting connections between the co-regulated genes.

Statistical analysis

Statistical analyses of the data were performed using the Student's *t* test and a minimum of three replications were performed for each analysis. Statistics relevant to the microarray technique are described in detail in these sections.

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Figure Legends

Figure 1. Comparison of each TLR mRNA expression among the three primary epithelial cell cultures. The ratio of each TLR mRNA to the GAPDH mRNA in HGEC-1 was arbitrarily assigned the value of 1. The ratio of TLR mRNA to GAPDH mRNA in all the other cells was normalized to this. Data is representative of three independent experiments performed in triplicate. Values are means $\pm$ SD. The nine human TLR mRNAs except TLR8 mRNA were detected in all HGECs studied.

Figure 2. Comparative expression of TLR2 and 4mRNA in cell types. Marked differences in TLR2 and TLR4 mRNA expression. The HGECs showed much less mRNA expressions of TLR4 than the HGFs and OBA-9 suggesting that OBA-9 is not a good human gingival epithelial cell model for TLR studies. **This data implies that variations in TLR molecular abundance profiles are both subject-specific (patient) and cell-type specific (HGEC vs HGF).** Each epithelial cell appears to have its own characteristic TLR mRNA expression pattern which is maintained through subculture and consistent between experiments. Data is representative of the results from two independent experiments. Values are means $\pm$ SD of three cultures.

Figure 3. Cytokine responses to P. gingivalis and TNFα challenged HGEC-2 and HGEC-3 cells. Primary epithelial cultures and cell lines at the fourth or fifth passage were challenged with heat-killed *P. gingivalis* (5×10^7 cells/ml) or TNFα (10 ng/ml). RNA and DNA (for SNP analysis) was extracted from each culture. Cell culture supernatant were separated for protein assay by Luminex 100 technology for 22 cytokines (IL-1α, IL-1β, IL-2, -3, -4, -5, -6, -7, -8, -10, -12p40, -12p70, -13, -15, GM-CSF, IFNγ, TNFα, RANTES, MCP-1, MIP-1α and Eotaxin) (Upstate Cell Signaling Solutions, Lake Placid, NY). The IL-6 and IL-8 cytokine responses were most discriminatory between HGEC-2 (E2) and -3 (E3) and are shown here. These figures demonstrate the general hierarchy of responses are TNFα>Pg, that E2 (the wild-type HGEC-2) is much more responsive than E3 (the heterozygote HGEC-3) and that IL-8 is much more abundant than IL-6.

Figure 4. Comparison of MIP-3α and BD-2 responses in epithelial cells: The mRNA levels of MIP-3α and BD-2 were increased in all the cells tested following microbial or cytokine challenge, except that the HGEC-3 *P. gingivalis* BD-2 increase was half that of HGEC-2 which is fully consistent with the literature suggesting that both TLR2 and -4 are involved in increasing BD-2 levels [26]. The consistency of the various cells MIP-3α mRNA responses to *P. gingivalis* and TNFα challenge and the BD-2 mRNA response to TNFα challenge indicates that the HGEC are fully functional except for the TLR4 function in the Asp299Gly heterozygote.

Figure 5. Gene expression profiling. Changes in the gene expression profile are depicted for the HGEC 2 and 3 cells (TLR4 wild type and heterozygote respectively) challenged with *P. gingivalis* and TNFα. TNFα exposure resulted in similar gene expression profiles for both primary epithelial cell cultures, *P. gingivalis* challenge however resulted in quite different patterns in gene expression for HGEC-2 and -3, supporting HGEC-3 hypo-responsiveness to *P. gingivalis*.

Figure 6. Informational network of linked genes for P. gingivalis stimulated HGEC-3 (the Asp299Gly TLR4 heterozygote primary epithelial cell culture) and HGEC-2 (the wild-type primary epithelial cell culture). To elucidate the computational read-out of altered gene

expression profiles with respect to cell receptor, cytokine and signal transduction changes, informational pathways were made using BioRag (Bioresource for array genes - www.biorag.org) to predict connections between the co-regulated cell receptor, cytokine and signal transduction genes. The BioRag (and PathwayAssist v3.0, Stratagene – data not shown) network identification program revealed a strong evidence-based interactome focused on NF-**K**B and revealed that the HGEC-2 TLR4 wild-type response to *P. gingivalis* (lower network diagram) involved IL-1α and TNFα upregulation (orange) and especially IL-8 (red). Heavy lines indicate more numerous associations reported for a particular linkage. The heterozygote (top) network is quite different from the wild-type (bottom) and confirms the consistency of the protein and gene hypo-responsiveness.

