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Molecular characterization of *Treponema denticola* infection-induced bone and soft tissue transcriptional profiles

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SUMMARY

***Treponema denticola* is associated with subgingival biofilms in adult periodontitis and with acute necrotizing ulcerative gingivitis. However, the molecular mechanisms by which *T. denticola* impacts periodontal inflammation and alveolar bone resorption remain unclear. Here, we examined changes in the host transcriptional profiles during a *T. denticola* infection using a murine calvarial model of inflammation and bone resorption. *T. denticola* was injected into the subcutaneous soft tissue over the calvaria of BALB/c mice for 3 days, after which the soft tissues and the calvarial bones were excised. RNA was isolated and analysed for transcript profiling using Murine GeneChip® arrays. Following *T. denticola* infection, 2905 and 1234 genes in the infected calvarial bones and soft tissues, respectively, were differentially expressed ($P \leq 0.05$). Biological pathways significantly impacted by *T. denticola* infection in calvarial bone and calvarial tissue included leukocyte transendothelial migration, cell adhesion (immune system) molecules, cell cycle, extracellular matrix–receptor interaction, focal adhesion, B-cell receptor signaling and transforming growth factor-β signaling pathways resulting in proinflammatory, chemotactic effects, and T-cell stimulation. In conclusion, localized *T. denticola* infection differentially induces transcription of a broad array of host genes, the profiles of which differed between inflamed calvarial bone and soft tissues.**

INTRODUCTION

Periodontitis is an immune-inflammatory chronic disease initiated by complex subgingival biofilm and resulting in irreversible destruction of alveolar bone and connective tissue attachment in the periodontium. *Treponema denticola*, a principal oral helical-shaped anaerobic spirochete, has been routinely isolated from human subgingival plaque and is

strongly associated with changes in the subgingival ecology characteristic of disease sites (Sela, 2001; Ellen & Galimanas, 2005; Holt & Ebersole, 2005). Interestingly, the proportion of *T. denticola* increases significantly in periodontal disease biofilms and is typically detected together with other pathogens *Porphyromonas gingivalis* and *Tannerella forsythia* (Socransky & Haffajee, 2005). Furthermore, *T. denticola* has been linked with endodontic infections, orofacial abscesses and periapical radiolucencies (Baumgartner *et al.*, 2003; Siqueira & Rocas, 2004). *T. denticola* as a dominant member of pathogenic biofilms at sites of periodontal disease may contribute to the disease processes by elaborating components that can mediate adherence to mucosal surfaces, enable penetration into epithelial cells, affect host systems through specific cleavage of cell surface receptors, inhibit host defense mechanisms, elicit gingival tissue inflammation, and induce alveolar bone resorption. For example, chymotrypsin-like protease, phospholipase C, oligopeptidase, endopeptidase and cystalysin are defined factors with possible or confirmed roles in pathogenicity (Fenno & McBride, 1998; Chi *et al.*, 2003; Choi *et al.*, 2003; Ellen & Galimanas, 2005). The continuous induction of proinflammatory cytokines by host cells triggered by periopathogens is thought to be responsible for the destruction of tooth-supporting tissues and alveolar bone in diseased periodontal sites compared with healthy or inactive sites (Stashenko *et al.*, 1991; Ejeil *et al.*, 2003). Moreover, a recent study documented dissemination of *T. denticola* to the spleen, heart and brain following dental pulp infection in mice (Foschi *et al.*, 2006). *In vitro* studies have shown that components of *T. denticola* can induce a range of proinflammatory cytokines, including interleukin 1α (IL-1α), IL-1β, tumor necrosis factor-α (TNF-α), IL-6 and IL-8, (Nixon *et al.*, 2000) which are potential molecular effectors of connective tissue destruction and resorption of alveolar bone in periodontitis (Gemmell & Seymour, 1998). Conversely, *in vitro* studies have also shown that dentilisin, a major surface protease and virulence factor of *T. denticola*, can reduce proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, (Miyamoto *et al.*, 2006). However, the *in vivo* role of these inflammatory molecules, as well as the broader aspects of the host response to *T. denticola* in the periodontium, remain to be elucidated. Nonetheless, the capacity of *T. denticola* to disrupt the normal activities of several immune response participants is well documented.

Microarray analysis of the transcriptional host responses following exposure to bacterial and viral pathogens has become a powerful approach to improve understanding of the molecular basis of the host response to infections. Host response characterization has identified gene transcripts such as proinflammatory and anti-inflammatory responses uniquely affected by pathogens such as *Streptococcus pneumonie* and *Pseudomonas aeruginosa* (Joyce *et al.*, 2009; McConnell *et al.*, 2010). 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), 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.*, 2005). Microarray antibody analyses showed that *T. denticola* lipo-oligosaccharide induced changes in the phosphorylation state and/or expression of gingival fibroblast intracellular signaling proteins, including Fos (Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor), MKK1 (mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) protein-serine kinase 1), MKK2 (MAPK/ERK protein-serine kinase 2), MKK3/6 (MAP kinase protein-serine kinase 3/6), nuclear factor-*k*B p50 (nuclear transcription factor), and nuclear factor-*k*B p65 (Tanabe *et al.*, 2008). The aim of the present study was to profile transcript abundance in the calvarial bone and overlying soft tissues to identify genes whose expression differed in response to localized *T. denticola* infection in mice using the calvarial model of inflammation and bone resorption. We performed a genome-wide transcriptional analysis of the calvarial bone and overlying soft tissues isolated from *T. denticola*-infected and mock-infected mice. Microarray data analysis identified several hundred probe sets and subsequently numerous pathways which were significantly changed in *T. denticola*-infected mice.

METHODS

Mice

BALB/c female mice 8–10 weeks of age (Harlan, Indianapolis, IN) were routinely acclimatized for at least 1 week before use. Mice were infected with *T. denticola* ATCC 35404 as described below following isoflurane inhalation anesthesia. All mouse infection procedures were performed in accordance with the approved guidelines set forth by the Institutional Animal Care and Use Committee at the University of Kentucky (Lexington, KY).

