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Polymicrobial periodontal pathogen transcriptomes in calvarial bone and soft tissue

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SUMMARY

Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* are consistently associated with adult periodontitis. This study sought to document the host transcriptome to a *P. gingivalis*, *T. denticola*, and *T. forsythia* challenge as a polymicrobial infection using a murine calvarial model of acute inflammation and bone resorption. Mice were infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* over the calvaria, after which the soft tissues and calvarial bones were excised. A Murine GeneChip® array analysis of transcript profiles showed that 6997 genes were differentially expressed in calvarial bones ($P < 0.05$) and 1544 genes were differentially transcribed in the inflamed tissues after the polymicrobial infection. Of these genes, 4476 and 1035 genes in the infected bone and tissues were differentially expressed by upregulation. Biological pathways significantly impacted by the polymicrobial infection in calvarial bone included leukocyte transendothelial migration (LTM), cell adhesion molecules, adherens junction, major histocompatibility complex antigen, extracellular matrix–receptor interaction, and antigen processing and presentation resulting in inflammatory/cytokine/chemokine transcripts stimulation in bone and soft tissue. Intense inflammation and increased activated osteoclasts were observed in calvarias compared with sham-infected controls. Quantitative real-time RT-PCR analysis confirmed that the mRNA level of selected genes corresponded with the microarray expression. The polymicrobial infection regulated several LTM and extracellular membrane pathway genes in a manner distinct from mono-infection with *P. gingivalis*, *T. denticola*, or *T. forsythia*. To our knowledge, this is the first definition of the polymicrobially induced transcriptome in calvari-

al bone and soft tissue in response to periodontal pathogens.

INTRODUCTION

Polymicrobial infections induce distinct pathogenic characteristics compared with mono-infection with a single pathogen (Brogden, 2002; Brogden *et al.*, 2005). The predominant polymicrobial infection of humans is expressed clinically as periodontal disease, a complex, immuno-inflammatory condition, with multiple bacterial species responsible for triggering the destructive host responses. A predominant consortium identified in most adult periodontitis patients consists of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (red complex pathogens) (Socransky *et al.*, 1998; Socransky & Haffajee, 2005). With modern metagenomic microbiome technologies, it is clear that periodontopathic biofilms comprise a wide array of bacteria, many uncultivable and so minimally studied (Chen *et al.*, 2010). Nevertheless, this consortium is strongly associated with disease lesions and is nearly always co-isolated or identified in biofilm samples from adult periodontitis lesions. Additionally, these pathogens elaborate numerous virulence determinants that may mediate adherence to mucosal surfaces (Lamont & Jenkinson, 1998; Holt *et al.*, 1999), enable penetration into gingival epithelial cells (Lamont *et al.*, 1995; Tribble & Lamont, 2010), inhibit host defense mechanisms (Holt & Ebersole, 2005), create a more anaerobic environment, and elicit synergistic gingival/periodontal tissue destruction and alveolar bone resorption (Kesavalu *et al.*, 2007; Hajishengallis, 2009). We have reported that *P. gingivalis*, *T. denticola*, and *T. forsythia* not only exist as a pathogenic consortium in periodontitis lesions, but also exhibit synergistic virulence resulting in immuno-inflammatory alveolar bone resorption in rodent models (Kesavalu *et al.*, 2007). Our recent mouse microarray studies have determined the *in vivo* transcriptional profiles of host soft tissue and calvarial bone following acute infection with *P. gingivalis* (Meka *et al.*, 2010), *T. denticola* (Bakthavatchalu *et al.*, 2010a), and *T. forsythia* (Bakthavatchalu *et al.*, 2010b) in a calvarial model of inflammation and bone resorption. A recent gingival transcriptome study in human patients demonstrated that the microbial content of the periodontal pocket is a determinant of gene expression in the adjacent gingival tissues, and provided new insights into the differential ability of periodontal species to elicit a local host response (Papapanou *et al.*, 2009). In addition, a gene ontology analysis of healthy and diseased gingival tissues from patients with advanced periodontitis identified 61 differentially expressed pathways of genes, including those regulating apoptosis, antimicrobial immune response, and antigen presentation (Demmer *et al.*, 2008). However, global host gene expression in response to defined polymicrobial infection *in vivo* remains to be defined.

This investigation is to determine the transcriptome profiles to *P. gingivalis*, *T. denticola*, and *T. forsythia* as a polymicrobial infection. To better understand the distinct local gene expression profiles, we performed a genome-wide transcriptional analysis of the calvarial bone and overlying soft tissues. The analysis focused on altered biological gene pathways that were significantly changed during the polybacterial infection, which were compared with those found after mono-infection with *P. gingivalis*, *T. denticola* or *T. forsythia*, yielding distinct results.

METHODS

Mice

Female BALB/c mice 8–10 weeks of age (Harlan, Indianapolis, IN) were routinely acclimatized before polymicrobial infection. BALB/c mice were infected with bacteria as described below following isoflurane inhalation anesthesia. Polymicrobial infection and sham-infection procedures for mice were performed in accordance with the approved guidelines of the Institutional Animal Care and Use Committee at the University of Kentucky (Lexington, KY).

