

Porphyromonas gingivalis infection-induced tissue and bone transcriptional profiles

A. Meka^{1,*}, V. Bakthavatchalu², S. Sathishkumar^{2,†}, M.C. Lopez³, R.K. Verma¹, S.M. Wallet^{1,4}, I. Bhattacharyya⁵, B.F. Boyce⁶, M. Handfield⁴, R.J. Lamont⁴, H.V. Baker³, J.L. Ebersole² and L. Kesavalu^{1,4}

¹ Department of Periodontology, College of Dentistry, University of Florida, Gainesville, FL, USA

² Center for Oral Health Research, University of Kentucky, Lexington, KY, USA

³ Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, FL, USA

⁴ Department of Oral Biology, University of Florida, Gainesville, FL, USA

⁵ Department of Oral and Maxillofacial Surgery, Division of Pathology, University of Florida, Gainesville, FL, USA

⁶ Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY, USA

Correspondence: Kesavalu N. Lakshmyya, Department of Periodontology, D11-24, College of Dentistry, University of Florida, Gainesville, FL 32610, USA Tel.: +1 352 273 6500; fax: +1 352 273 6192; E-mail: kesavalu@dental.ufl.edu

*Present addresses: 1 DNA Way, South San Francisco, CA 94080, USA.

†Genetic ID NA, Inc, 501 Dimick Drive, Fairfield, Iowa 52556, USA.

Keywords: bone; calvarial tissue; gene expression; microarray; *Porphyromonas gingivalis*

Accepted 13 October 2009

SUMMARY

Porphyromonas gingivalis has been associated with subgingival biofilms in adult periodontitis. However, the molecular mechanisms of its contribution to chronic gingival inflammation and loss of periodontal structural integrity remain unclear. This investigation aimed to examine changes in the host transcriptional profiles during a *P. gingivalis* infection using a murine calvarial model of inflammation and bone resorption. *P. gingivalis* FDC 381 was injected into the subcutaneous soft tissue over the calvaria of BALB/c mice for 3 days, after which the soft tissues and calvarial bones were excised. RNA was isolated from infected soft tissues and calvarial bones and was analysed for transcript profiles using Murine Gene-Chip[®] arrays to provide a molecular profile of the events that occur following infection of these tissues. After *P. gingivalis* infection, 6452 and 2341 probe sets in the infected soft tissues and calvarial bone, respectively, were differentially expressed ($P \leq 0.05$). Biological pathways significantly impacted by *P. gingivalis* infection in tissues and calvarial bone included cell adhesion

(immune system) molecules, Toll-like receptors, B-cell receptor signaling, transforming growth factor- β cytokine family receptor signaling, and major histocompatibility complex class II antigen processing pathways resulting in proinflammatory, chemotactic effects, T-cell stimulation, and downregulation of antiviral and T-cell chemotactic effects. *P. gingivalis*-induced inflammation activated osteoclasts, leading to local bone resorption. This is the first *in vivo* evidence that localized *P. gingivalis* infection differentially induces transcription of a broad array of host genes, the profiles of which differed between inflamed soft tissues and calvarial bone.

INTRODUCTION

Periodontitis is a chronic immuno-inflammatory infectious disease initiated by complex microbial biofilms resulting in destruction of periodontal tissues, resorption of alveolar bone, and exfoliation of teeth. *Porphyromonas gingivalis*, a gram-negative black-pigmented anaerobe, is an important member of the

pathogenic biofilm at sites of periodontal disease (Lamont & Jenkinson, 1998; Holt *et al.*, 1999). It may contribute to the disease process by a variety of mechanisms, including production of numerous components that mediate adherence to mucosal surfaces, enable penetration into epithelial cells, inhibit host defense mechanisms, and elicit gingival tissue inflammatory destruction (Lamont & Jenkinson, 1998), resulting in local alveolar bone destruction (Holt *et al.*, 1999). *In vitro* studies have shown that components of *P. gingivalis* can induce a range of proinflammatory cytokines, including interleukin-1 α (IL-1 α), IL-1 β , tumor necrosis factor- α (TNF- α), IL-6, and IL-8, which have been proposed as potential molecular effectors of connective tissue destruction and resorption of alveolar bone in periodontitis (Gemmell *et al.*, 1998; Kinane *et al.*, 2001). However, robust data on the *in vivo* role of these biomolecules, as well as the broader aspects of the host response in the periodontium, remain to be defined.

The use of microarrays to survey transcriptional host responses after exposure to microbial pathogens has become a powerful approach to improve understanding of the molecular basis of the host response to bacterial infections, which is critical for limiting tissue destruction. Host response profiling has identified transcripts uniquely affected by the pathogens *Listeria monocytogenes*, *Brucella abortus*, and *Mycobacterium tuberculosis*. Recent microarray studies have determined *in vitro* responses of host cells to challenge with *P. gingivalis* or its virulence components in primary human coronary artery endothelial cells and human aortic endothelial cells (Chou *et al.*, 2005), a stromal cell line (Ohno *et al.*, 2006), human monocytes (Zhou & Amar, 2006), a macrophage-like human cell line U937 (Oshikawa *et al.*, 2004), human periodontal ligament-derived cells (Kasamatsu *et al.*, 2005), human periodontal ligament and gingival fibroblasts (Han & Amar, 2002), gingival fibroblasts from healthy and inflammatory gingival tissues (Wang *et al.*, 2003), and human gingival epithelial cells (Handfield *et al.*, 2005). A recent review reported on transcriptional profiling of gingival epithelial cell responses to challenge by four different microorganisms (Handfield *et al.*, 2008). Only one previous report has described *ex vivo* gene expression changes in mice in response to infection with *P. gingivalis* and this was limited to spleen CD4 and CD8 cells (Gemmell *et al.*, 2006). As a consequence,

there remains a lack of information concerning *P. gingivalis* induction of gene expression when the microorganism interacts with host cells and tissues *in vivo*. The aim of the present study was to define changes in the transcriptome of calvarial bone and overlying soft tissues in response to localized *P. gingivalis* infection in mice using a calvarial model of acute inflammation and bone resorption.

METHODS

Animals

BALB/c female mice 8–10 weeks of age (Harlan, Indianapolis, IN) were routinely acclimatized for at least 1 week before use. They were infected with *P. gingivalis* as described below following isoflurane inhalation anesthesia. All procedures were performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee at the University of Kentucky.

Microorganisms and mouse infection

P. gingivalis FDC 381 was cultured and maintained for the animal infections, which were initiated within 15–30 min of bacterial preparation, as described previously (Kesavalu *et al.*, 1992) in studies showing that *P. gingivalis* induces an inflammatory infiltrate, osteoclast activation, and increased messenger RNA (mRNA) expression of proinflammatory cytokines in calvarial tissues (Zubery *et al.*, 1998; Kesavalu *et al.*, 2002). *P. gingivalis* was injected at 1.5×10^9 cells ($n = 10$) into the soft tissues overlying the calvaria of the mice. Bacteria (suspended in 10 μ l of reduced transport fluid) were injected into the subcutaneous tissue over the right side of the parietal bone and anterior to the lamboid suture once daily for 3 days using a Hamilton syringe (Hamilton Co., Reno, NV). A control group ($n = 10$) was injected with vehicle (reduced transport fluid) once daily for 3 days. Mice were sacrificed 8 h after the last injection using CO₂ asphyxiation and cervical dislocation. The calvarial bones and overlying soft tissues from five mice in each group were excised, snap frozen in liquid nitrogen, and stored at -80°C until RNA isolation. The bone samples consisted predominantly of mature lamellar bone containing osteocytes, with associated osteoblasts and osteoclasts and small amounts of

bone marrow. The soft tissue consisted predominantly of the cutaneous epithelium, with subcutaneous and dermis fibroblasts/connective tissue and vascular tissue and cells, and subcuticular fat. The calvarial bones with intact overlying tissue of the remaining five mice were fixed in 10% formalin and embedded in paraffin for histological assessment of bone changes, as described previously (Zubery *et al.*, 1998).