Figure 7. GM-CSF levels following various 4 hour challenges for four primary epithelial cell cultures. While all epithelial cells show similar increases following TNFα exposure the response to *P. gingivalis* differs for heterozygotes and wild-type epithelial cells. HGEC-3, -9 and -12 are clearly hypo-responsive and HGEC-3 and HGEC-12 are Asp299Gly TLR4 heterozygotes (with HGEC-9 hypo-responsive for as yet undetermined causes), while HGEC-1, -2 and -5 are of the wild-type for Asp299Gly. This demonstrates consistent differences in cytokine production (GM-CSF) related to TLR-4 SNP carriage when challenged by *P. gingivalis.* IL-6 and IL-8 show a similar response pattern (data not shown).

Figure 8. Demonstration that macrophages do not require TLR4 receptor-ligation as a trigger for cytokine response to challenge.

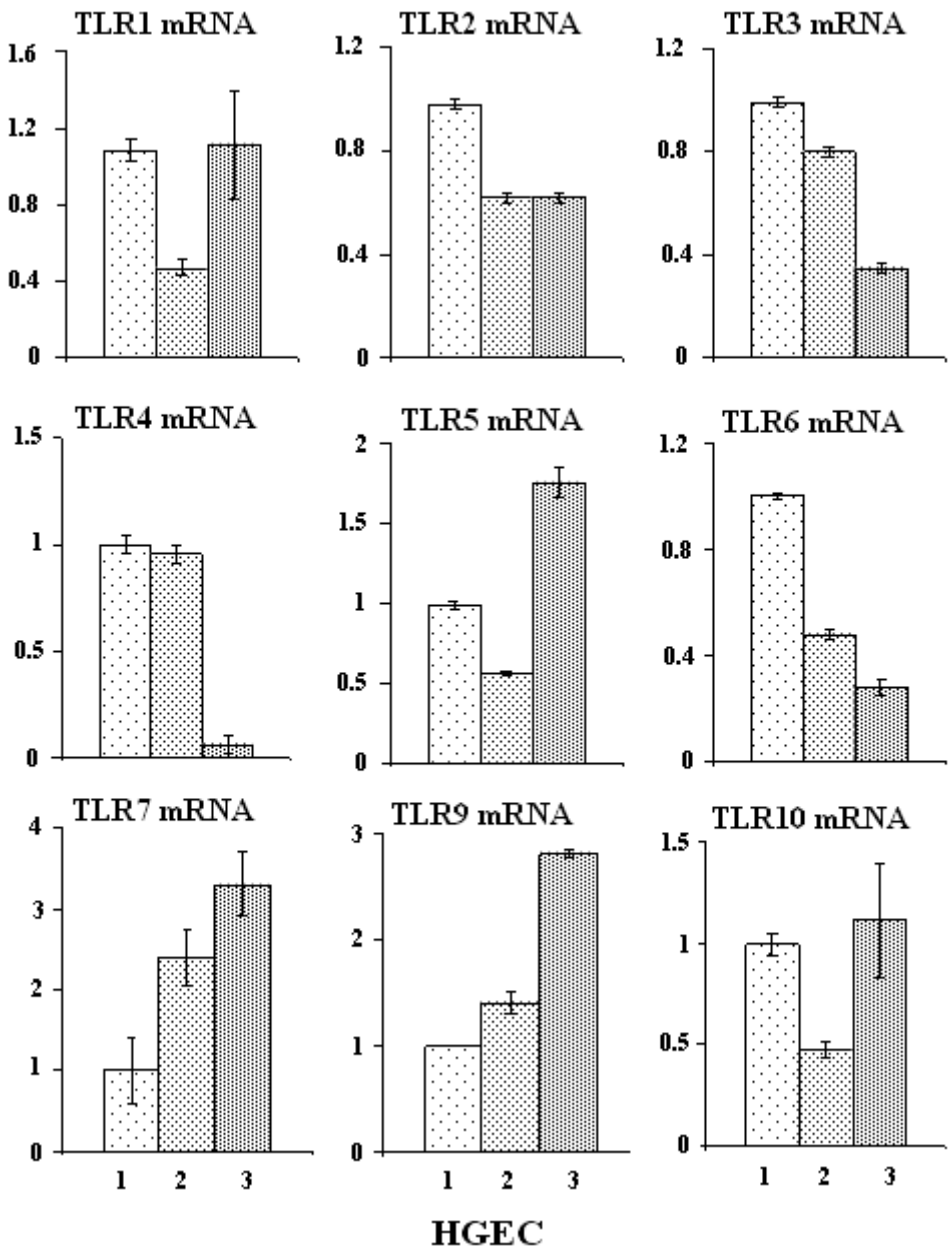

TLR1 mRNA
TLR2 mRNA
TLR3 mRNA
TLR4 mRNA
TLR5 mRNA
TLR6 mRNA
TLR7 mRNA
TLR9 mRNA
TLR10 mRNA
1 2 3
HGEC

Figure 1

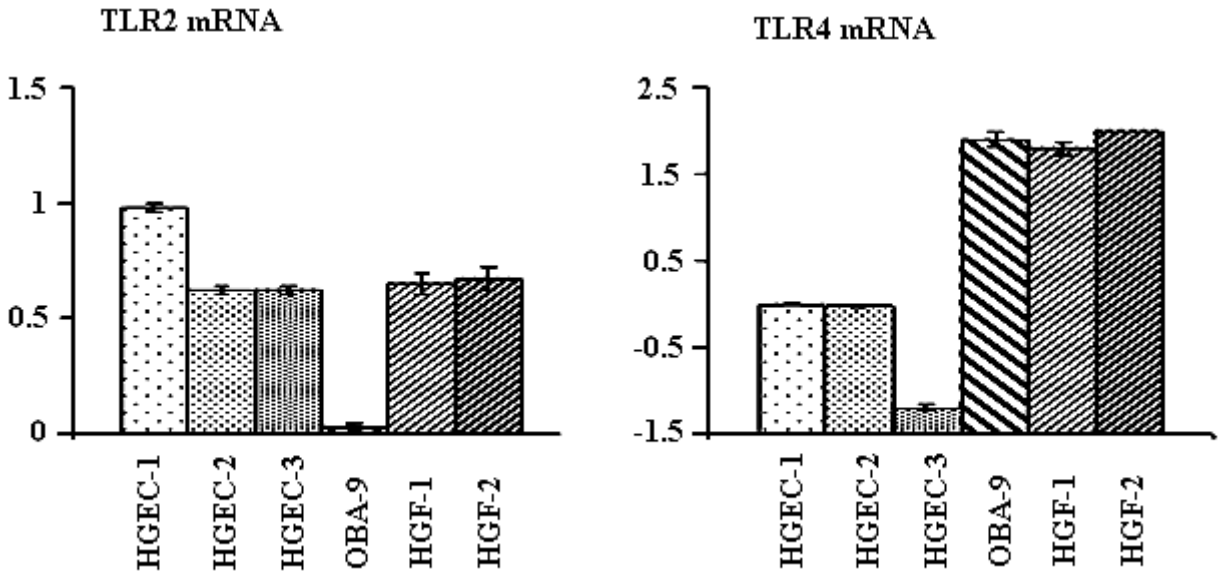

TLR2 mRNA
1.5
1
0.5
0
HGEC-1
HGEC-2
HGEC-3
OBA-9
HGF-1
HGF-2
TLR4 mRNA
2.5
1.5
0.5
-0.5
-1.5
HGEC-1
HGEC-2
HGEC-3
OBA-9
HGF-1
HGF-2