Bacterial strains and growth conditions

Porphyromonas gingivalis FDC 381, *T. denticola* ATCC 35404, and *T. forsythia* ATCC 43037 were grown under anaerobic conditions at 37°C as previously described (Bakthavatchalu *et al.*, 2010a,b; Meka *et al.*, 2010).

Polymicrobial inocula and mouse infection

We prepared the polymicrobial inoculum based on our previously published report (Kesavalu *et al.*, 2007), and consistent with the detection of these species in subgingival biofilm in adult periodontitis, synergism in biofilm formation between *P. gingivalis* and *T. denticola* (Kuramitsu *et al.*, 2005), and specific molecular interactions between *T. denticola* and *T. forsythia* (Nishihara & Koseki, 2004). For polymicrobial infection, members of the *P. gingivalis* + *T. denticola* + *T. forsythia* consortium were individually prepared as described previously (Bakthavatchalu *et al.*, 2010a,b; Meka *et al.*, 2010). Briefly, *P. gingivalis* was gently mixed in a vortex for 1–2 min with an equal volume of *T. denticola*, and allowed to interact for 5 min. Subsequently, *T. forsythia* was added to the *P. gingivalis* + *T. denticola* tubes, mixed gently for 1–2 min, and allowed to interact for an additional 5 min to allow interactions among these species. Bacterial culture, growth phase, viability, enumeration, interaction times, suspension medium, and infection procedures were all standardized as described elsewhere (Kesavalu *et al.*, 2007). The *P. gingivalis*, *T. denticola*, and *T. forsythia* suspended in reduced transport fluid were injected at 1.5×10^9 into the soft tissues overlying the calvariae of the mice ($n = 10$ mice; Zubery *et al.*, 1998; Meka *et al.*, 2010; Bakthavatchalu *et al.*, 2010a,b). Polymicrobial cultures (5×10^8 cells of each) were injected (suspended in 30 μl reduced transport fluid) into the soft tissues overlying the calvariae of the mice (Bakthavatchalu *et al.*, 2010a,b; Meka *et al.*, 2010). The sham-infected control group ($n = 10$ mice) was injected with 30 μl reduced transport fluid once daily for 3 days. Bacterial infection, mouse euthanasia, and collection and preparation of calvarial bone and soft tissue for histology were performed as previously described (Bakthavatchalu *et al.*, 2010a,b; Meka *et al.*, 2010).

Total RNA isolation and mouse GeneChip hybridization

RNA was isolated from the calvarial soft tissue and calvaria bone from each mouse [polymicrobial (*P. gingivalis/T. denticola/T. forsythia*) infected and control mice, $n = 5$ in each group] with Trizol reagent (Invitrogen, Carlsbad, CA) (Meka *et al.*, 2010). Quantification of RNA yield was carried out by spectrophotometric analysis and the absorbance at 260 and 280 nm was checked to determine the RNA purity and concentration. For GeneChip analyses, individual RNA samples were further purified with Qiagen RNeasy columns (Qiagen, Valencia, CA). All calvarial tissue and bone samples were processed individually and RNA samples were not pooled. The biotin-labeled complementary RNA was synthesized by *in vitro* transcription, fragmented and hybridized on a mouse GeneChip MG-MOE430A (Affymetrix, Santa Clara, CA), following the protocol described in the GeneChip Expression Analysis Technical manual (Affymetrix). After hybridization, the GeneChip arrays were stained and scanned in an Affymetrix GCS 3000 7G Scanner as previously described (Bakthavatchalu *et al.*, 2010a; Meka *et al.*, 2010).

Murine microarray data analysis

The polymicrobial murine microarray raw data were normalized, evaluation of the dataset was performed by both unsupervised and supervised analyses and hierarchal clustering analysis was carried out, following which, differences between the various treatment tissue classes and determination of fold-change of significantly impacted genes were determined as described previously (Dennis *et al.*, 2003; Draghici *et al.*, 2003; Feezor *et al.*, 2003; Bakthavatchalu *et al.*, 2010a; Meka *et al.*, 2010). PATHWAY EXPRESS was used to compile a list of impacted pathways and to populate Kyoto Encyclopedia of Genes & Genomes (KEGG) pathways according to the gene regulation uncovered by microarray. Pathways were prioritized and further investigated (see http://vortex.cs.wayne.edu/faq.htm).

Real-time reverse transcription-polymerase chain reaction analysis

Expression of selected genes, that showed significant differential expression to infection compared to controls in the microarray analyses, was confirmed by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) analysis (LightCycler FastStart DNA Master SYBR Green I, Roche, Indianapolis, IN) as described previously (Bakthavatchalu *et al.*, 2010a; Meka *et al.*, 2010). Six representative upregulated genes based on a broad overview of the different functional categories such as extracellular

matrix, cell adhesion, cell proliferation, immune and defense responses, transport, and other categories from both polymicrobial challenged soft tissues and calvarial bone were evaluated. These genes were: defensin B (*Defb3*), small proline rich (*Sprr2d*), and matrix metalloproteinases 13 (*Mmp13*) from calvarial tissue and chemokine (C-X-C Motif) ligand 7 (*Cxcl7*), matrix metalloproteinases 9 (*Mmp9*), and peptidoglycan recognition protein 1 (*Pglyrp1*) from calvarial bone.