RNA isolation and mouse GeneChip hybridization

Total RNA was isolated from the frozen calvarial tissue and calvarial bone from each mouse (*P. gingivalis*-infected and control animals, $n = 5$ in each group) with Trizol reagent (Invitrogen, Carlsbad, CA). Quantification of RNA yield was by spectrophotometric analysis and the absorbance at 260 and 280 nm was checked to determine the RNA concentration and purity. Quality of the RNA was determined by gel electrophoresis. For GeneChip analyses, RNA samples were further purified with Qiagen RNeasy columns (Qiagen, Valencia, CA). Equal amounts of RNA from samples were labeled and hybridized on a GeneChip following the protocol described in the GeneChip Expression Analysis Technical manual (Affymetrix, Santa Clara, CA). Briefly, reverse transcription was performed on 8 μ g total RNA using SuperScript reverse transcriptase enzyme (Invitrogen) and oligo-(dT)₂₄ primer containing a T7 RNA polymerase promoter. RNA samples were not pooled. The biotin-labeled complementary RNA was then synthesized by *in vitro* transcription, fragmented and hybridized on mouse GeneChip MG-MOE430A (Affymetrix). After hybridization, the GeneChip arrays were stained and scanned in an Affymetrix GCS 3000 7G Scanner. The probe sets represent genes or DNA sequences within genes. Some genes are represented by more than one probe set in the microarray, and hence probe sets are not uniquely correlated to genes. However, for ease of discussion, we use the terms 'probe sets' and 'genes' interchangeably (Feezor *et al.*, 2003).

Microarray data analysis

The microarray data were normalized and a model-based apparent gene expression matrix was generated using the algorithms implemented in dChip (Li &

Wong, 2001). For unsupervised analysis the dataset was analysed as a whole, including arrays hybridized with material derived from both calvarial soft tissue and calvarial bone, and in separate analyses with either calvarial soft tissue or calvarial bone-derived specimens. In all cases an initial variation filter was applied to identify probe sets whose hybridization signal intensity varied the most across the respective datasets. For this purpose probe sets were selected that displayed a coefficient of variation > 1 . The signal intensities of these probe sets were then mean centered and variance normalized to 1 on a probe set-wise basis. The variance normalized data were then subjected to hierarchical clustering using the algorithms implemented in dChip (Eisen *et al.*, 1998; Li & Wong, 2001).

For supervised analyses the model-based expression matrix was imported into BRB Array tools for high-level analysis. The expression matrix was log transformed and statistical analyses were performed in log space. Initially, the dataset was divided into tissue and treatment classes. Differences between the various treatment tissue classes were determined using a modified *t*-test with a random variance model and a significance threshold of $P < 0.05$. To assess the ability of probe sets significant at $P < 0.05$ to distinguish between the treatment classes, 'leave-one-out-cross-validation' studies were performed in which each array was left out in turn, a classification model was built, and the ability of the classifier to correctly identify the array left out (not used in building the classification model) was determined using several prediction models (nearest neighbor analysis, nearest centroid analysis and other models implemented in BRB Array tools) (Feezor *et al.*, 2003; Handfield *et al.*, 2005, 2008; Hasigawa *et al.*, 2008). In cases where the misclassification rate was above zero the significance of the classifier's performance was assessed using Monte Carlo simulations with 100 random permutations of the dataset. The performance of the classifier was considered significant at the $P \leq 0.05$ threshold (Hasigawa *et al.*, 2008).

Genes were categorized into Gene Ontology (GO) and annotated using NetAffx[®] (Santa Clara, CA, USA), an analysis web interface from Affymetrix and Database for Annotation, Visualization, and Integrated Discovery – Expression Analysis Systematic Explorer (DAVID EASE) (Dennis *et al.*, 2003; Hosack *et al.*, 2003). The mean fold change of expression of

P. gingivalis infection to uninfected (control) gene expression was calculated using the mean of the five replicates. The Gene Ontology database organizes genes into hierarchical categories based on biological process, molecular function and subcellular location. Onto-Express (microarray tool developed at the Intelligent Systems and Bioinformatics Laboratory, Wayne State University, Detroit, MI) constructs functional profiles; using Gene Ontology terms, for biological process, cellular component and molecular function (Draghici *et al.*, 2006). Gene ontology trees were populated using Pathway Express (Khatri & Draghici, 2005), available at <http://vortex.cs.wayne.edu/projects.htm>.

Quantitative RT-PCR analysis

Selected genes that showed significant differential expression in microarray analysis were confirmed for expression by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) analysis (LightCycler FastStart DNA Master SYBR Green I, Roche). Selected upregulated genes from both calvarial soft tissue and bone [cytochrome P450, family 2, gamma 1 (*Cyp2g1*), aldo-keto reductase family 1, member C3 (*Akr1c18*), metallothionein 1 (*Mt1*), nephronectin (*Npnt*), matrilin-2 (*Matn2*), insulin-like growth factor binding protein 4 (*Igfbp4*)] were evaluated. Total RNA (250 ng) from pooled control and *P. gingivalis* infection samples were reverse transcribed to complementary DNA (cDNA) using the transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Briefly, total RNA was mixed with the anchored oligo-(dT)₁₈ primer (2.5 µm) in a final volume of 13 µl and denatured for 10 min at 65°C and immediately cooled on ice. Then, 5× transcriptor RT reaction buffer, protector RNase inhibitor, deoxynucleotide mix, and transcriptor reverse transcriptase were added to the following final concentrations of 1×, 20 U, 1 mM each, and 10 U, respectively, in a final volume of 20 µl. Transcriptor cDNA synthesis reactions were carried out for 30 min at 55°C, and at 85°C for 5 min for enzyme inactivation. These cDNAs were used for qRT-PCR analyses. The qRT-PCR was performed using 2.0 µl cDNA in a 20-µl reaction for 10 min at 95°C and 40 cycles of 10 s at 95°C, annealing at 60°C followed by amplification at 72°C for 8–10 s. For the determination of the melting

curve, the temperature was increased 0.1°C every 1 s from 55°C to 95°C. All PCRs were repeated two or three times. Using the relative quantification application in the LightCycler analysis software (Roche), the fold changes of the different gene expressions were calculated based on the expression of the target gene vs. the expression of the housekeeping gene *ActB* of the same sample. The mouse β-actin (*ActB*) gene was used as an internal control based on its constant level of expression across groups in microarray analysis. Standard curves were generated for each gene. To determine the specificity of DNA quantification, a melting curve was generated for each gene.

Relative standard curves

Constructing a standard curve with serial dilutions of known template concentration for each target was not feasible. Therefore, dilutions (1 : 50, 1 : 100, 1 : 250, and 1 : 500) of cDNA for each sample type (control soft tissue and calvarial bone, infected soft tissue and calvarial bone) for a given target gene were used to construct a relative standard curve for that target along with *ActB*. Template concentrations for reactions in the relative standard were given arbitrary values of 1 (undiluted), 0.02, 0.01, 0.004, and 0.002. The undiluted cDNA from the sample was analysed as unknown. The fold changes in gene expression of the different target genes were calculated as ratios of the relative expression of the target gene to the relative expression of *ActB* of the same sample. Each PCR (20 µl) contained 2 µl of the respective cDNA dilution, 1× LightCycler FastStart DNA Master SYBR Green I mix, 0.5 µM primers and 4 mM MgCl₂. The amplification was performed under the following conditions: a preincubation step of 1 min at 95°C, followed by 45 cycles of 10 s at 95°C, 5 s at 60°C and 8–10 s at 72°C (temperature ramp was 20°C/s). Amplification was followed by melting curve analysis using the program run for one cycle at 95°C with 0-s hold, 55°C with 15-s hold, and 95°C with 0-s hold at the step acquisition mode at 0.1°C/s. A negative control without cDNA template was run with every assay to assess the overall specificity. Unless otherwise mentioned, each assay was repeated once or twice. A relative value for the target concentration in each reaction was determined on the basis of the dilution using the LightCycler software, version 4. The concentration of *ActB* was used

to control for input RNA because it is considered a stable housekeeping gene (Piana *et al.*, 2008). The *ActB* concentration was determined once for each cDNA sample and used to normalize all other genes tested from the same cDNA sample. The relative change in gene expression was recorded as the ratio of normalized target concentrations in the undiluted cDNA.