Figure 2

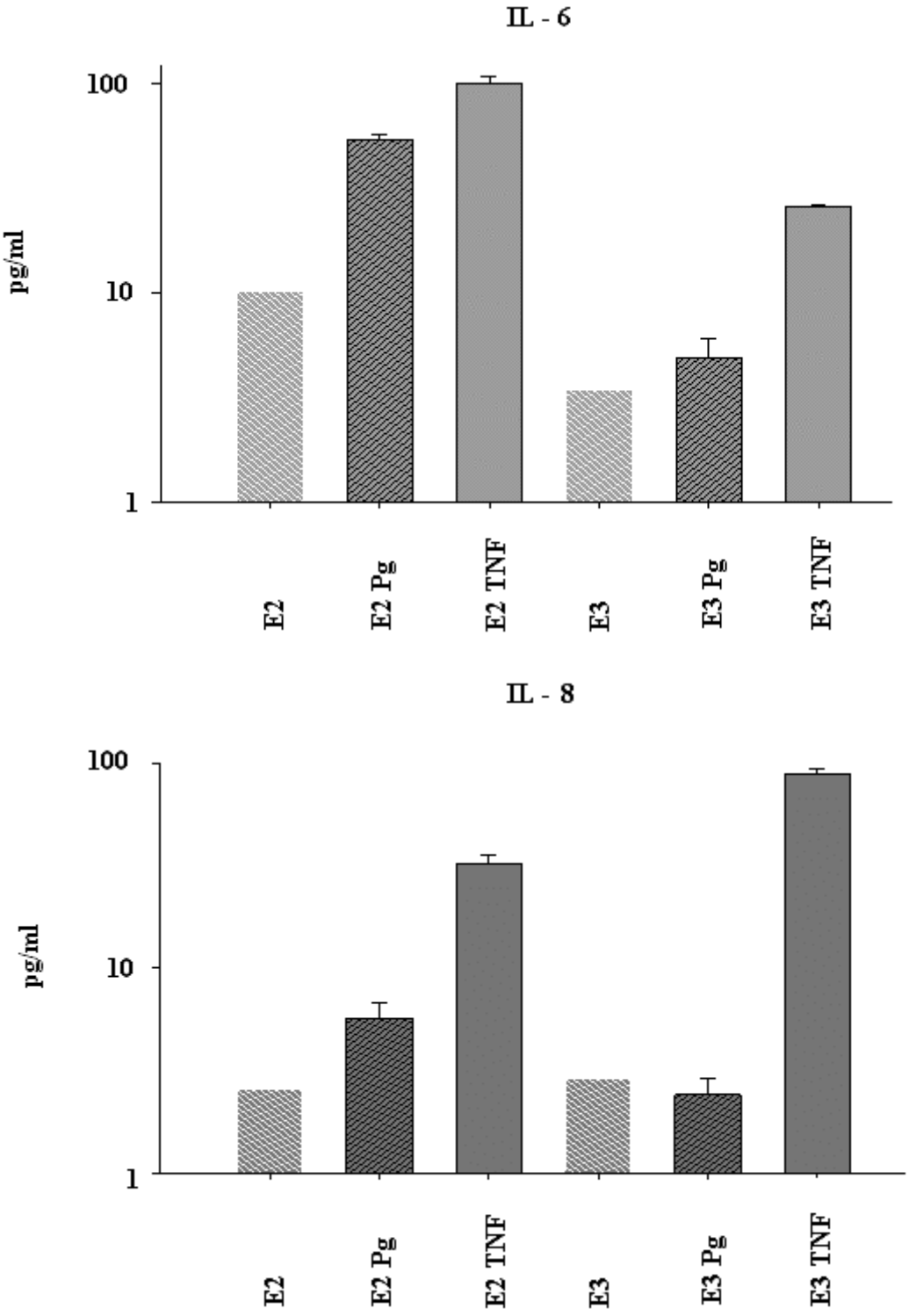

IL - 6
100
10
1
pg/ml
E2
E2 Pg
E2 TNF
E3
E3 Pg
E3 TNF
IL - 8
100
10
1
pg/ml
E2
E2 Pg
E2 TNF
E3
E3 Pg
E3 TNF