Calvarial bone histology

The mouse polymicrobial infected and sham-infected calvariae were fixed in 10% neutral phosphate-buffered formalin, decalcified, embedded, sectioned, stained, and analyzed for osteoclasts as described previously for mono-infection with *P. gingivalis* (Meka *et al.*, 2010), *T. denticola* (Bakthavatchalu *et al.*, 2010a), and *T. forsythia* (Bakthavatchalu *et al.*, 2010b).

Statistical analysis

Polymicrobial transcriptome murine microarray data were analyzed as described above (Bakthavatchalu *et al.*, 2010a, 2010b; Meka *et al.*, 2010). *P*-values of 0.05 or less were considered significant. The qRT-PCR data from two independent experiments were combined and results were presented 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, GSE 29670.

RESULTS

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

To analyze host global gene transcription following the polymicrobial infection, the mouse gene chip MOE430A containing 22,690 probe sets, with 17,809 and 17,908 probe sets provided positive readable signals in soft tissue and bone, respectively, to polymicrobial infection in comparison to sham-infected control. Significant differences were observed in mean gene expression levels of 6997 and 1544 probes sets in bone and soft tissue in response to polymicrobial infection ($P < 0.05$), respectively, in comparison to sham-infected control. Of the significantly regulated genes, 4476 probe sets were upregulated and 2521 genes were downregulated in calvarial bone. In contrast, 1035 genes were upregulated and 459 were downregulated in calvarial soft tissue samples following the polymicrobial infection compared with control tissues. The results of this gene expression analysis indicate that polymicrobial infection stimulated greater changes in the transcriptome of upregulated and downregulated genes in calvarial bone compared with the overlying inflamed soft tissues. The majority of genes with altered expression in calvarial bone to polymicrobial infection were primarily associated with basic cellular functions [transcription, cell proliferation, cell cycle, cell adhesion, extracellular matrix (ECM), transport, apoptosis] for maintaining tissue integrity.

The significantly altered probe sets following a polybacterial challenge were analyzed by the PATHWAY EXPRESS tool as previously described (Draghici *et al.*, 2007, Khatri *et al.*, 2007). Probe sets significant at $P \leq 0.05$ to differentiate between the polymicrobial infection and sham-infected groups were confirmed by LOOCV analysis (see Supplementary material, Tables S1 and S2). Pathways significantly impacted by *P. gingivalis*, *T. denticola*, and *T. forsythia* at the $P \leq 0.05$ level in calvarial bone and soft tissue types included: Leukocyte Transendothelial Migration (LTM) (Figs 1 and 2), Cell Adhesion Molecules (CAM) (see Supplementary material, Fig. S1), Adherens Junction (AJ) (see Supplementary material, Fig. S2), major histocompatibility complex (MHC) Antigen I and II Processing and Presentation (APP) (see Supplementary material, Fig. S3), and ECM–receptor interaction (Fig. 3). Table 1 shows calvarial bone and soft tissue pathways generated from this analysis that were predominantly affected in order of their impact factors. The *P. gingivalis*, *T. denticola*, and *T. forsythia* significantly impacted 23 pathways in calvarial bone and eight in soft tissue with an impact factor >5. The high impact factors associated with these pathways predict that the effects of *P. gingivalis*, *T. denticola*, and *T. forsythia*-induced gene expression changes in the bone or tissue should have a significant biological effect downstream. There are more than five pathways including LTM, CAM,