Bone histology

The calvariae were fixed in 10% phosphate-buffered formalin and decalcified in 14% ethylenediaminetetraacetic acid (Zubery *et al.*, 1998). The anterior half of frontal bones and most of the occipital bones were trimmed off, and the parietal bones were cut coronally into two pieces. These half calvariae were then embedded in paraffin with the edges cut coronally placed at the bottom of the cassettes, and four non-consecutive levels were cut, providing eight coronal sections through each calvaria. Sections (4 µm thick) were stained with hematoxylin and eosin. Histomorphometric analysis of osteoclasts was carried out on two sections from calvaria from each animal. This was accomplished by counting the number of osteoclasts throughout the length of the calvarial bone in each section. These bone sections contained the largest number of bone marrow spaces and hence the greatest length of bone surface was available for assessment of resorption.

Antibody analysis

Serum from mice infected with *P. gingivalis* or from control uninfected mice was used to determine immunoglobulin G (IgG) antibody titers to *P. gingivalis* using a standard enzyme-linked immunosorbent assay protocol (Kesavalu *et al.*, 1992), and provided an additional marker of infection. Briefly, *P. gingivalis* was grown in liquid culture as described above, cells were harvested by centrifugation, and the pellets were washed with sterile phosphate-buffered saline (Kesavalu *et al.*, 1992). The cells were treated overnight with 0.5% buffered formal saline (FK cells), washed, and diluted to an optical density at 600 nm of 0.3 as coating antigen. Purity and sterility were confirmed using blood agar plating and the antigens were stored at -20°C. Diluted serum (1 : 100) from infected mice was incubated in wells of antigen-coated microtiter plates for 2 h on a rotator at room temperature, along with a puri-

fied mice IgG (Sigma, St Louis, MO) standard to create a gravimetric curve for antibody quantification. After washing, affinity-purified goat anti-mouse IgG (Fc-specific) or goat anti-mouse IgM (µ-chain-specific) conjugated to alkaline phosphatase (1 : 5000) was added to the plates and incubated for 2 h. Subsequently, rabbit anti-goat IgG alkaline phosphatase (Sigma) was added to the wells, incubated for 2 h, washed, and the assay was developed with *p*-nitrophenolphosphate (Sigma). The assay reactions were stopped by the addition of 3 M NaOH and analysed at an optical density of 405 nm using a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA, USA). The serum antibody level of infected mice was quantified using known standard curves. Antigen-coated wells in the absence of serum provided a background negative control. Mean serum IgG antibody levels were derived from triplicate determinations for each mouse serum.

Statistical analyses

Microarray data were analysed as described above. *P* values of 0.05 or less were considered significant. The qRT-PCR data from two independent experiments were combined and results were expressed as means ± SD.

RESULTS

Ontology of gene expression changes in murine calvarial soft tissue and bone

The mouse gene chip MOE430A contains 22,690 probe sets, and 11,391 and 1677 probe sets (*P* < 0.001) were detected above background in soft tissue and calvarial bone, respectively. Mean gene expression levels of 6452 and 2341 probe sets were significantly different in soft tissue and bone in response to the *P. gingivalis* infection (*P* < 0.05). In addition, expression of 5517 probes was upregulated and 935 probes were significantly downregulated in calvarial soft-tissue infection with *P. gingivalis* (data not shown). In contrast, expression of 1900 probes was upregulated and 441 probes were downregulated significantly in calvarial bone at the site of infection with *P. gingivalis* (data not shown). *P. gingivalis* acute infection stimulated changes in a wide array of genes (6452), particularly in the calvarial soft tissue and more than 139, 40, and 15 genes were > 10-, 25-, and 50-fold upregulated, respectively (data

not shown). In contrast, all of the 1900 genes upregulated in bone to *P. gingivalis* acute infection were altered less than five-fold. These results also demonstrate a low level of similarity (overlap) in upregulated and downregulated genes within the tissues examined.

To mine the array data for biologically relevant information, an ontology analysis of known metabolic pathways was performed using individual comparisons of the *P. gingivalis* infected tissue or bone samples to the corresponding baseline uninfected state. A pairwise comparison was imposed by the ontology algorithms described above. For this analysis, signal intensities were renormalized across all samples, and supervised analyses were repeated as described above. To populate biological pathways to the maximal extent and so enhance their predictive power, a

significance level of $P < 0.05$ was adopted. A total of 6452 genes were differentially expressed in the tissue samples, whereas 2341 genes were differentially expressed in bone samples compared with uninfected controls. The ability of probe sets significant at $P < 0.05$ to correctly identify between treatment groups was confirmed by leave-one-out-cross-validation analysis (Supporting Information, Table S1). The classifiers performed flawlessly and correctly predicted the treatment group with 100% accuracy. Probe sets at the $P < 0.05$ significance level were analysed by the Pathway Express (PE) algorithm. Pathways significantly impacted by *P. gingivalis* in bone tissue types included, Antigen Processing and Presentation (Fig. 1), Toll-like Receptor Signaling (Figs 2 and 3), Cell Adhesion (immune system)