Bacteria and mouse infection

The *T. denticola* ATCC 35404 was cultured and maintained for the animal infections, which were within 15–30 min of bacterial preparation, as described previously (Kesavalu *et al.*, 1997, 1999). *T. denticola* was injected at 1.5×10^9 cells ($n = 10$ mice) into the soft tissues overlying the calvaria of the mice. Bacteria (suspended in 10 μl of reduced transport fluid) were injected into the subcutaneous tissue over the right side of the parietal bone and anterior to the lambdoid suture once daily for 3 days using a Hamilton syringe (Hamilton Co., Reno, NV). An uninfected control group ($n = 10$ mice) was injected with reduced transport fluid once daily for 3 days. Mice were sacrificed 8 h after the last injection by CO_2 asphyxiation and cervical dislocation. The calvarial bone 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 paraffin embedded for histological assessment of calvarial bone resorption, as described before (Zubery *et al.*, 1998).

RNA isolation and mouse GeneChip hybridization

Total RNA was isolated from the frozen calvarial soft tissue and calvarial bone from each mouse (*T. denticola*-infected and control mice, four or five mice per group) with Trizol reagent (Invitrogen, Carlsbad, CA) (Meka *et al.*, 2010). RNA yield was quantified 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 using an Agilent bioanalyser (Agilent Technologies, Santa Clara, CA). 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 μg total RNA using SuperScript reverse transcriptase enzyme (Invitrogen) and oligo-$(dT)_{24}$ primer containing a T7 RNA polymerase promoter. All biological samples were processed individually and samples were not pooled. The biotin-labelled complementary RNA was then synthesized by *in vitro* transcription, fragmented and hybridized to the 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 data set 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 data sets. 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 was normalized to 1 on a probe-set-wise basis. The variance-normalized data

were then subjected to hierarchal 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 data set was divided into tissue and treatment classes. Differences between the various treatment tissue classes were determined using a modified *f*-test with a random variance model and a significance threshold of $P \leq 0.05$. To assess the ability of probe sets significant at $P \leq 0.05$ to distinguish between the treatment classes, 'leave-one-out-cross-validation' (LOOCV) 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; Hasegawa *et al.*, 2008). In cases where the misclassification rate was above zero, the significance of the classifier's performance were assessed using Monte Carlo simulations with 2000 random permutations of the data set. The performance of the classifier was considered significant at the $P \leq 0.05$ threshold (Hasegawa *et al.*, 2008).

The geometric means of each class calculated by BRB Array tools was used to determine the fold change of significantly impacted genes of *T. denticola*-infected bone or tissue compared with uninfected (control) samples. 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; Hasegawa *et al.*, 2007; Meka *et al.*, 2010), available at http://vortex.cs.wayne.edu/projects.htm.

Quantitative real-time reverse transcription–polymerase chain reaction analysis

Selected genes that showed significant differential expression in microarray analysis, was confirmed by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) analysis (LightCycler FastStart DNA Master SYBR Green I, Roche, Indianapolis, IN). Thirteen upregulated genes were selected based on a broad overview of different functional categories such as extracellular matrix, cell adhesion, bone remodeling, defense and immune response, response to stress, and cytokine/chemokine responses from both calvarial soft tissues and bone. These genes were: defensin-β (*Defb3*), small proline rich 2 (*Sprr2i*), chemokine (CC motif) receptor-like 2 (*Ccrl2*), prostaglandin-endoperoxidase synthase 2 (*Ptgs2*), metallothionein 4 (*Mt4*), calvarial bone integrin binding sialoprotein (*Ibsp*), matrix metalloproteinase 13 (*Mmp13*), WNT1-inducible signaling protein (*Wisp1*), cathepsin K (*Ctsk*), acid phosphatase 5 (*Acp5*), tissue inhibitor of proteinase 1 (*Timp1*), matrilin-2 (*Matn2*) and matrix metalloproteinase 9 (*Mmp9*). Total RNA (250 ng) from pooled control and *T. denticola* infection samples was reverse transcribed to complementary DNA (cDNA) using the transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions as previously described (Meka *et al.*, 2010). Using the relative quantification application in the LightCycler analysis software (Roche Diagnostics, Indianapolis, IN), the fold changes of the different gene expressions were calculated based on the expression of the target gene versus the expression of the house-keeping gene, *ActB* (β-actin), of the same sample. Mouse *ActB* gene was used as an internal control based on its constant level of expression across groups in cDNA microarray analysis (Piana *et al.*, 2008). Standard curves were generated for each gene. The melting curve profiles were examined to verify a single peak for each sample, indicating primer specificity, and the transcript copy number was calculated. RNA extracts were prepared in duplicate from independent experiments, and cDNA samples were loaded in triplicate.

Calvarial bone histology

The mouse calvariae were fixed in 10% phosphate-buffered formalin and decalcified in 14% ethylenediaminetetraacetic acid (Zubery *et al.*, 1998). The anterior half of mouse 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. Calvarial 5-μm thin sections were stained with hematoxylin and eosin. Histomorphometric analysis of osteoclasts was carried out on two sections from the calvaria of 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 bone resorption.

Immunoglobulin G antibody analysis

Treponema denticola infected and sham-infected control mice sera was used to determine immunoglobulin G (IgG) antibody titers to *T. denticola* using a standard enzyme-linked immunosorbent assay protocol as previously described (Kesavalu *et al.*, 1999; Meka *et al.*, 2010), and provided an additional marker of infection. The optical density was measured at 405 nm (OD_{405}) using a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA). The infected mice serum antibody concentration was quantified using a gravimetric standard curve. The standard curve consisted of purified mice IgG standard (Sigma, St Louis, MO), which were coated onto wells of the microtiter polystyrene plates, detected and developed as described above (Meka *et al.*, 2010). Antigen-coated wells in the absence of serum provided a background negative control. Mean serum IgG antibody concentrations to whole cells of *T. denticola* 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 separate experiments were combined and results were expressed as means ± SD.

Microarray data accession numbers

The array results have been deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession numbers GSE17110, 19855.

RESULTS

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

The mouse gene chip MOE430A contains 22,690 probe sets, with 17,322 and 16,757 probe sets ($P < 0.001$) providing positive readable signals in soft tissue and calvarial bone, respectively, to *T. denticola* infection. Significant differences were observed in mean gene expression levels of 2905 and 1234 probe sets in bone and soft tissue in response to infections, respectively. Of the significantly regulated genes, approximately two-thirds were upregulated (1946 and 810) and the remaining one-third were downregulated (959 and 424) for both soft tissue and calvarial bone samples. The results of this initial gene profile analysis demonstrate that *T. denticola* infection stimulated greater changes in the transcriptome of bone compared with soft tissue. The majority of genes with altered expression in calvarial bone with *T. denticola* were primarily associated with basic cellular functions for maintaining integrity of the tissues.