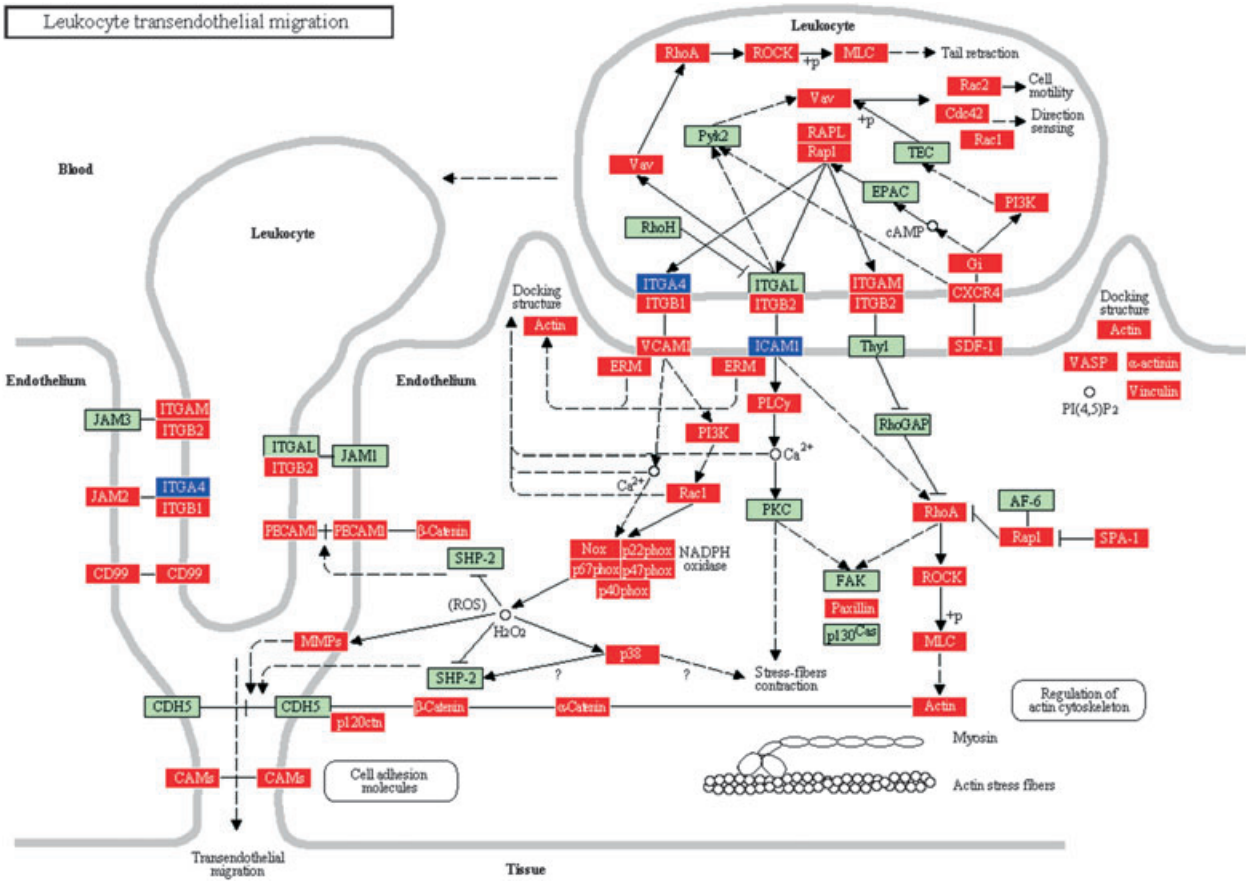

Figure 1 Leukocyte transendothelial migration (LTM) pathway containing genes differentially regulated by polymicrobial infection with *Porphyromonas gingivalis* + *Treponema denticola* + *Tannerella forsythia* in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Genes shown in red are upregulated, genes shown in blue are downregulated, and green indicates no change in gene expression at the $P < 0.05$ significance level. +p = phosphorylation event; −p = dephosphorylation event; ? = receptors that are yet to be identified; and O = other molecule. 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.

AJ, Phosphatidylinositol Signaling system and ribosome that were significantly impacted and overlapped in both the calvarial bone and soft tissue (Table 1).

We have selected LTM and ECM-interaction pathways to examine how *P. gingivalis* (Meka *et al.*, 2010) or *T. denticola* (Bakthavatchalu *et al.*, 2010a) or *T. forsythia* mono-infection (Bakthavatchalu *et al.*, 2010b) impacted regulation of pathway genes compared with a polybacterial infection in bone and soft tissues. The *T. denticola* mono-infection impacted (impact factor: 306) the LTM pathway to a greater extent than mono-infection with *T. forsythia* or polymicrobial infection in calvarial bone and soft tissue. Polymicrobial infection regulated more LTM pathway genes (49/119) in bone than mono-infection with *P. gingivalis* or *T. denticola* or *T. forsythia*. *Porphyromonas gingivalis* regulated more of the LTM pathway (39/115) genes in soft tissue than the polymicrobial infection-induced genes (Table 2). Three LTM pathway genes [claudin 1, chemokine (C-X-C motif) ligand 12, Mmp 2] were regulated following both mono-infection and the polymicrobial infection in bone and a few LTM pathway genes (CD99 antigen, integrin beta 1, junctional adhesion molecule 2, vav 1 oncogene) were uniquely regulated only following the polymicrobial infection in bone (Table 3) and not in tissue (see Supplementary material, Table S3).

Similarly, the *T. forsythia* mono-infection impacted the ECM receptor-interaction pathway (impact factor: 23) more than *T. denticola* and the polymicrobial infection in bone, whereas the polymicrobial infection regulated more ECM receptor-interaction pathway