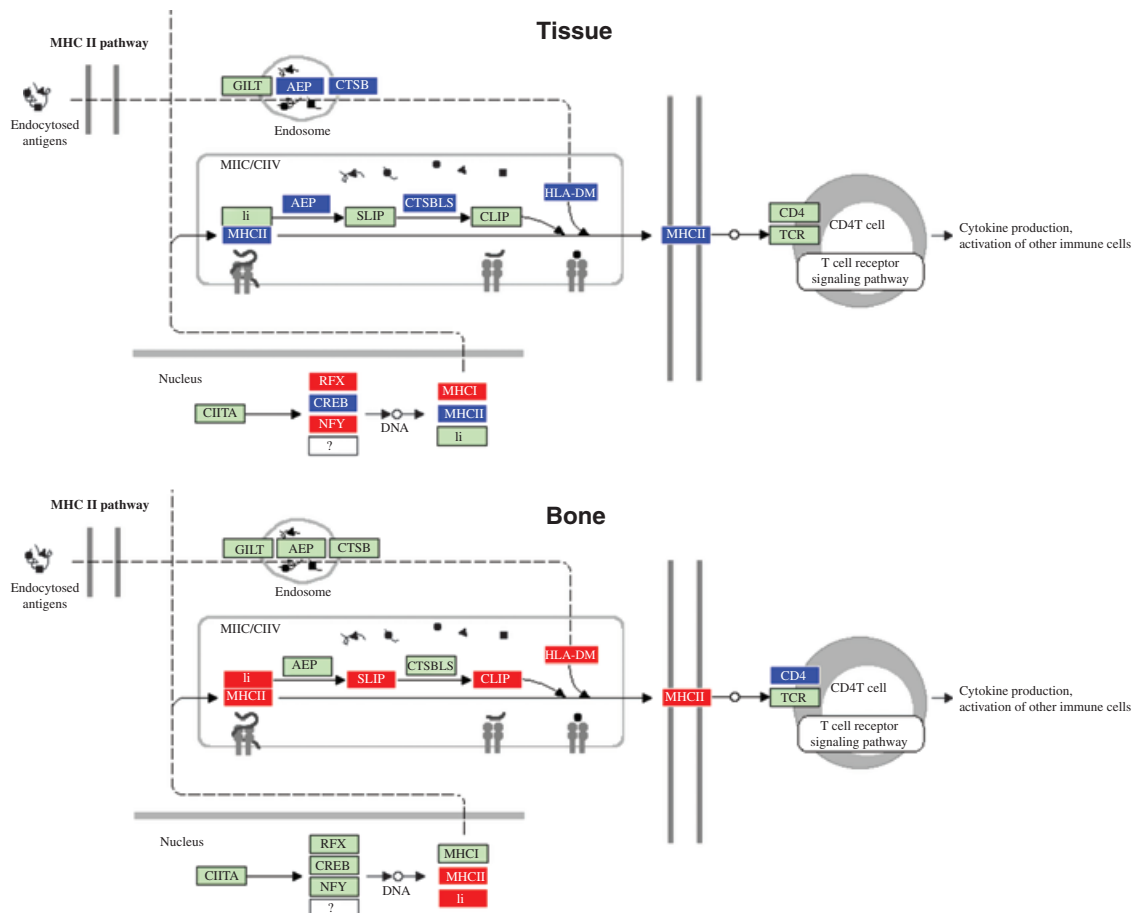


Figure 1 Major histocompatibility complex (MHC) class II antigen-processing pathways containing *Porphyromonas gingivalis* differentially regulated genes in calvarial tissue and calvarial bone at $P < 0.05$, adopted from Pathway Express and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates upregulation, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation of T-cell receptor signaling pathway, and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

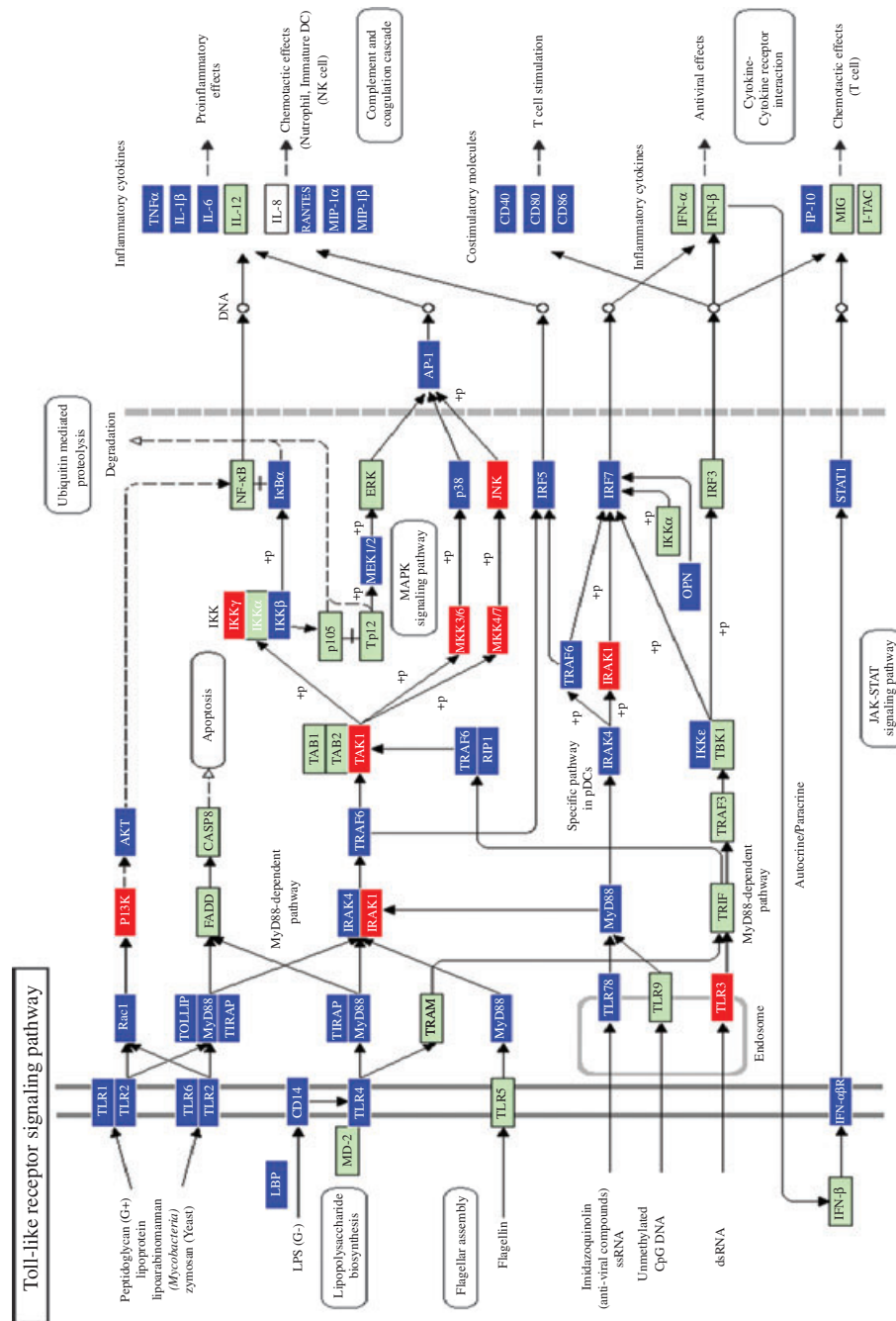


Figure 2 Toll-like receptor signaling pathways containing *Porphyromonas gingivalis* differentially regulated genes in calvarial tissue at $P \leq 0.05$, adopted from Pathway Express and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates upregulation, blue indicates repression, and green indicates no change in gene expression. +P indicates phosphorylation, and -P indicates dephosphorylation. An arrow indicates a molecular interaction resulting in proinflammatory, chemotactic effects, and downregulation of antiviral and T-cell chemotactic effects.