The ability of probe sets significant at $P \leq 0.05$ to correctly identify differences between treatment groups was confirmed by LOOCV analysis (Tables S1 and S2). The classifiers correctly predicted the treatment group with 60–89% accuracy. The significance of the LOOCV analysis was determined by Monte Carlo simulations with 2000 random permutations of the data set. These significantly regulated probe sets were analysed using the PATHWAY EXPRESS tool, which builds a list of all associated pathways. PATHWAY EXPRESS uses several algorithms to determine the significance of each pathway compared with that exported by chance alone, as well as the downstream impact of measured gene expression changes for each pathway, expressed as impact factor (Khatri & Draghici, 2005; Hasegawa *et al.*, 2007; Meka *et al.*, 2010). Pathways significantly impacted by *T. denticola* at the $P < 0.05$ level in bone and soft tissue types included: Cell Adhesion (immune system comprising antigen-presenting cells, T cells, B cells) Molecules (Fig. 1), Leukocyte Transendothelial Migration (actin cytoskeleton, leukocyte) (Figs 2 and 3), and Cell Cycle (MAPK signaling pathway and apoptosis) (Figs 4 and 5). Table 1 shows soft-tissue and calvarial bone pathways

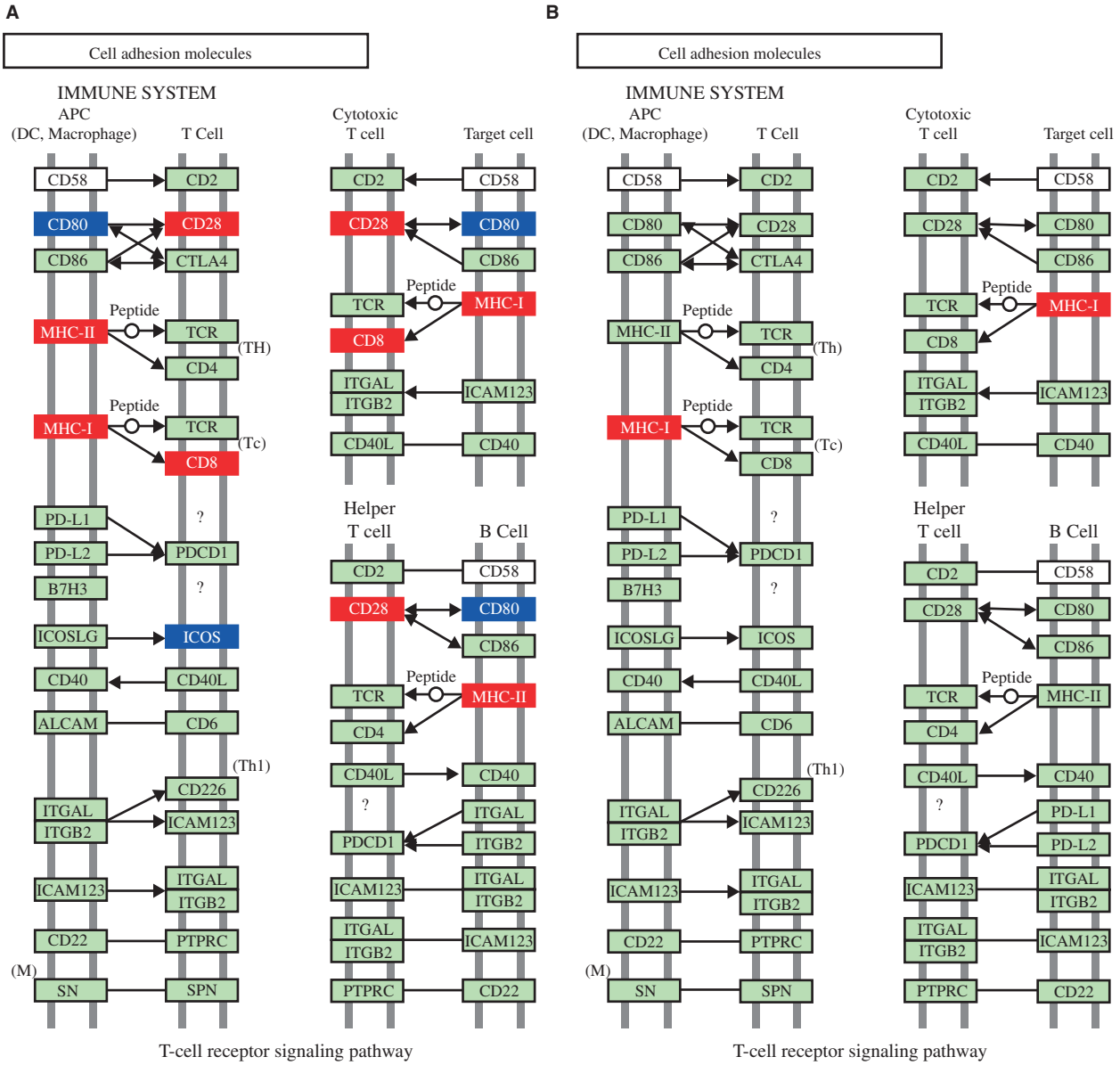

Figure 1 Cell adhesion (immune system) molecules pathway containing *Treponema denticola* differentially regulated genes in calvarial bone (A) and tissue (B) compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation of dendritic cells, macrophages, T cells (T-cell receptor signaling pathway), and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

generated in this analysis that were predominantly affected in order of their impact factors (Khatri & Draghici, 2005; Hasegawa *et al.*, 2007; Meka *et al.*, 2010). *T. denticola* significantly impacted seven pathways in bone and four in tissue with an impact factor > 5. The high impact factors associated with these pathways predict that the effects of *T. denticola*-induced gene expression changes in the calvarial bone or soft tissue will have a significant biological effect downstream. Cell adhesion molecules, Leukocyte transendothelial migration pathways and Cell cycle pathways were significantly impacted or overlapped in both the calvarial bone and soft tissue (Table 1); for this reason, they were chosen for further study to compare and contrast the impact of *T. denticola* on two distinct tissue types.