Figure 3

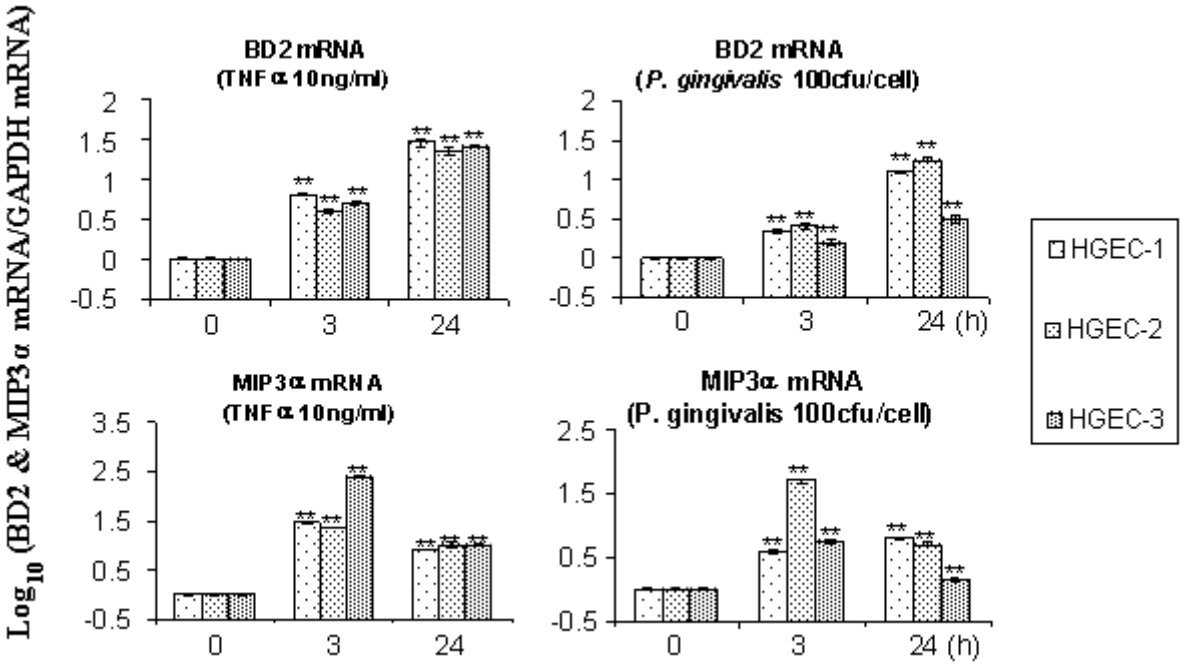

BD2 mRNA
(TNFα 10ng/ml)
BD2 mRNA
(P. gingivalis 100cfu/cell)
MIP3α mRNA
(TNFα 10ng/ml)
MIP3α mRNA
(P. gingivalis 100cfu/cell)
Log10 (BD2 & MIP3α mRNA/GAPDH mRNA)
0
3
24
24 (h)
HGEC-1
HGEC-2
HGEC-3