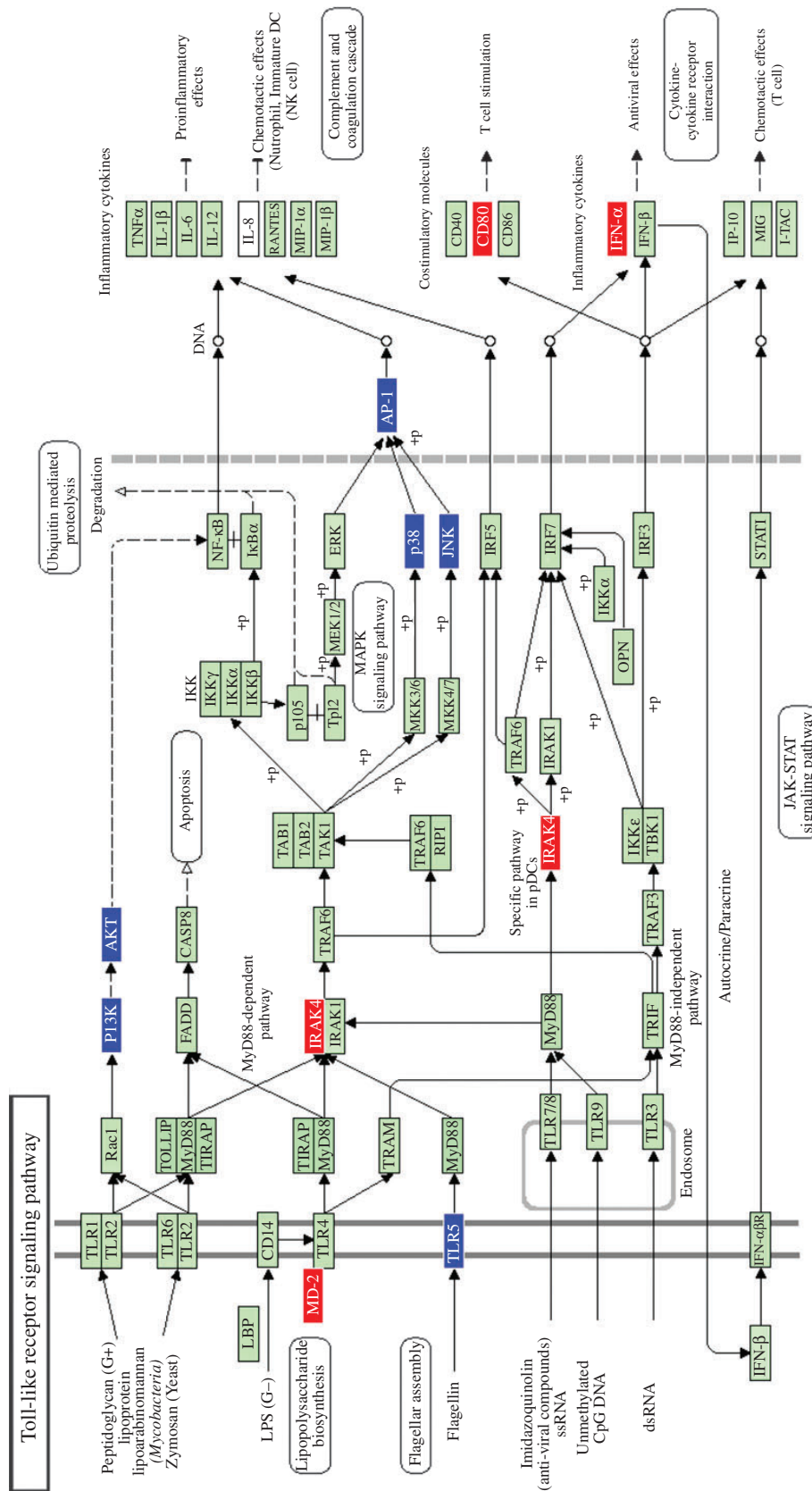


Figure 3 Toll-like receptor signaling pathways containing *Porphyromonas gingivalis* differentially regulated genes in calvarial bone at $P \leq 0.05$. Red indicates upregulation, blue indicates repression, and green indicates no change in gene expression. +P indicates phosphorylation, and -P indicates dephosphorylation. An arrow indicates a molecular interaction resulting in proinflammatory, chemotactic effects, T-cell stimulation, and downregulation of antiviral and T-cell chemotactic effects.

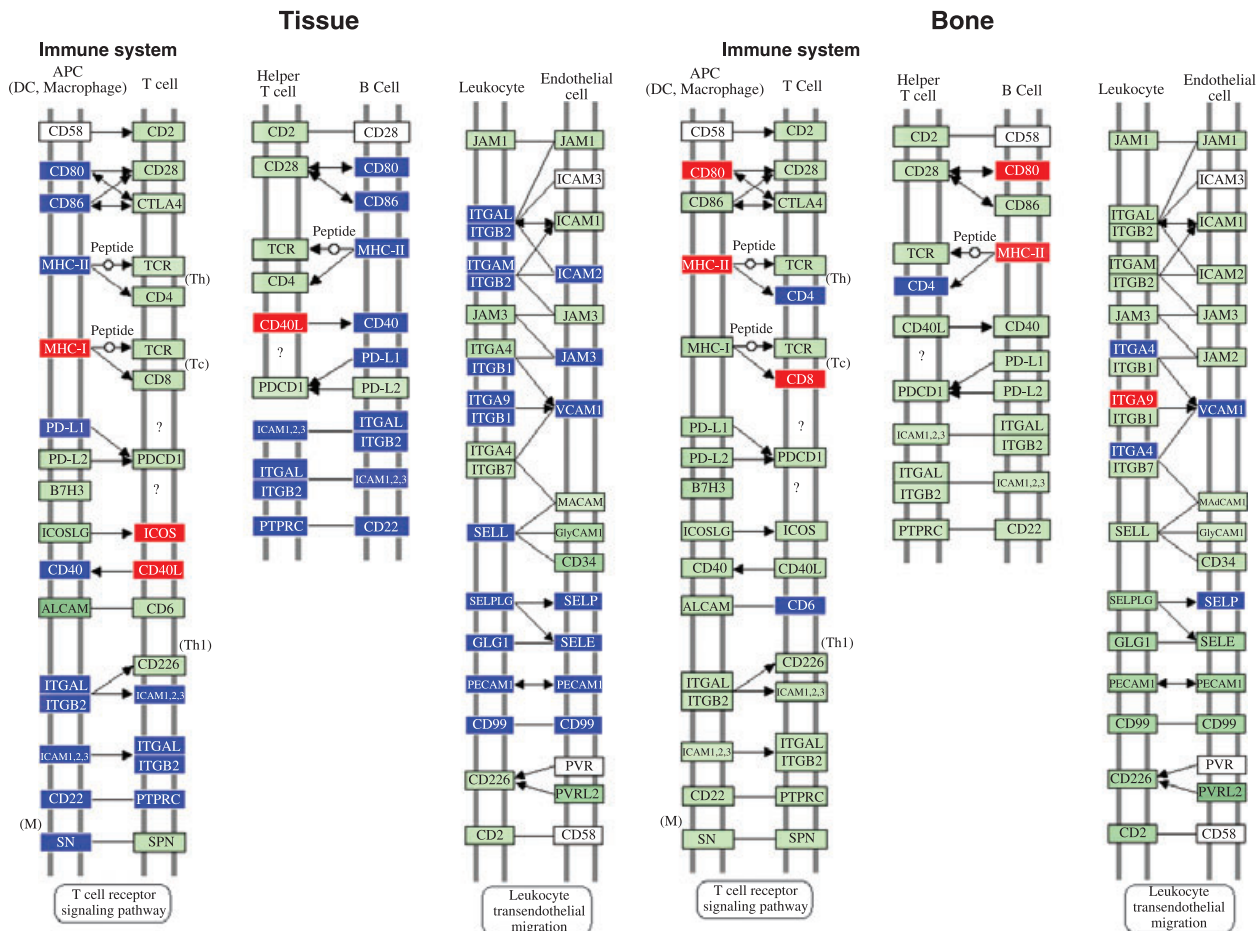


Figure 4 Cell adhesion (immune system) molecules pathway containing *Porphyromonas gingivalis* differentially regulated genes in calvarial tissue at $P \leq 0.05$, adapted from Pathway Express and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates upregulation, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation, and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

Molecules (Fig. 4), and Cytokine–Cytokine Receptor Interaction (Fig. 5). Table 1 shows soft tissues and calvarial bone pathways generated in this analysis that were predominantly affected in order of their impact factors. *P. gingivalis* significantly impacted 25 pathways in tissue and two in bone with an impact factor >10 . Moreover, Toll-like receptor, B-cell receptor, cytokine–cytokine receptor, and mitogen-activated protein kinase signaling pathways are other major pathways impacted in calvarial soft tissue. Cell adhesion molecules and phosphatidylinositol signaling system pathways were significantly impacted or overlapped in both the calvarial tissue and bone. Interestingly, few classical proinflammatory genes, e.g. IL-1, TNF, IL-6, were altered by the localized *P. gingivalis* infections using these criteria. However,

various genes related to bone remodeling activities [e.g. *Ptger3* (prostaglandin E receptor 3), *Bmp1* (bone morphogenetic protein 1), *Bmp2*, *Bmp4*, *PTHrP* (parathyroid hormone-related protein), *Tuft1* (tuftelin 1), *Ptger4* (prostaglandin E receptor 4)] and tissue extracellular matrix [e.g. *Lama3* (laminin alpha 3 chain precursor), *Timp1* (tissue inhibitor of proteinase 1), *Timp3*] were upregulated in the localized inflamed tissue. These data also emphasize that this localized infection by *P. gingivalis* appeared to upregulate genes in the overlying infected tissue that could negatively impact physiological and remodeling processes in the underlying bone. A few inflammatory/innate immune/acute-phase response genes, including serum amyloid A, S100A9 calcium-binding protein, and chemokine ligand 2, (e.g. *S100a9*,