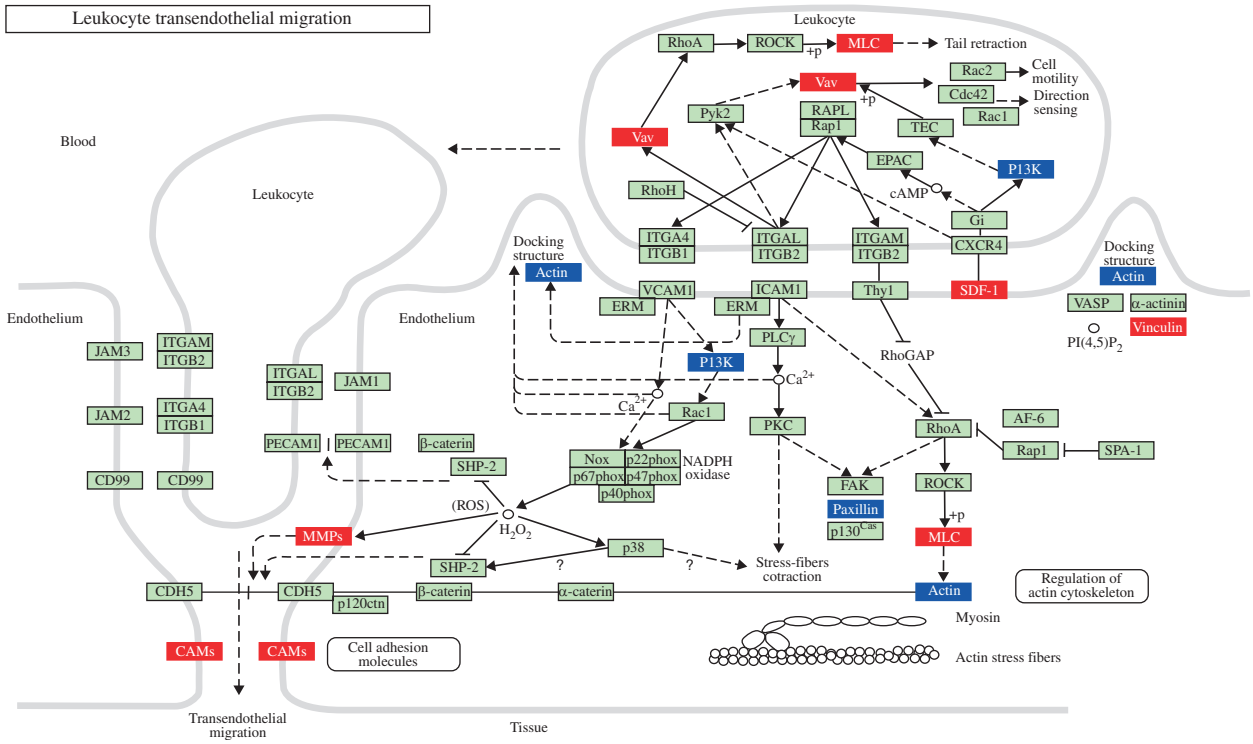

Figure 2 Leukocyte transendothelial migration pathway containing *Treponema denticola* differentially regulated genes in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in transendothelial migration, leukocyte activation, regulation of actin cytoskeleton and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

Inflammatory and immune response gene expression profiles

As studies of infection with periodontal pathogens have emphasized the chronic inflammatory nature of the challenge leading to localized tissue destruction, we also focused on examination of gene profiles related to inflammatory and immune responses. The proinflammatory cytokines IL-1β (2.5-fold increase), IL-6 (2.4-fold increase), TNF (2.4-fold increase), and other cytokines such as IL-7, IL-15, IL-17, IL-18, IL-23 involved in induction of inflammation and alveolar bone resorption were modestly upregulated in soft tissue and calvarial bone following *T. denticola* infection. However, we found that the expression of defensin beta 3 (*defb3*), *Il1b*, *Il7* (Interleukin-7), C1q and TNF 1 (*C1qtnf1*), chitinase 3-like 3 (*Chi3l3*), chemokine (C-C motif) receptor 2 (*Ccr2*), leukotriene B (*Ltb*), S100 calcium binding protein A11 (*S100a11*), and inflammatory/cytokine/chemokine genes in soft tissue and calvarial bone were modestly upregulated, with the exception of *defb3*, which was robustly induced at 15-fold to *T. denticola* infection. Again, we observed changes in anti-inflammatory cytokines: IL-1RA (0.68-fold decrease), IL-4 (1.9-fold increase), TGFβ (2.5-fold increase) in both soft tissue and calvarial bone. There was moderate expression of Th1 (IFN-γ:1.5-fold increase) and Th2 cytokine messenger RNA expression (IL-4, 1.9-fold increase). Matrix metalloproteinases 13, 9, 14 and 23 (*Mmp13*, *Mmp9*, *Mmp14*, *Mmp23*) and Cathepsin K gene were upregulated in calvarial bone. Probe sets representing tissue inhibitor of metalloproteinase (TIMP1, TIMP2 and TIMP3) demonstrated a modest upregulation during *T. denticola* infection.

Calvarial histology

The sham-infected control mice showed neither edema nor minimal inflammation in the soft tissue