Figure 4

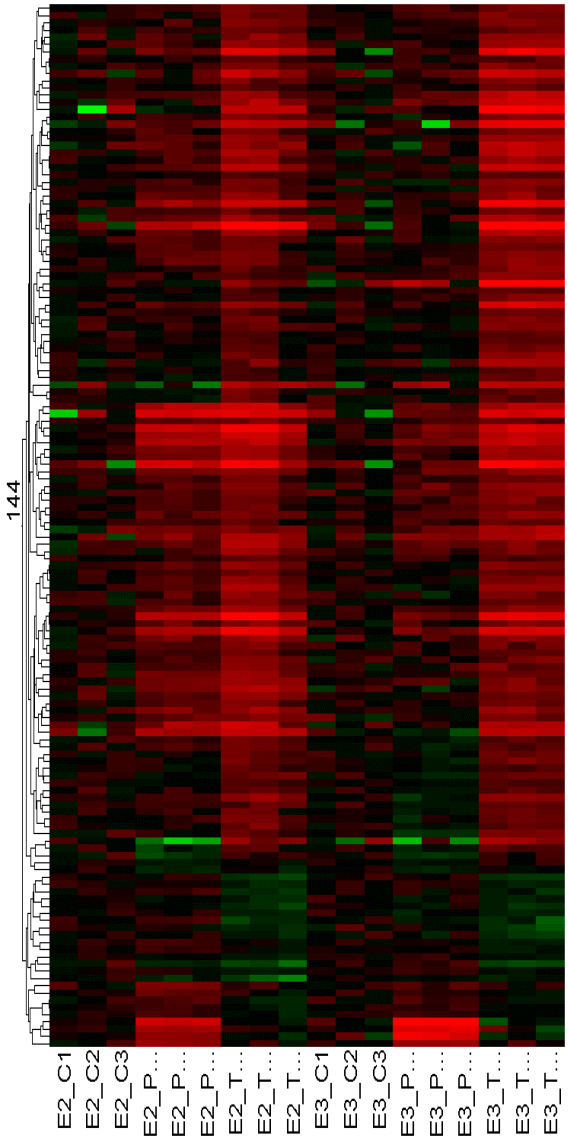
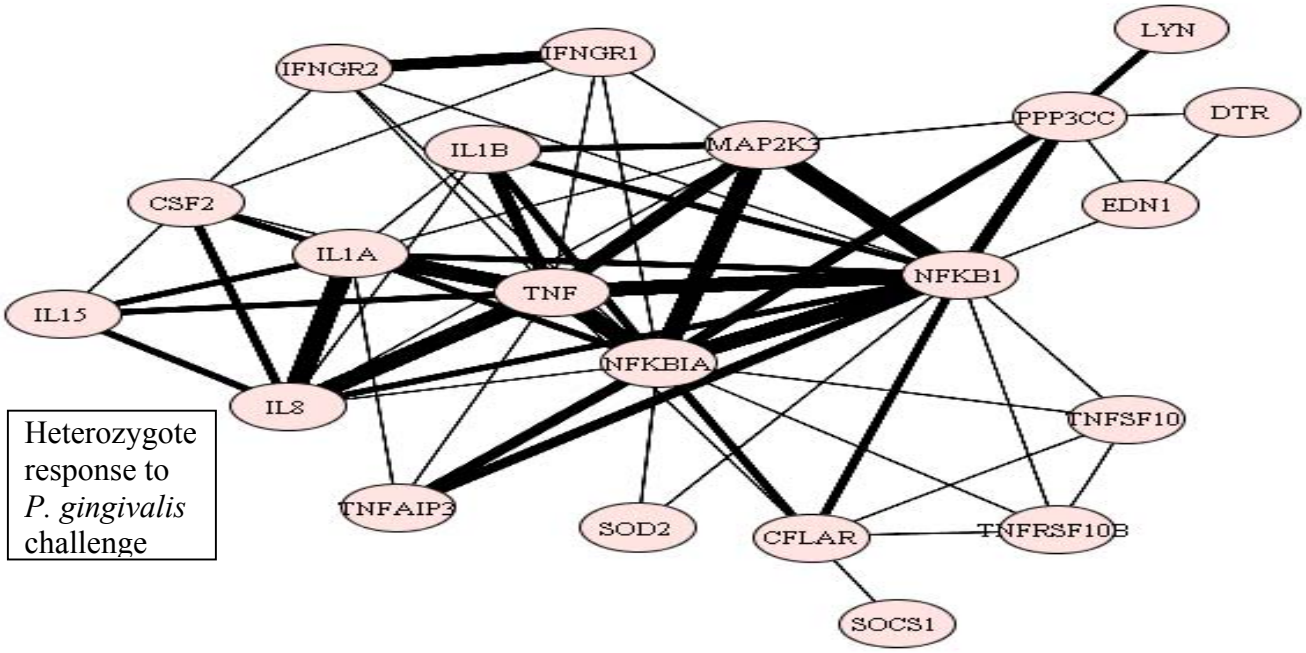