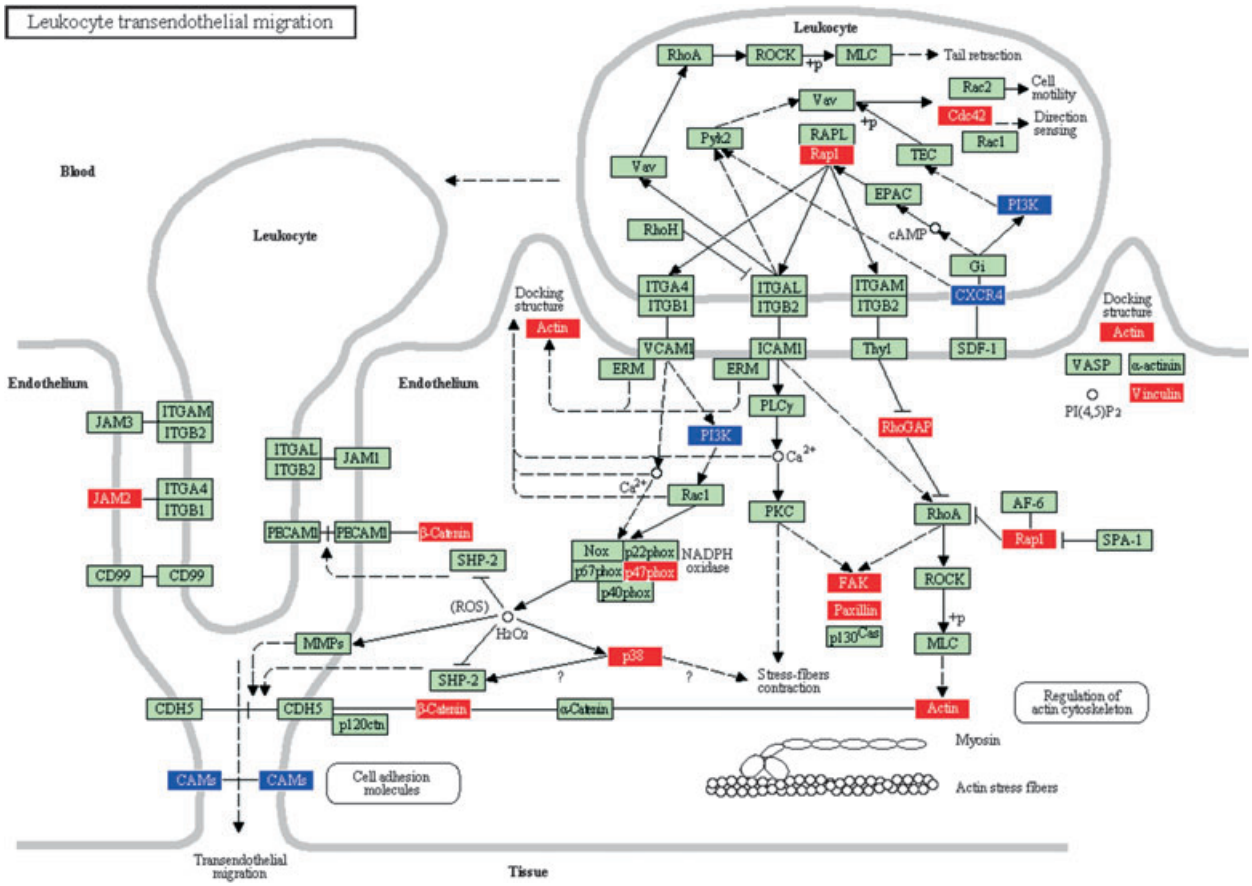

Figure 2 Leukocyte transendothelial migration (LTM) pathway containing genes differentially regulated by polymicrobial infection with *Porphyromonas gingivalis* + *Treponema denticola* + *Tannerella forsythia* in calvarial soft tissue compared with sham-infected controls at $P \leq 0.05$. An arrow indicates 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.

genes (45/81) in bone than a *T. denticola* mono-infection and the *P. gingivalis* infection did not significantly impact ECM genes in calvarial bone. In contrast, neither the mono-infection nor polymicrobial infection significantly impacted ECM receptor-interaction pathway genes in soft tissue (Table 4). Five ECM pathway genes (collagen type XI alpha 1, integrin alpha V, syndecan 1, thrombospondin 1, tenascin C) were robustly regulated following both mono-infection and polymicrobial infection in bone and a few ECM pathway genes (CD36 antigen, integrin beta 1, integrin beta 5, laminin gamma 1, syndecan 4) were uniquely regulated only after the polymicrobial infection in bone (Table 5) and not in tissue (see Supplementary material, Table S4). The significant ECM protein gene transcription of *Mmp9* (16-fold), laminin B1 subunit 1 *(Lamb1-1*; four-fold), *Mmp13* (five-fold), *Mmp14* (four-fold), *Mmp8* (three-fold), *Mmp3* (five-fold), *Mmp2* (three-fold), *Mmp23* (two-fold), *Ctgf* (connective tissue growth factor: four-fold), *Fmod* (fibromodulin: four-fold), *Fn1* (fibronectin 1: five-fold), *Matn2* (matrilin 2: two-fold), *Ecm1* (four-fold), *Timp1*, (seven-fold) *Timp2*, (two-fold) and *Timp3* (two-fold) were altered in calvaria.

Inflammatory and immune response gene expression profiles

Studies of infection with periodontal pathogens have examined acute and chronic inflammatory responses to microbial challenge leading to localized tissue destruction. Thus, we also focused on examination of gene profiles related to inflammatory and immune responses to the polymicrobial infection. Several inflammation/cytokine/chemokine transcripts including *Cxcl7* (12-fold), *Cxcl12* (four-fold), *Ccl4* (three-fold),