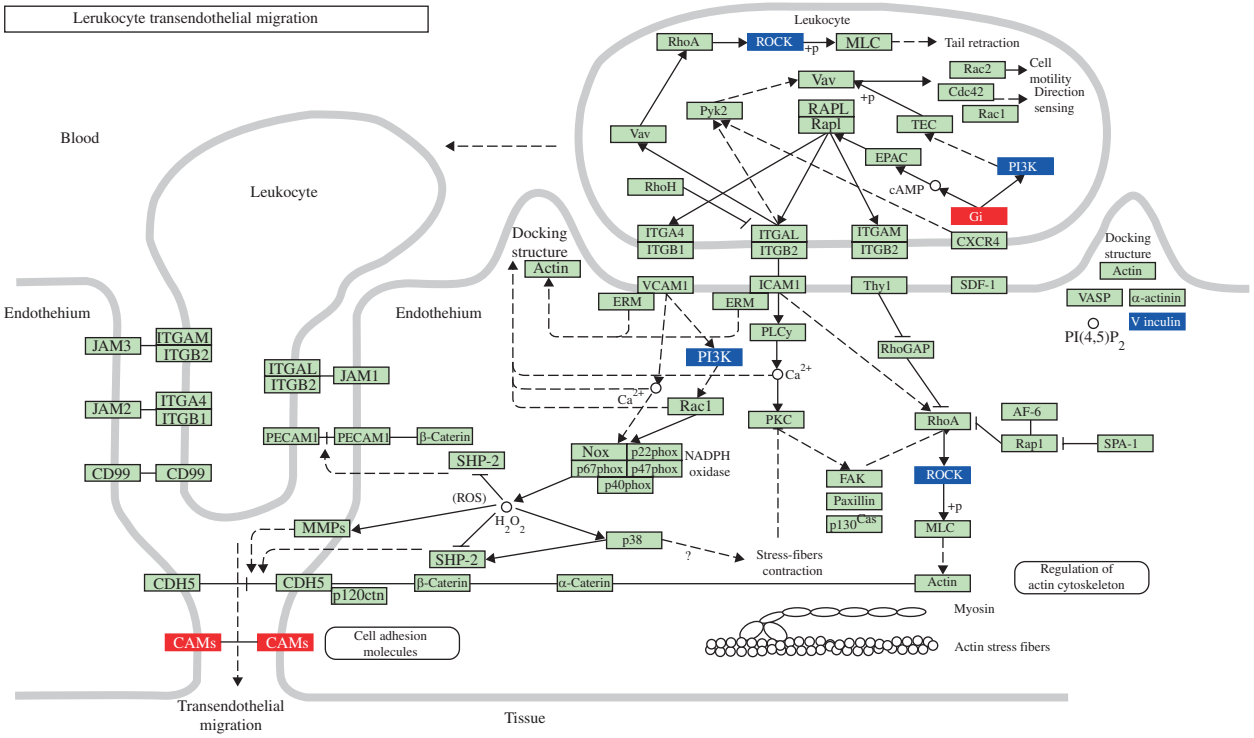

Figure 3 Leukocyte transendothelial migration pathway containing *Treponema denticola* differentially regulated genes in calvarial soft tissue compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in transendothelial migration, leukocyte activation, regulation of actin cytoskeleton and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

over the calvaria (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 *T. denticola*, but not in the sham-infected control mice. The *T. denticola*-infected mice did not develop local abscesses, ulceration of the overlying skin, or any evidence of infection of neighboring sites. Histological examination revealed edema and an intense mixed inflammatory cell infiltrate of variable intensity in the calvarial tissues, and this large infiltrate consisted of polymorphonuclear leukocytes, lymphocytes and macrophages (Fig. 6B). In addition, significantly increased numbers of osteoclasts were seen inside the calvaria compared with sham-infected 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). Activated osteoclasts were also present in some mice on the upper periosteal surface, consistent with more bone resorption.

Validation of microarray gene expression

The *T. denticola*-induced transcript expression results from the microarray studies were confirmed by qRT-PCR for selected genes, including *Defb3*, *Sprr2i*, *Ccrl2*, *Ptgs2*, *Mt4* in soft tissue and *Ibsp*, *Mmp13*, *Wisp1*, *Ctsk*, *Acp5*, *Timp1*, *Matn2*, *Mmp9* in calvarial bone using aliquots of the pooled RNA samples that were evaluated in the microarrays (Table S3). Transcripts of β-actin were used as an expression control and the qRT-PCR analyses were performed at least twice for each gene. The qRT-PCR results confirmed the microarray data and the selected upregulated genes in microarray showed corresponding increased expressions with qRT-PCR analysis, although expression levels differed between the two techniques.

Immune response in calvarial infection

To provide additional documentation of calvarial infection and to demonstrate an immunological

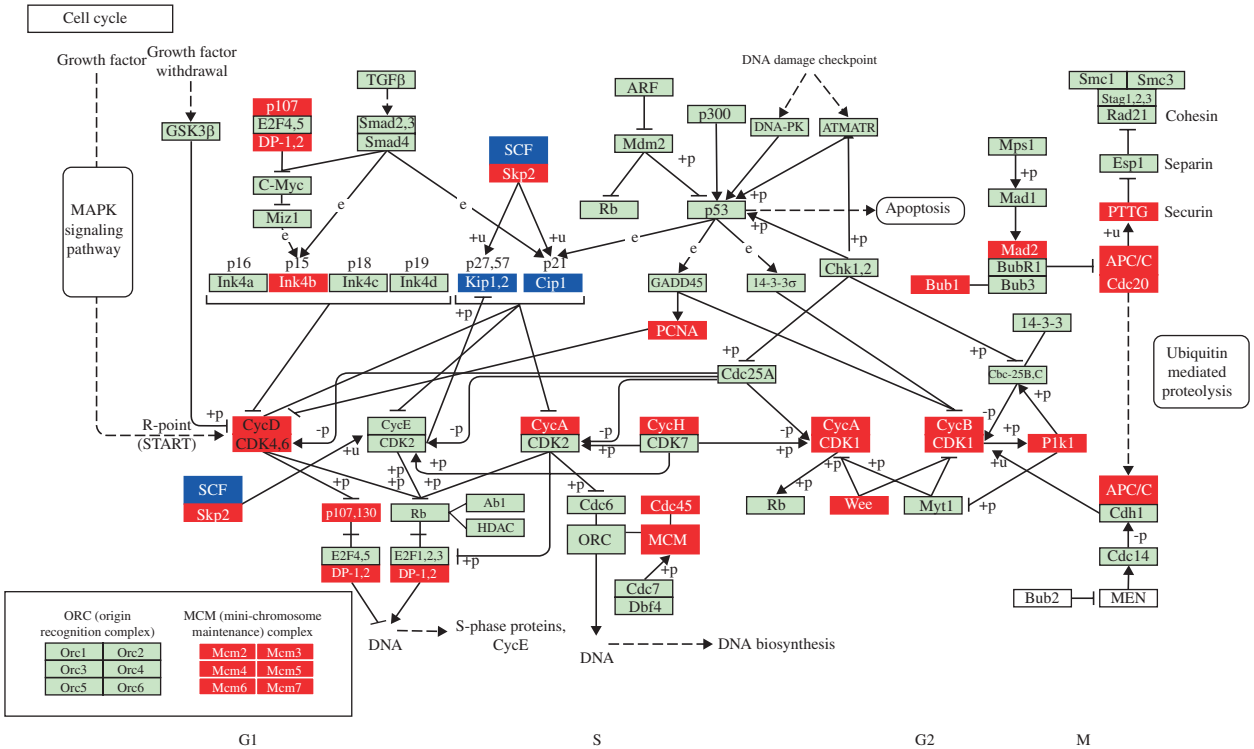

Figure 4 Cell cycle pathway containing *Treponema denticola* differentially regulated genes in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation of mitogen-activated protein kinase (MAPK) signaling pathway, transforming growth factor-β (TGF-β) cytokine, apoptosis, ubiquitin-mediated proteolysis and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

response to *T. denticola*, we evaluated the levels of anti-*T. denticola* whole-cell-specific IgG antibody in mice sera collected at the end of the 3 days of acute infection. As expected, *T. denticola* subcutaneous infection for 3 days did not induce *T. denticola*-specific serum IgG antibody responses.