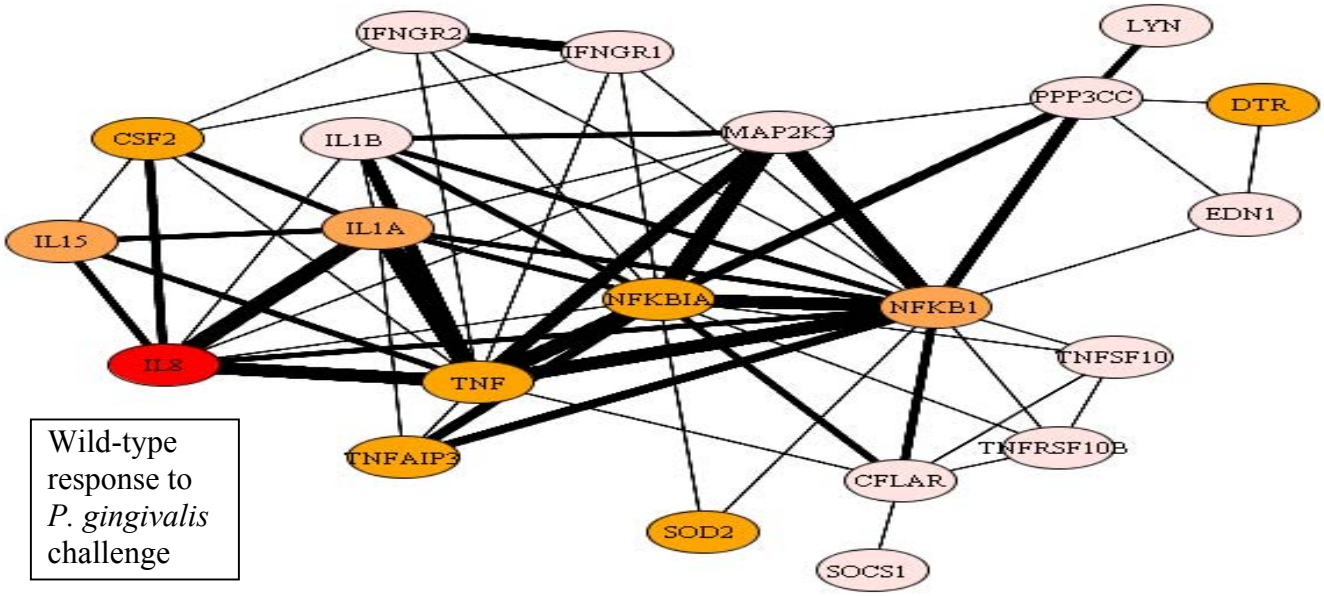


Figure 5.