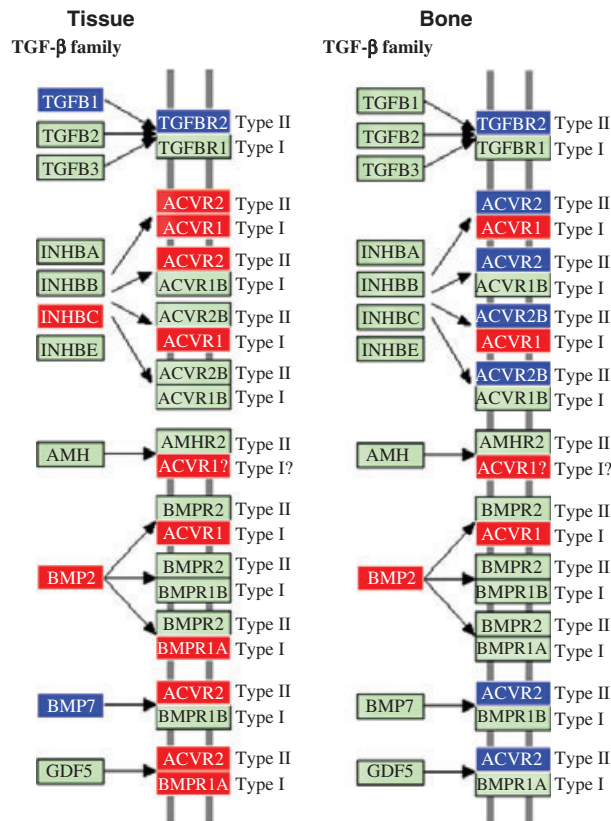


Figure 5 Transforming growth factor- β (TGF- β) cytokine family receptor interaction (cytokine–cytokine receptor interaction) pathway containing *Porphyromonas gingivalis* differentially regulated genes in calvarial tissue and bone at $P \leq 0.05$. Red indicates upregulation, blue indicates repressed, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation.

S100a8, *Saa3*, *Cp*, and *Ccl2*) were greatly upregulated in inflamed soft tissues.

Histology

No edema and minimal inflammation were present in the soft tissue over the calvaria of the control uninfected mice (Fig. 6A). Calvarial soft-tissue swelling occurred at the injection site within 24 h of the first injection and increased in size after 48 and 72 h in almost all of the mice injected with *P. gingivalis*, but not in control uninfected mice. None of the *P. gingivalis*-infected mice developed local abscesses, ulceration of the overlying skin, or any evidence of spread of infection to other sites. Histological examination revealed moderate edema and an intense mixed inflammatory cell infiltrate of

Table 1 Ontology analysis of calvarial tissue and bone gene pathways impacted by infection with *Porphyromonas gingivalis*¹

Impacted pathway ²	Impact factor ³	No. of input genes/no. of pathway genes ⁴
Calvarial tissue		
Adherens junction	47.991	18/74
Cell adhesion molecules	41.085	36/157
Antigen processing and presentation	36.401	17/99
Circadian rhythm	27.498	3/14
Toll-like receptor signaling pathway	27.299	45/101
B-cell receptor signaling pathway	24.705	31/66
Apoptosis	19.262	32/81
Cytokine–cytokine receptor interaction	18.667	63/242
Leukocyte transendothelial migration	18.487	39/115
Natural killer cell mediated cytotoxicity	17.046	37/127
Fc ϵ RI signaling pathway	14.506	28/78
Phosphatidylinositol signaling system	14.256	12/70
Insulin signaling pathway	13.955	40/137
Regulation of actin cytoskeleton	13.618	52/206
Chronic myeloid leukemia	13.599	27/77
MAPK signaling pathway	13.085	64/258
ErbB signaling pathway	13.08	28/87
Jak-STAT signaling pathway	12.531	39/151
Focal adhesion	12.308	49/192
T cell receptor signaling pathway	11.354	28/105
GnRH signaling pathway	11.088	30/95
Acute myeloid leukemia	10.871	21/58
Glioma	10.663	21/65
Hematopoietic cell lineage	10.643	27/85
Pancreatic cancer	10.042	23/73
Calvarial bone		
Cell adhesion molecules	112.874	12/157
Phosphatidylinositol signaling system	33.315	4/70

¹The calvarial tissue and bone gene pathways were determined by Pathway Express (Khatri & Draghici, 2005; Hasegawa et al., 2008).

²Kyoto Encyclopedia of Genes and Genome Pathways (<http://www.genome.jp/kegg/>).

³The impact factor measures the pathways most affected by changes in gene expression in calvarial tissue and bone by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these perturbations throughout the pathway. Only pathways with an impact factor > 10 are included in this table.

⁴Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.

ErbB, the ErbB family of RTKs (Receptor tyrosine kinases) consists of four receptors: ErbB-1, (also called epidermal growth factor receptor (EGFR)), ErbB-2 (also called HER2 or Neu), ErbB-3 and ErbB-4; Fc ϵ RI, the high-affinity IgE receptor. The interaction of IgE with the high affinity receptor for IgE (designated FcRI) is central to IgE dependent anaphylaxis; GnRH, gonadotropin-releasing hormone; Jak-STAT, Janus kinase–signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase.

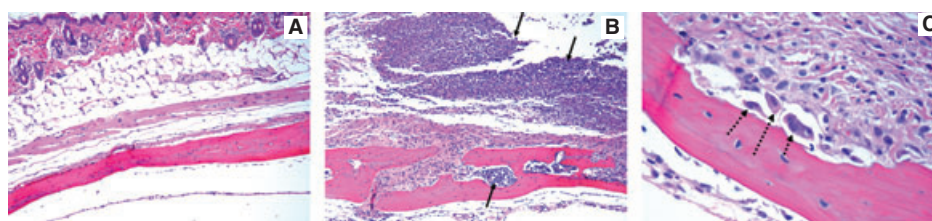


Figure 6 Effects of local injection of *Porphyromonas gingivalis* on mouse calvaria. Live *P. gingivalis* bacteria (1.5×10^9) were injected once daily for 3 days into the subcutaneous tissues overlying the calvaria of mice. All photomicrographs of slides stained with hematoxylin and eosin. (A) No edema and minimal inflammation present in the soft tissue over the calvaria of the control mouse (magnification $\times 10$). (B) Section from *P. gingivalis*-infected mouse reveals enlarged bone marrow spaces with an intense inflammatory cell infiltrate (solid black arrows) and edema present in the adjacent soft tissue and within the marrow spaces (magnification $\times 10$). (C) Multiple osteoclasts (dashed black arrows) present in resorption lacunae. (magnification $\times 40$).

variable intensity in the calvarial tissues and this large infiltrate consisted of polymorphonuclear leukocytes, lymphocytes, and macrophages (Fig. 6B). Microabscesses and areas of soft-tissue necrosis were also evident in sections and where the necrosis abutted the parietal bone. Osteoclasts were also present in some mice on the upper periosteal surface, consistent with more aggressive resorption. In addition, significantly increased numbers of osteoclasts were seen inside the calvaria compared with uninfected controls (data not shown), and these caused an increase in the size of the bone marrow spaces because of increased endosteal bone resorption (Fig. 6C). *P. gingivalis* cells were not seen in Gram-stained calvarial sections from mice with moderate soft-tissue inflammatory infiltrates. We therefore assumed that the bacteria were removed by host immune cells during the 8 h between the last injection and sacrifice.