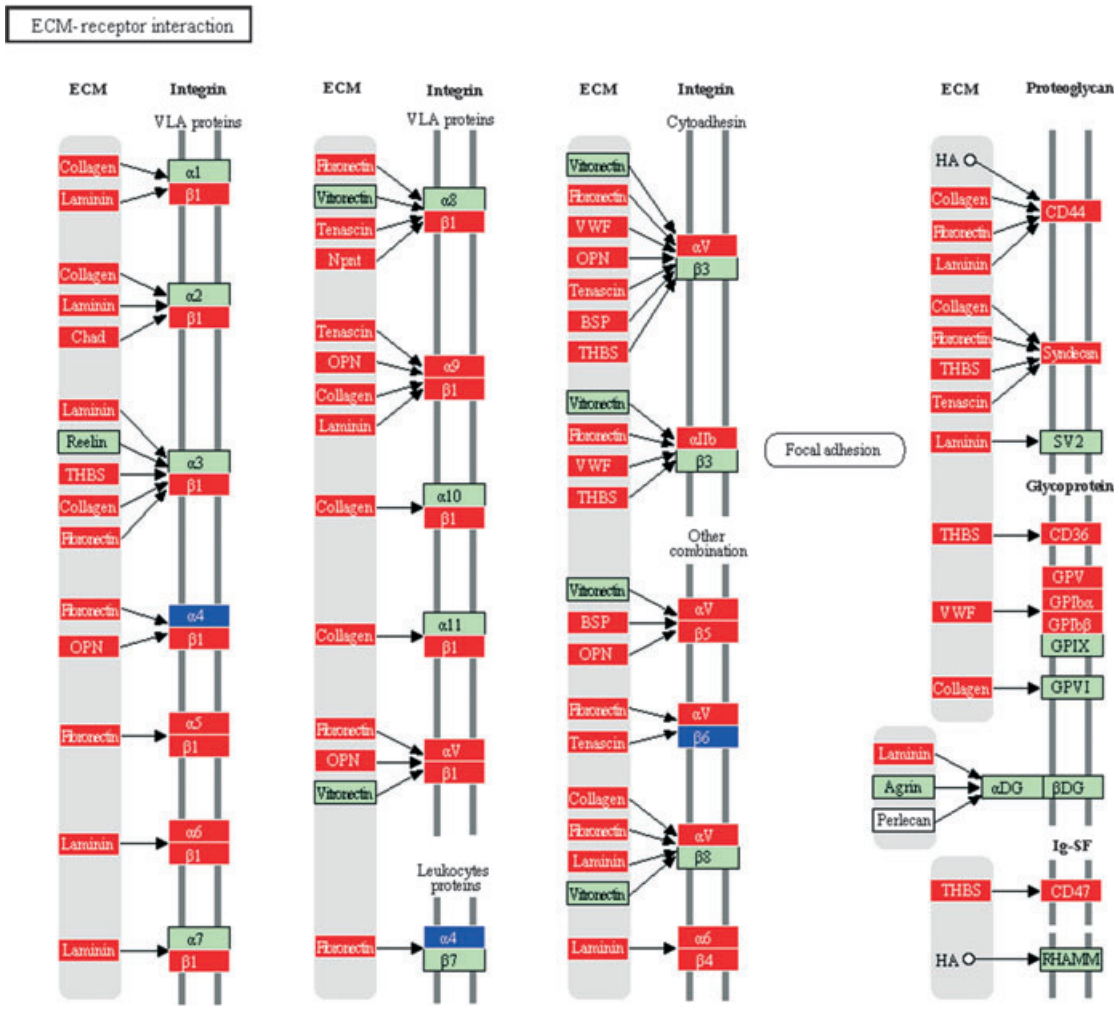

Figure 3 Extracellular membrane (ECM) –receptor interaction pathway containing genes differentially regulated by polymicrobial infection with *Porphyromonas gingivalis* + *Treponema denticola* + *Tannerella forsythia* in calvarial bone compared with sham-infected controls at $P \leq 0.05$. An arrow indicates a molecular interaction resulting in ECM–receptor activation, regulation of integrin (VLA proteins, leukoproteins, cytoadhesin, focal adhesion, proteoglycan, glycoprotein).

Ccl9, *Ccl12*, *Ccl17*, *Ccl20*, interleukin-1 receptor (Ilr2: five-fold), tumor necrosis factor receptor, lymphotoxin B, chitinase 3-Like 3, and neutrophil cytosolic factor were significantly altered in calvarial bone. The significantly upregulated immune and defense response genes included *Orm1* (orosomucoid 1: seven-fold), *Hp* (Haptoglobulin: eight-fold), *C1qtnf1* (C1q And Tumor Necrosis Factor Related Protein 1: six-fold), *Bst1* (Bone Marrow Stromal Cell Antigen 1: seven-fold), *Saa1* (Serum Amyloid A 1: two-fold), *Ifi30* (Interferon Gamma Inducible Protein 30: two-fold), and *Ifitm3* (Interferon Induced Transmembrane Protein 3: eight-fold). Other altered genes that increased expression were associated with cell cycle and proliferation [Ribonucleotide Reductase M2 (*Rrm2*), eight-fold; cyclin B (*Ccnb2*), 11-fold; Transforming Growth Factor, Beta 1 (*Tgfb1*), three-fold], transport [Lactotransferrin (*Ltf*), 12-fold; Solute Carrier Family 6 (*Slc6*) five-fold; Ceruloplasmin (*Cp*), seven-fold], cell adhesion [CEA-Related Cell Adhesion Molecule (*Cecam1*), 10-fold; CD44 Antigen (*Cd44*) eight-fold; Laminin B1 Subunit 1 (*Lamb1-1*) four-fold], apoptosis [Peptidoglycan Recognition Protein 1 (*Pglyrp1*) 14-fold], bone remodeling and ossification [Integrin Binding Sialoprotein (*Ibsp*) 22-fold; Secreted Phosphoprotein 1 (*Spp1*) six-fold], and other category genes [acid phosphatase 5 tartrate resistant (*Acp5*) 11-fold; immunoglobulin superfamily member 6 (*Igsf6*) eight-fold].