GO Biological Process, Molecular Function Gene Category	List Hits	List Total	Bonferroni
immune response	30	143	0.000
response to biotic stimulus	32	143	0.000
defense response	30	143	0.000
cytokine activity	15	142	0.000
response to external stimulus	40	143	0.000
response to pest/pathogen/parasite	21	143	0.001
inflammatory response	13	143	0.001
innate immune response	13	143	0.002
response to wounding	15	143	0.006
receptor binding	19	142	0.045



Heterozygote response to *P. gingivalis* challenge



Wild-type response to *P. gingivalis* challenge

Figure 6

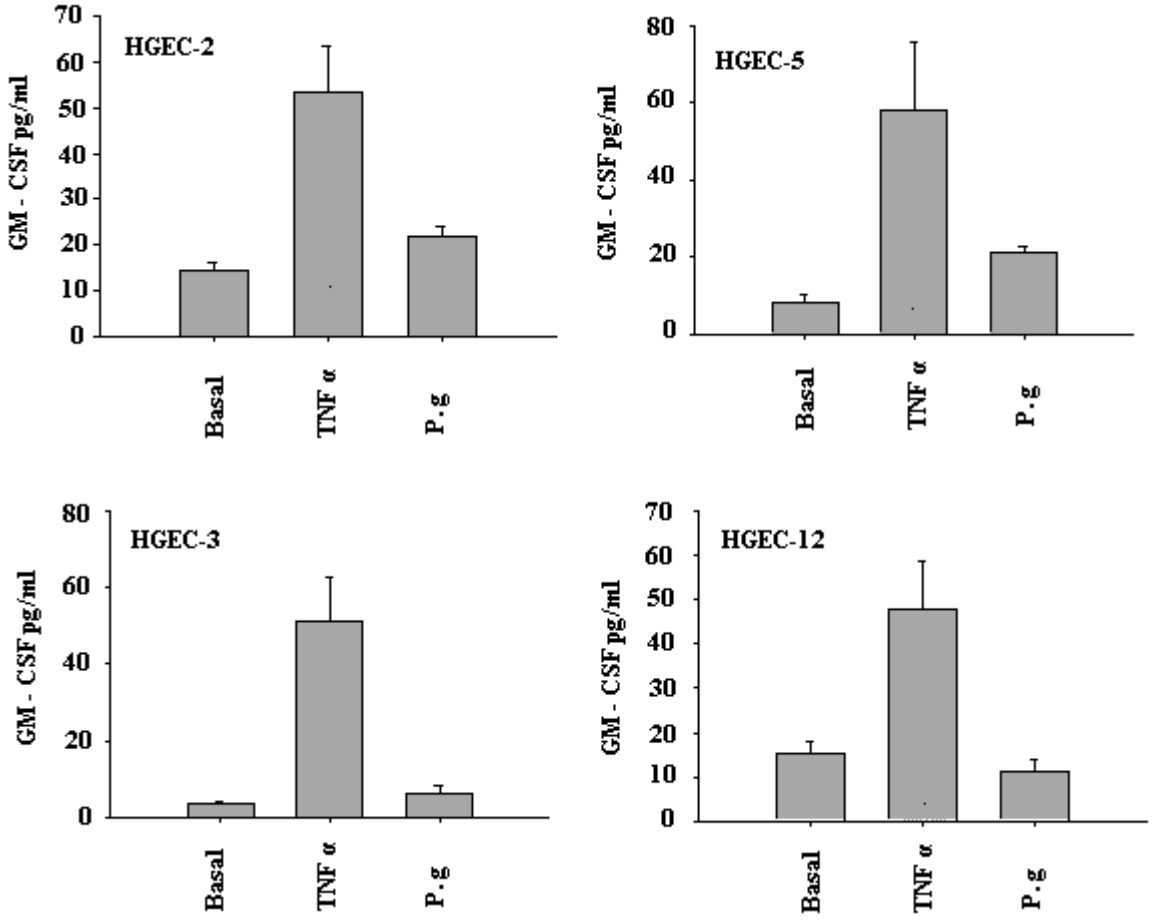

HGEC-2
GM - CSF pg/ml
70
60
50
40
30
20
10
0
Basal
TNF α
P. g
HGEC-5
GM - CSF pg/ml
80
60
40
20
0
Basal
TNF α
P. g
HGEC-3
GM - CSF pg/ml
80
60
40
20
0
Basal
TNF α
P. g
HGEC-12
GM - CSF pg/ml
70
60
50
40
30
20
10
0
Basal
TNF α
P. g