Validation of microarray gene expression

Porphyromonas gingivalis-induced transcript expression results from the microarray studies were analysed by qRT-PCR for selected genes, including *Cyp2g1*, *Akr1c18*, *Mt1*, *Npnt*, *Matn2*, and *Igfbp4* in soft tissue and in calvarial bone from aliquots of the pooled RNA samples used for the microarray (Supporting Information, Table S2). Transcripts of β -actin were used as an expression control and the qRT-PCR analyses were performed at least twice for each gene. The expression levels of *Akr1c18*, *Mt1*, *Npnt*, *Matn2*, and *Igfbp4* genes by qRT-PCR were higher than the microarray expression level and the expression levels of *Cyp2g1* gene by qRT-PCR were lower than the microarray expression levels. The magnitude

of the expression levels of these genes quantified using qRT-PCR generally did not show equivalent expression change with the microarray fold-change outcomes.

Serum IgG antibody in calvarial infections

To provide additional documentation of calvarial infection and to demonstrate an immunological response to *P. gingivalis*, we evaluated the levels of *P. gingivalis*-specific IgG antibody in mouse sera collected at the end of the 3 days of the acute infection period. As expected, *P. gingivalis* infection for 3 days (acute infection) did not induce *P. gingivalis*-specific serum IgG antibody responses that were not different from those observed in control uninfected mice.

DISCUSSION

Microarray technology continues to evolve as a powerful tool to study host–pathogen interactions and to expand our understanding of the molecular pathogenesis of diseases induced by pathogens (Collazo *et al.*, 2002; Dennis *et al.*, 2003; Feezor *et al.*, 2003; Handfield *et al.*, 2005; Demmer *et al.*, 2008). Here, we evaluated the gene transcription profiles of host soft tissue and bone in a calvarial bone resorption model during a localized acute infectious challenge with the oral pathogen *P. gingivalis*. In the soft tissue *P. gingivalis* downregulated several cytokines involved in bone resorption including IL-1 β , IL-1 α , IL-6, and TNF- α , whereas in bone none of these cytokines was differentially regulated. These results are consistent with previous results in a murine model that showed *P. gingivalis* infection did not significantly upregulate bone resorptive cytokines in

gingival tissue (Sasaki *et al.*, 2004). Moreover, under the conditions of infection in the current study, *P. gingivalis* did not appear to stimulate the receptor activated nuclear factor- κ ligand (RANKL) bone resorption pathway (Wada *et al.*, 2006), as TNF receptor-associated factor 6 and nuclear factor- κ B were downregulated in tissue and were not altered in bone.

One possible explanation for this lack of increase in expression levels of osteoclastogenic cytokines is that the bones were harvested 8 h after the last injection of bacteria following 3 days of injections and that expression levels had been elevated, but returned to normal levels by the time the bones were sampled. Further studies will be required to examine this possibility. One means by which *P. gingivalis* may impact bone turnover is through downregulation of the anti-inflammatory cytokine IL-10. Mice that are IL-10^{-/-} exhibit a hyperinflammatory phenotype and display increased susceptibility to *P. gingivalis*-induced periodontal bone loss (Sasaki *et al.*, 2004). In addition, downregulation of intercellular adhesion molecule 1 and P-selectin in tissue may contribute to bone loss as knockout mice deficient in cellular adhesion molecules show increased *P. gingivalis*-induced alveolar bone loss (Baker *et al.*, 2000). Another possible mechanism for proinflammatory cytokine-independent alveolar bone loss is through downregulation of activin receptor type II (ACVR2), which occurred in bone samples. Activins are dimeric growth and differentiation factors which belong to the transforming growth factor- β superfamily (Chang *et al.*, 2002). Activins can be stimulated by a subset of bone morphogenetic proteins (Tsuchida *et al.*, 2008), and will bind and activate the SMAD family of transcriptional regulators, which control osteoblast expansion, differentiation, and bone formation (Lian *et al.*, 2006; Li, 2008).

A major difference between the tissue and bone samples was in the impact on the antigen processing and presentation pathway. In bone, *P. gingivalis* upregulated several components of the major histocompatibility complex class II (MHCII) pathway, including MHCII itself, which processes endocytosed antigens and leads to expansion of CD4-positive T cells including T helper cells. In addition, in bone samples IL-18 was upregulated, which in combination with a lack of IL-12 upregulation, is indicative of a T helper type 2 protective response (Orozco *et al.*, 2007). By contrast, in tissue the MHCII pathway was downregulated, which would be predicted to decrease the level

of stimulation of CD4 T cells and consequently lower the levels of cytokines and B cells. Indeed, several T helper-B cell interacting molecules were downregulated in tissue including CD80, CD86, CD40, and CD22. Leukocyte migration through the endothelium was also potentially abrogated by reduced expression of many interacting components. Leukocyte adhesion components that were downregulated included integrin α -L/ β -2 (ITGA1/ITGB2), which together form a receptor for intercellular adhesion molecule-1, and are involved in a variety of immune phenomena, including leukocyte-endothelial cell interaction (Smith *et al.*, 2007). Moreover, E-selectin (SELE) and P-selectin (SELP) were downregulated, further contributing to compromised leukocyte recruitment (Ley & Kansas, 2004). Indeed P/E-selectin-deficient mice showed increased bacterial colonization of the oral cavity (Niederman *et al.*, 2001). Recognition of *P. gingivalis* microbe-associated molecular pathogens in calvarial tissue may also be compromised and there was downregulation of Toll-like receptors 1, 2, 4 (along with CD14 and LBP), and 6 and the downstream signaling molecule MyD88. In some strains of mice knockout of Toll-like receptor four contributes to alveolar bone loss following infection with *P. gingivalis* (Costalonga *et al.*, 2009). The significant increase in osteoclast numbers and full thickness calvarial bone resorption defects observed in response to *P. gingivalis* infection were similar to the effects that we observed in the calvaria of mice following local injections of IL-1 (Boyce *et al.*, 1989), as well as with other periodontal pathogens, *C. rectus* and *F. nucleatum* (Zubery *et al.*, 1998).

In conclusion, the present study reports findings from a comprehensive gene expression profile of inflamed soft tissue and calvarial bone that accompanied a localized, acute infection of mice with *P. gingivalis*. Importantly, many of the most highly affected genes in tissues are related to biological processes not historically emphasized within the literature related to periodontal disease initiation and progression (Kinane *et al.*, 2001; Tatakis & Kumar, 2005). It is likely that host responses and their contribution to protection or to tissue destruction are highly context dependent, and vary according to parameters such as dose of infecting organism, duration of infection, and genetic background. The substantive impact of *P. gingivalis* in downregulating host inflammatory/inmate immune responses in this model supports the

need for additional studies to explore the role of selected genes in the periodontal infections, including the effect of *P. gingivalis* on host response patterns as a component of the polymicrobial infection in periodontal disease.

ACKNOWLEDGEMENTS

We thank Drs Arnold Stromberg, Cidambaram Srinivasan, Christopher Saunders, and Matt Hersh (Department of Statistics, University of Kentucky, Lexington, KY) for initial data management and microarray data analysis. We also thank Dr Quey-Chen and Ms Donna Wall (University of Kentucky, Lexington, Microarray Facility) for microarray technical assistance. This study was supported by National Institute of Dental and Craniofacial Research DE015720 (L.K), and NIAMS AR43510 (B.F.B).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Leave-one-out cross validation.