DISCUSSION

We used a cDNA microarray to study the gene transcriptional profiles of host soft tissue and calvarial bone during a localized acute infectious challenge with the oral pathogen *T. denticola* in BALB/c mice. Diverse functional classes of genes were altered by *T. denticola*, suggesting that numerous cellular processes were transcriptionally modulated during the course of the infection. In the murine calvarial bone resorption model, microarray gene expression analysis revealed activation of 810 genes and repression of 424 genes in inflamed soft tissue in response to infection with *T. denticola*, compared with 1946 and 959 genes that were up- and downregulated in the underlying calvarial bone, respectively. In addition, the number of host genes upregulated by *T. denticola* over the course of infection in both bone and soft tissue was twice the number downregulated, suggesting that induction of gene expression is the predominant response to the infection. The breadth of functional categories and gene families that were altered in response to the localized infection in calvarial bone included Cell Proliferation, Cell Cycle, Transport, Transcription, Defense and Immune Response, Cell Adhesion, Extracellular Matrix, and Stress, indicating that *T. denticola* elicits a multitude of specific gene expression changes during infection. In soft tissue, functional categories and gene families that were altered in response to the localized infection included Cell Cycle, Cell Proliferation, Transcription Factors, Transport, Defense and Immune Response, and Extracellular Matrix.

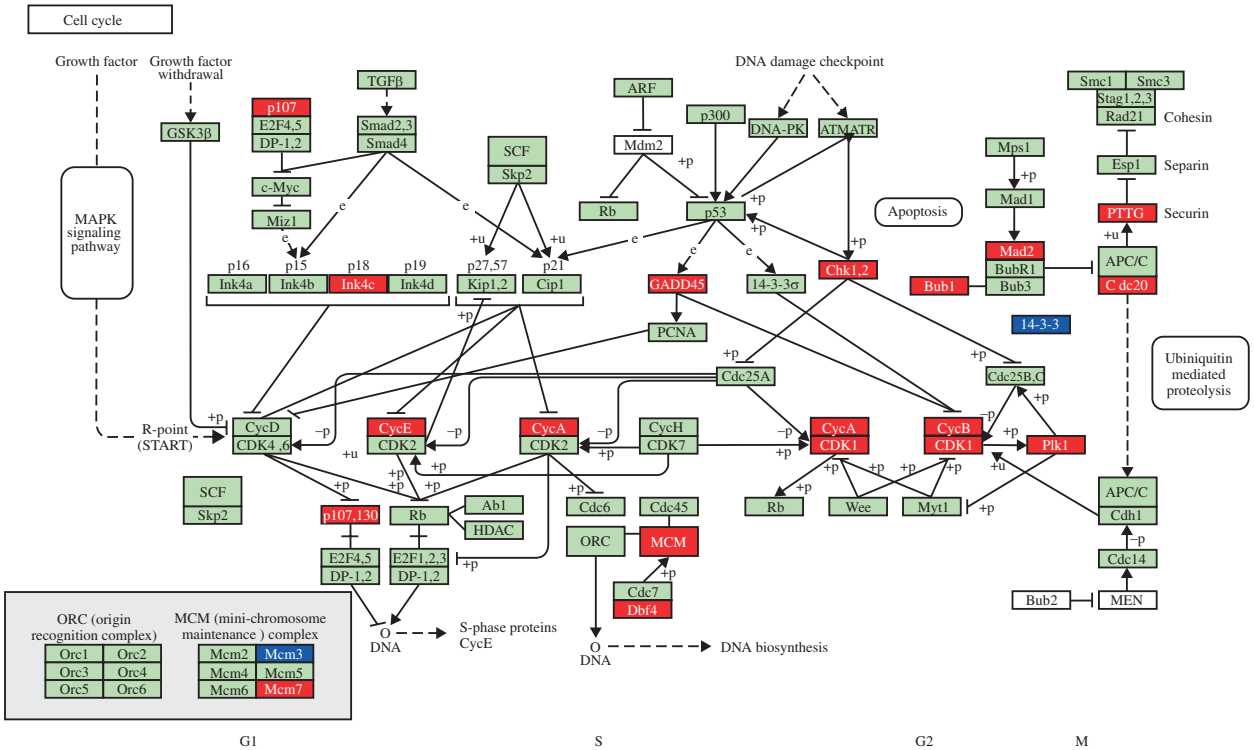

Figure 5 Cell Cycle pathway containing *Treponema denticola* differentially regulated genes in calvarial soft tissue compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation of mitogen-activated protein kinase (MAPK) signaling pathway, transforming growth factor-β (TGF-β) cytokine, apoptosis, ubiquitin-mediated proteolysis and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

We expected that *T. denticola* would induce a robust inflammatory gene expression profile, which is a fundamental host defense/protective mechanism coordinated and controlled by cytokines, chemokines and oxidative burst effectors (Eskra *et al.*, 2003). We found that proinflammatory cytokines IL-1β, IL-6, TNF, and other cytokines such as IL-7, IL-15, IL-17, IL-18 and IL-23 involved in the induction of inflammation and calvarial bone resorption were modestly upregulated in soft tissue and calvarial bone following *T. denticola* infection. We also found expression of *Il1b*, *Il7*, *C1qtnf1*, *Chi3l3*, *Ccr2*, *Itgb2*, *Itgam*, *Cxcl7*, *Ltb*, *S100a11* and *Cxcl12* inflammatory/cytokine/chemokine genes in soft tissue and calvarial bone was modestly upregulated.