Table 1 Ontology analysis of calvarial bone and soft tissue pathways impacted by polymicrobial infection with *Porphyromonas gingivalis* + *Treponema denticola* + *Tannerella forsythia*[1]

Impacted pathway[2]	Impact Factor[3]	Input genes/no. of pathway genes[4]	*P* value
Calvarial bone			
Leukocyte transendothelial migration	55.967	49/119	0.000E0
Cell adhesion molecules	45.448	49/159	0.000E0
Adherens junction	31.999	37/77	3.510E-13
Antigen processing and presentation	17.26	37/100	5.829E-7
Phosphatidylinositol signaling system	15.717	28/75	2.497E-6
Ribosome	13.463	55/115	2.058E-5
DNA replication	12.825	26/36	3.723E-5
ECM-receptor interaction	11.145	45/81	1.755E-4
Focal adhesion	9.608	94/199	7.127E-4
B-cell receptor signaling pathway	7.748	35/71	3.776E-3
Apoptosis	6.122	41/93	1.563E-2
Natural killer cell mediated cytotoxicity	5.945	47/109	1.819E-2
Cell cycle	5.81	54/124	2.041E-2
Toll-like receptor signaling pathway	5.591	48/101	2.459E-2
VEGF signaling pathway	5.518	36/76	2.616E-2
Calvarial tissue			
Leukocyte transendothelial migration	79.756	14/119	0.000E0
Cell adhesion molecules	71.416	13/159	0.000E0
Adherens junction	22.027	13/77	6.282E-9
Phosphatidylinositol signaling system	14.206	7/75	1.029E-5
Ribosome	10.313	21/115	3.756E-4
PPAR signaling pathway	7.475	14/76	4.806E-3
mTOR signaling pathway	7.117	11/55	6.584E-3
Insulin signaling pathway	5.621	20/140	2.397E-2

[1]The calvarial bone and tissue gene pathways were determined by PATHWAY EXPRESS (Draghici *et al.* 2007; Khatri *et al.* 2007).

[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 soft tissue 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 >5 are included in this table.

[4]Number of regulated genes in a pathway/total number of genes currently mapped to this pathway. First three pathways that are impacted both in bone and tissue are indicated in bold.

ECM, extracellular matrix; VEGF, vascular endothelial growth factor; PPAR, peroxisome proliferator-activated receptor; mTOR, mammalian target of rapamycin.

The majority of genes upregulated during a polymicrobial infection in soft tissue were associated with inflammatory, immune and defense response, transport, ECM, cell cycle and differentiation, and cell adhesion and 'other' genes. Inflammatory, immune and defense response transcripts *Cxcl7*, *Saa1*, *Ncf1*, *Selp*, and *Orm1* were modestly induced in soft tissue, whereas defensin beta 3 (*Defb3*) was induced significantly at 58-fold. The transcription of ECM protein genes *Mmp12* and *Mmp13* was again modestly induced in soft tissue. A potentially interesting observation was, that the top five genes upregulated in response to polymicrobial infection in soft tissue, *Defb3*, *Sprr2d*, *Sprr2i*, *Sprr2f*, and *Sprr2h* were not enhanced at all in calvarial bone, suggesting tissue-specific unique gene upregulation. These comparative analyses of transcription clearly demonstrate that the polymicrobial infection induced transcript alterations distinctively different in soft tissue and bone.

Real-time RT-PCR confirmations

The polymicrobial infection-induced mRNA expression defined by the microarray studies were confirmed by qRT-PCR, including *Defb3*, *Sprr2d*, and *Mmp13* in soft tissue and *Cxcl7*, *Mmp9*, and *Pglyrp1*

Table 2 Ontology analysis of leukocyte transendothelial migration (LTM) pathway impacted by mono-infection and polymicrobial infection[1]

Impacted LTM pathway[2]	Impact factor[3]	Input genes/no. of pathway genes[4]	*P* value
Calvarial bone			
Porphyromonas gingivalis	2.912	18/115	2.127E-1
Treponema denticola	306.541	14/119	0.000E0
Tannerella forsythia	85.099	37/119	0.000E0
P. gingivalis/T. denticola/ T. forsythia	55.967	49/119	0.000E0
Calvarial tissue			
P. gingivalis	18.487	39/115	1.194E-2
T. denticola	220.561	5/119	0.000E0
T. forsythia	148.247	35/119	0.000E0
P. gingivalis/T. denticola/ T. forsythia	79.756	14/119	0.000E0

[1]The calvarial bone and tissue gene pathways were determined by PATHWAY EXPRESS (Draghici *et al.* 2007; Khatri *et al.* 2007).
[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 soft tissue to mono-infection (*P. gingivalis* or *T. denticola* or *T. forsythia*) and polymicrobial infection with *P. gingivalis* + *T. denticola* + *T. forsythia* by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these perturbations throughout the pathway.
[4]Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.

in calvarial bone using aliquots of the pooled RNA samples that were evaluated in the microarrays (see Supplementary material, Table S5). The expression levels of *Mmp13* and *Mmp9* by qRT-PCR were higher than microarray expression levels and the expression levels of *Defb3*, *Sprr2d*, *Cxcl7*, and *Pglyrp1* by qRT-PCR were lower than microarray expression levels. Temporal qRT-PCR data confirmed the direction of change of mRNA levels of expression of selected genes in calvarial soft tissue and bone, indicating our microarray data reliability as well as the high sensitivity of the mouse DNA microarray technology. However, the magnitude of the fold-change in levels differed between the two analyses, a common phenomenon largely the result of differences in dynamic range and threshold detection between microarray and qRT-PCR technologies in agreement with other studies (Cobb *et al.*, 2002; Higgins *et al.*, 2003).