Table S2. Comparison of expression of selected genes in calvarial tissue and calvaria by microarray and real-time quantitative polymerase chain reaction.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

REFERENCES

- Baker, P.J., DuFour, L., Dixon, M. and Roopenian, D.C. (2000) Adhesion molecule deficiencies increase *Porphyromonas gingivalis*-induced alveolar bone loss in mice. *Infect Immun* **68**: 3103–3107.
- Boyce, B.F., Aufdemorte, T.B., Garrett, I.R., Yates, A.J.P. and Mundy, G.R. (1989) Effects of interleukin-1 on bone turnover in normal mice. *Endocrinology* **125**: 1142–1150.
- Chang, H., Brown, C.W. and Matzuk, M.M. (2002) Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* **23**: 787–823.
- Chou, H.H., Yumoto, H., Davey, M. *et al.* (2005) *Porphyromonas gingivalis* fimbria-dependent activation of inflammatory genes in human aortic endothelial cells. *Infect Immun* **73**: 5367–5378.
- Collazo, C.M., Yap, G.S., Hieny, H. *et al.* (2002) The function of gamma interferon-inducible GTP-binding protein IGTP in host resistance to *Toxoplasma gondii* is Stat1 dependent and requires expression in both hematopoietic and nonhematopoietic cellular compartments. *Infect Immun* **70**: 6933–6939.
- Costalonga, M., Batas, L. and Reich, B.J. (2009) Effects of Toll-like receptor 4 on *Porphyromonas gingivalis*-induced bone loss in mice. *J Periodontol Res* **44**: 537–542.
- Demmer, R.T., Behle, J.H., Wolf, D.L. *et al.* (2008) Transcriptomes in healthy and diseased gingival tissues. *J Periodontol* **79**: 2112–2124.
- Dennis, G. Jr, Sherman, B.T., Hosack, D.A. *et al.* (2003) DAVID: database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* **4**: P3.
- Draghici, S., Khatri, P., Eklund, A.C. and Szallasi, Z. (2006) Reliability and reproducibility issues in DNA microarray measurements. *Trends Genet* **22**: 101–109.
- Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) 1998. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**: 14863–14868.
- Feezor, R.J., Oberholzer, C., Baker, H.V. *et al.* (2003) Molecular characterization of the acute inflammatory response to infections with gram-negative versus gram-positive bacteria. *Infect Immun* **71**: 5803–5813.
- Gemmell, E., Winning, T.A., Bird, P.S. and Seymour, G.J. (1998) Cytokine profiles of lesional and splenic T cells in *Porphyromonas gingivalis* infection in a murine model. *J Periodontol* **69**: 1131–1138.
- Gemmell, E., Drysdale, K.E. and Seymour, G.J. (2006) Gene expression in splenic CD4 and CD8 cells from BALB/c mice immunized with *Porphyromonas gingivalis*. *J Periodontol* **77**: 622–633.
- Han, X. and Amar, S. (2002) Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J Dent Res* **81**: 399–405.
- Handfield, M., Mans, J.J., Zheng, G. *et al.* (2005) Distinct transcriptional profiles characterize oral epithelium–microbiota interactions. *Cell Microbiol* **7**: 811–823.
- Handfield, M., Baker, H.V. and Lamont, R.J. (2008) Beyond good and evil in the oral cavity: insights into host–microbe relationships derived from transcriptional profiling of gingival cells. *J Dent Res* **87**: 203–223.

- Hasigawa, Y., Tribble, G.D., Baker, H.V., Mans, J.J., Handfield, M. and Lamont, R.J. (2008) Role of *Porphyromonas gingivalis* SerB in gingival epithelial cell cytoskeletal remodeling and cytokine production. *Infect Immun* **76**: 2420–2427.
- Holt, S.C., Kesavalu, L., Walker, S. and Genco, C.A. (1999) Virulence factors of *Porphyromonas gingivalis*. *Periodontol 2000* **20**: 168–238.
- Hosack, D.A., Dennis, G. Jr, Sherman, B.T., Lane, H.C. and Lempicki, R.A. (2003) Identifying biological themes within lists of genes with EASE. *Genome Biol* **4**: R70.
- Kasamatsu, A., Uzawa, K., Shimada, K. *et al.* (2005) Elevation of galectin-9 as an inflammatory response in the periodontal ligament cells exposed to *Porphyromonas gingivalis* lipopolysaccharide *in vitro* and *in vivo*. *Int J Biochem Cell Biol* **37**: 397–408.
- Kesavalu, L., Ebersole, J.L., Machen, R.L. and Holt, S.C. (1992) *Porphyromonas gingivalis* virulence in mice: induction of immunity to bacterial components. *Infect Immun* **60**: 1455–1464.
- Kesavalu, L., Chandrasekar, B. and Ebersole, J.L. (2002) *In vivo* induction of proinflammatory cytokines in mouse tissue by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* **17**: 177–180.
- Khatrri, P. and Draghici, S. (2005) Ontological analysis of gene expression data: current tools, limitations, and open problems. *Bioinformatics* **21**: 3857–3895.
- Kinane, D.F., Podmore, M., Murray, M.C., Hodge, P.J. and Ebersole, J.L. (2001) Etiopathogenesis of periodontitis in children and adolescents. *Periodontol 2000* **26**: 54–91.
- Lamont, R.J. and Jenkinson, H.F. (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* **62**: 1244–1263.
- Ley, K. and Kansas, G.S. (2004) Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol* **4**: 325–335.
- Li, B. (2008) Bone morphogenetic protein-Smad pathway as drug targets for osteoporosis and cancer therapy. *Endocr Metab Immune Disord Drug Targets* **8**: 208–219.
- Li, C. and Wong, W.H. (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA* **98**: 31–36.
- Lian, J.B., Stein, G.S., Javed, A. *et al.* (2006) Networks and hubs for the transcriptional control of osteoblastogenesis. *Rev Endocr Metab Disord* **7**: 1–16.
- Niederman, R., Westernoff, T., Lee, C. *et al.* (2001) Infection-mediated early-onset periodontal disease in P/E-selectin-deficient mice. *J Clin Periodontol* **28**: 569–575.
- Ohno, T., Okahashi, N., Kawai, S. *et al.* (2006) Proinflammatory gene expression in mouse ST2 cell line in response to infection by *Porphyromonas gingivalis*. *Microbes Infect* **8**: 1025–1034.
- Orozco, A., Gemmell, E., Bickel, M. and Seymour, G.J. (2007) Interleukin 18 and periodontal disease. *J Dent Res* **86**: 586–593.
- Oshikawa, M., Sugano, N., Koshi, R., Ikeda, K. and Ito, K. (2004) Differential gene induction in macrophage-like human cells by two types of *Porphyromonas gingivalis*: a microarray study. *J Oral Sci* **46**: 9–14.
- Piana, C., Wirth, M., Gerbes, S., Viernstein, H., Gabor, F. and Toegel, S. (2008) Validation of reference genes for qPCR studies on caco-2 cell differentiation. *Eur J Pharm Biopharm* **69**: 1187–1192.
- Sasaki, H., Okamatsu, Y., Kawai, T., Kent, R., Taubman, M. and Stashenko, P. (2004) The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss. *J Periodontol Res* **39**: 432–441.
- Smith, A., Stanley, P., Jones, K., Svensson, L., McDowall, A. and Hogg, N. (2007) The role of the integrin LFA-1 in T-lymphocyte migration. *Immunol Rev* **218**: 135–146.
- Tatakis, D.N. and Kumar, P.S. (2005) Etiology and pathogenesis of periodontal diseases. *Dent Clin North Am* **49**: 491–516.
- Tsuchida, K., Nakatani, M., Uezumi, A., Murakami, T. and Cui, X. (2008) Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer. *Endocr J* **55**: 11–21.
- Wada, T., Nakashima, T., Hiroshi, N. and Penninger, J.M. (2006) RANKL–RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* **12**: 17–25.
- Wang, P.L., Ohura, K., Fujii, T. *et al.* (2003) DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* **305**: 970–973.
- Zhou, Q. and Amar, S. (2006) 2006. Identification of proteins differentially expressed in human monocytes exposed to *Porphyromonas gingivalis* and its purified components by high-throughput immunoblotting. *Infect Immun* **74**: 1204–1214.
- Zubery, Y., Dunstan, C.R., Story, B.M. *et al.* (1998) Bone resorption caused by three periodontal pathogens *in vivo* in mice is mediated in part by prostaglandin. *Infect Immun* **66**: 4158–4162.

Copyright of Molecular Oral Microbiology is the property of Wiley-Blackwell and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.