Several of the highly upregulated genes have not been reported in the periodontal literature, or specifically in response to *T. denticola* infection. The level of upregulation of the *Defb3* gene (15-fold) in inflamed soft tissue was striking. Beta-defensins are believed to contribute to the host defense system by controlling pathogens at the mucosal surface. As beta-defensins are predominantly expressed at epithelial surfaces, and their expression is inducible in response to inflammatory stimuli against both gram-positive and gram-negative bacteria, these molecules are thought to be an important component of the innate immune system (Maxwell *et al.*, 2003). Our finding that *Defb3* is highly upregulated during *T. denticola* infection in our model corroborates this hypothesis, because these antimicrobial peptides were induced to overcome a gram-negative bacterial infection.

The small proline-rich (Sprr) proteins are the primary constituents of the epidermal cornified envelope, which is the major barrier against the environment. These proteins are expressed in all squamous tissues of the skin, scalp and most of the epithelial lining of the proximal digestive tract including oral epithelium (Tesfaigzi & Carlson, 1999). High levels of *Sprr* genes are detected in various diseases (inflammatory dermatoses), cancers of the skin,

Table 1 Ontology analysis of calvarial bone and tissue gene pathways impacted by infection with *Treponema denticola*[1]

Impacted pathway[2]	Impact factor[3]	No. of input genes/no. of pathway genes[4]
Calvarial bone		
Leukocyte transendothelial migration	306.541	14/119
Cell adhesion molecules	180.322	20/159
ECM–receptor interaction	17.812	32/81
Focal adhesion	12.734	56/199
DNA replication	10.797	16/36
Cell cycle	8.546	33/124
TGF-beta signaling pathway	5.617	21/93
Calvarial tissue		
Cell adhesion molecules	307.089	3/159
Leukocyte transendothelial migration	220.561	5/119
Phosphatidylinositol signaling system	8.488	4/75
Cell cycle	6.798	17/124

[1]The calvarial tissue and bone gene pathways were determined by PATHWAY EXPRESS (Hasegawa *et al.*, 2007; Khatri & Draghici, 2005).
[2]Kyoto Encyclopedia of Genes and Genome Pathways (http://www.genome.jp/kegg/).
[3]The impact factor measures the pathways most affected by changes in gene expression in calvarial bone and tissue to *T. denticola* by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these perturbations throughout the pathway (Hasegawa *et al.*, 2007; Meka *et al.*, 2010). Only pathways with an impact factor > 5 are included in this table.
[4]Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.
ECM, extracellular matrix; TGF, transforming growth factor.

human papillomavirus infection, and these genes are upregulated under stress (De Heller-Milev *et al.*, 2000). A recent study identified SPRR1A as a novel stress-inducible downstream mediator of gp130 cytokines in cardiomyocytes and documented its cardioprotective effect against ischemic stress (Pradervand *et al.*, 2004). The upregulation of *Sprr2i* (4.8), *Sprr2h* (4.7), *Sprr2f* (2.3), *Sprr2a* (6.0), and *Sprr2d* (12.8), *Sprrl9* (3.2) (2- to 13-fold) expression in soft tissue indicates that they constitute an important, previously unrecognized, response to *T. denticola* infection and these gene products may be a major means by which the host controls *T. denticola*.

T. denticola infection-induced robust transcriptional changes in extracellular matrix (ECM) proteins expressed in calvarial bone and soft tissue. For example, matrix metalloproteinases 13, 9, 14 and 23

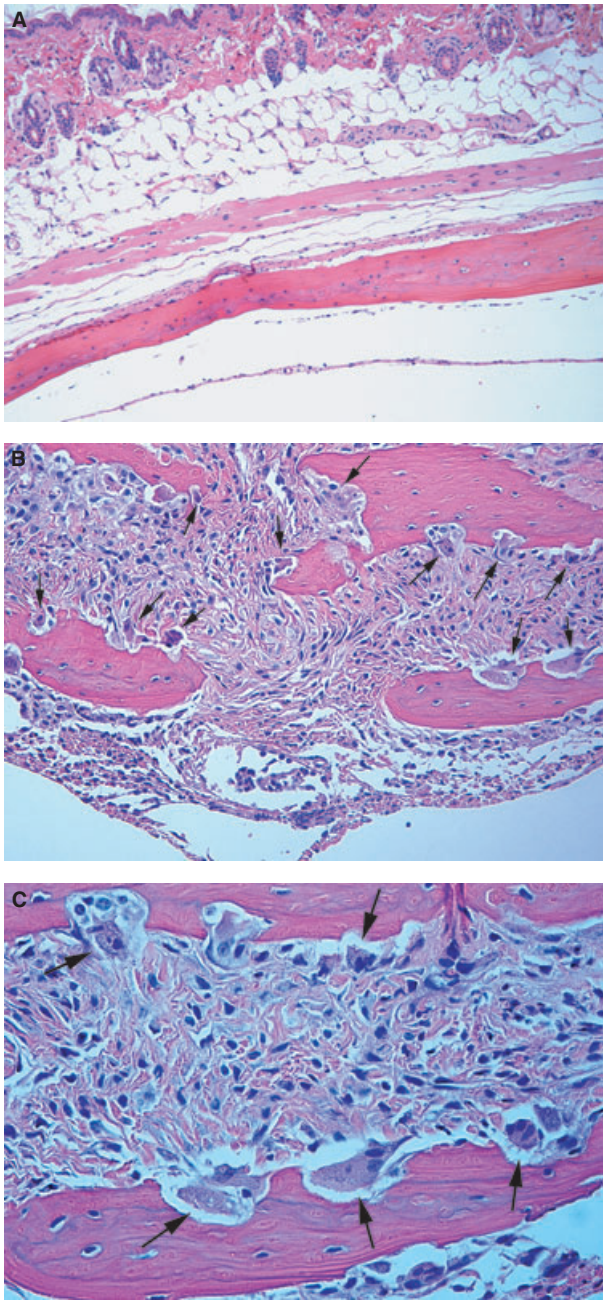

Figure 6 Effects of *Treponema denticola* local injection on mouse calvaria. Live *T. denticola* bacteria (1.5×10^9) were injected once daily for 3 days into the subcutaneous tissues overlying the calvaria of mice. All photomicrographs of slides stained with hematoxylin and eosin. (A) No edema and minimal inflammation present in the soft tissue over the calvaria of the sham-infected control mouse (10 ×). (B) Section from *T. denticola* infected mouse reveals disruption of the suture area with intense mixed inflammatory infiltrate, edema, fibrosis and vascularity. Numerous osteoclasts (black arrows) are seen throughout the bone surfaces. (20 ×). (C) Activated osteoclasts at higher magnification (40 ×).