Figure 7

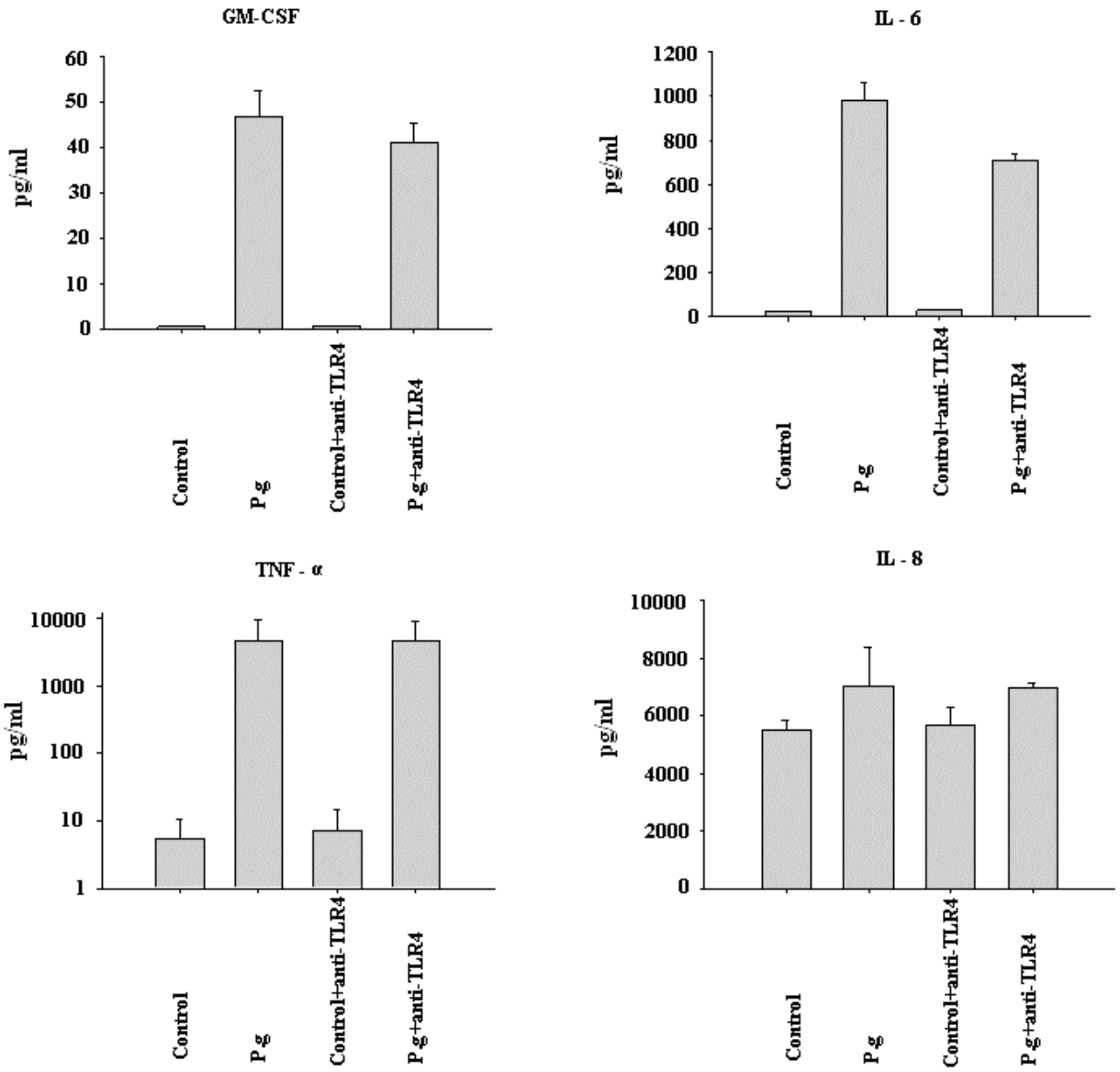

GM-CSF
pg/ml
60
50
40
30
20
10
0
Control
P.g
Control+anti-TLR4
P.g+anti-TLR4
IL - 6
pg/ml
1200
1000
800
600
400
200
0
Control
P.g
Control+anti-TLR4
P.g+anti-TLR4
TNF - α
pg/ml
10000
1000
100
10
1
Control
P.g
Control+anti-TLR4
P.g+anti-TLR4
IL - 8
pg/ml
10000
8000
6000
4000
2000
0
Control
P.g
Control+anti-TLR4
P.g+anti-TLR4

Figure 8