genes (*Mmp13*, *Mmp9*, *Mmp14*, *Mmp23*) were upregulated in calvarial bone, while tissue inhibitor of metalloproteinase (TIMP1, TIMP2 and TIMP3) was modestly upregulated during *T. denticola* infection. TIMPs form irreversible complexes with activated metalloproteinase, and the balance between these proteins controls extracellular matrix remodeling. TIMPs may also serve as an early indicator of the acute-phase response. Furthermore, several of the ECM proteins, chondroadherin, procollagen types I, III, V, fibronectin 1, fibromodulin, osteomodulin and biglycan, were elevated during *T. denticola* infection. The changes we found in ECM protein transcriptional expression levels are in agreement with those induced robustly by *Escherichia coli* lipopolysaccharide inoculation in a canine model of oral infection (Higgins *et al.*, 2003). Matrilin-2 (*Matn2*) is the largest member of the matrilin family of extracellular proteins, which have roles in the development and homeostasis of cartilage and bone. It is a secreted protein with calcium ion binding capabilities and is involved in matrix assembly. *Matn2* messenger RNA expression was increased two-fold in calvarial bone during *T. denticola* infection, but the significance of this change is unclear.

The composition of the ECM is known to impact multiple cellular activities including differentiation, proliferation and motility. Periostin (gene *Postn*; increased five- and two-fold in bone and soft tissue, respectively), also known as osteoblast-specific factor 2, is a protein secreted into the ECM and is widely expressed in various tissues. It binds to heparin, contributing to the induction of cell attachment and to the spreading of a variety of cell types, and also playing a role in cell adhesion. Periostin is highly expressed in the embryonic periosteum, periodontal ligaments, cardiac valves and placenta among other tissues. Moreover, *Postn* null mice exhibit incisor enamel defects and an early-onset periodontal disease-like phenotype, suggesting that periostin is critically required for maintenance of the periodontal ligament (Rios *et al.*, 2005, 2008). Changes in periostin (ECM protein) expression in bone and tissue in our model probably reflect its participation in the tissue remodeling of damaged calvarial bone and tissue after acute *T. denticola* infection.

Metallothionein (*Mt4*), a component of the acute-phase response and a reactive-oxygen scavenger was downregulated more than four-fold in calvarial bone and soft tissue during *T. denticola* infection. It appears to play a role in regulating zinc metabolism during the differentiation of epithelia and has been shown to sequester reactive oxygen species and reduce tissue damage in *Helicobacter pylori*-induced gastritis in mice (Tran *et al.*, 2007). Downregulation of the cysteine-rich metallothionein may therefore contribute to tissue damage during acute *T. denticola* infection.

Examination of the highly downregulated genes in inflamed soft tissues following local infection with *T. denticola* demonstrated a commonality regarding genes related to inflammatory/acute-phase responses (e.g. *Lypla3*, *Agt*). Lysophospholipase 3 (*LyPla3*) is an enzyme that removes lysophospholipids from cell membranes, and is present in many tissues but its precise role is largely unknown. Lysophospholipids are critical to cell survival and function and are associated with several diseases such as hyperlipidemia, inflammation and myocardial ischemia (Wang & Dennis, 1999). Angiotensinogen (*AGT*) has been the protein most consistently associated with hypertension and blood pressure regulation. Ethanolamine kinase 1 (*Etnk1*) is required for a pathway of phosphatidylethanolamine synthesis and is overexpressed in almost all cancer cells. The significance and linkage of these downregulated genes to *T. denticola* infection is unknown.

A major difference between the bone and tissue samples was in the impact on the cell adhesion molecules (CAM) pathway. In bone, *T. denticola* upregulated several components of the immune system pathway including major histocompatibility complex class I (MHCI) and MHCII, which processes endocytosed antigens and leads to expansion of CD8, CD23 (T cell, cytotoxic T cell, T helper–B cell interacting molecules), and downregulation of CD30. By contrast, in tissue only the MHCI was upregulated without the interaction of T-cell and B-cell molecules. Another major difference between the bone and tissue samples was in the impact on the leukocyte transendothelial migration pathway including activation of Vav (signal transducer protein), which subsequently activates myosin light chain phosphorylation, resulting in the retraction (downregulation) of the actin cytoskeleton. Moreover, leukocytes activate endothelial cell signals, with production of reactive oxygen species (ROS) in a Rac-mediated manner with subsequent activation of MMPs and CAMs. In contrast, in tissue,

only CAMs were activated, and downregulation of several components of the leukocyte transendothelial migration pathway such as Rho-associated kinase, phosphatidylinositol 3-kinase and Vinculin indicates decreased interaction of *T. denticola* in tissue. The cell cycle pathway was also strongly impacted in both bone and tissue following *T. denticola* infection. In bone, the MAPK signaling pathway leads to activation of cyclin D (CycD), CycA, CycB, CycH, key regulating enzyme cyclin-dependent kinases 4,6 (CDK), proliferating cell nuclear antigen, and cell cycle-regulating transcription factors DP-1,2 as well as downstream targets of CDKs include transcription factor E2F. The CDK inhibitors, such as p15Ink4b, p27Kip1 and p21Cip1, are involved in the negative regulation of CDK activities in cell cycle pathway and were impacted by *T. denticola* infection.

In conclusion, the present study provides findings from a comprehensive gene expression profile of murine inflamed soft tissue and calvarial bone that accompanies a localized, acute infection with *T. denticola*. Importantly, many of the most affected genes were related to biological processes not historically emphasized within the literature related to periodontal disease initiation and progression (Kinane *et al.*, 2001). Further studies are required to identify specific *T. denticola* virulence factors (dentilisin, lipo-oligosaccharide) that alter host gene expression and measurement of the host transcriptional response against a pathogenic strain compared with mutant strains lacking specific virulence factors. Furthermore, the substantive impact of *T. denticola* in downregulation of host inflammatory/innate immune responses in this model supports the need for additional studies to explore the role of selected genes in the periodontal disease process, as well as the effect of *T. denticola* on host response patterns as a component of a polybacterial infection in periodontal disease.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Table S1. Leave-one-out cross validation (calvarial bone).

Table S2. Leave-one-out cross validation (calvarial soft tissue).

Table S3. Comparison of expression of selected genes in calvarial tissue and calvariae by microarray and real-time quantitative polymerase chain reaction.

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