Calvarial histology

The uninfected control mice showed a lack of edema and minimal inflammation in the soft tissue over the calvaria at the site of injection (Fig. 4A). Soft tissue swelling occurred at the injection site and increased after 48 and 72 h in almost all of the mice injected with *P. gingivalis*, *T. denticola*, and *T. forsythia*, but not with control mice. None of the mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* had soft tissue abscesses, ulceration of the overlying skin, or any evidence of spread of infection to neighboring sites. Microscopic examination of the tissue sections showed prominent edema and a mixed inflammatory cell infiltrate consisting of aggregates of lymphocytes, polymorphonuclear leukocytes, and macrophages (Fig. 4B). Several multi-nucleated and activated osteoclasts were seen at the suture area on the inner aspect of the calvaria compared with controls. Activated osteoclasts were noted along the entire suture area corresponding to the areas of bone resorption (Fig. 4C,D).

DISCUSSION

This is the first study that has evaluated the global gene transcriptional profiles during a polymicrobial infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* in infected soft tissues and underlying bone. This type of seminal information is required to understand the complex synergistic interactions that can take place *in situ* affecting host cells, tissues, organs, and systems, as well as to provide unique insights into characteristics of the regulation of these interactions. Although the report of this transcriptome study does not include a total catalogue of specific differentially impacted biological pathways in calvarial bone and soft tissue, it targets a few pathways that were found to be highly significantly altered in expression. Recently, three studies have reported the use of microarrays to determine the polymicrobial sepsis 'transcriptome' *in vivo* and measured the broad-scale gene expression profiles for septic liver and spleen and compared these responses with controls using a well-accepted model of murine polymicrobial abdominal sepsis (Cobb *et al.*, 2002; Nemeth *et al.*, 2006; Weighardt *et al.*, 2006). Furthermore, a recent review summarized several studies that monitored the changes in gene expression that take place in host

Table 3 Leukocyte transendothelial migration (LTM) pathway genes impacted by polymicrobial and mono-infection in calvarial bone[1]

No.	Gene	LTM pathway genes[2]	*Pg + Td + Tf*	*P. gingivalis*	*T. denticola*	*T. forsythia*
1	Actb	actin, beta	2.03[3]	0	−1.69	0
2	Actg1	actin, gamma, cytoplasmic 1	1.63	0	0	1.57
3	Actn1	actinin, alpha 1	2.36	0	0	2.04
4	Actn3	actinin alpha 3	0	0	0	−1.50
5	Actn4		1.49	0	0	0
6	Arhgap5	Rho GTPase activating protein 5	0	−1.54	0	0
7	Cd99	CD99 antigen	1.53	0	0	0
8	Cdc42		1.69	1.46	0	1.42
9	Cdh5	cadherin 5	0	0	0	1.37
10	**Cldn1**	**claudin 1**	4.62	9.36	1.93	3.39
11	Cldn13	claudin 13	0	0	1.78	0
12	Cldn14	claudin 14	0	0	−1.40	0
13	Cldn19	claudin 19	0	0	2.63	0
14	Cldn3	claudin 3	0	0	0	2.69
15	Cldn5	claudin 5	0	−2.37	0	0
16	Cldn6	claudin 6	0	2.12	0	0
17	Cldn7	claudin 7	0	0	1.82	0
18	Ctnna1	catenin (cadherin associated protein), alpha 1	1.62	2.24	0	1.48
19	Ctnnb1	catenin (cadherin associated protein), beta 1	2.65	1.42	0	0
20	Ctnnd1	catenin (cadherin associated protein), delta 1	1.28	1.66	0	0
21	**Cxcl12**	**chemokine (C-X-C motif) ligand 12**	3.35	1.65	2.01	2.24
22	Cxcr4	chemokine (C-X-C motif) receptor 4	1.81	0	0	2.42
23	Cyba	cytochrome b-245, alpha polypeptide	5.15	0	0	3.63
24	Cybb	cytochrome b-245, beta polypeptide	9.68	0	0	7.53
25	Esam	endothelial cell-specific adhesion molecule	0	−1.46	−1.27	0
26	Ezr	ezrin	3.16	0	0	3.78
27	Gnai2	guanine nucleotide binding protein (G protein), alpha inhibiting 2	2.65	0	0	2.31
28	Gnai3	guanine nucleotide binding protein (G protein), alpha inhibiting 3	2.53	1.98	0	1.80
29	Icam1	intercellular adhesion molecule 1	−1.93	0	0	−1.40
30	Itga4	integrin alpha 4	−1.58	−1.57	0	−1.86
31	Itgam	integrin alpha M	4.92	0	0	0
32	Itgb1	integrin beta 1 (fibronectin receptor beta)	2.00	0	0	0
33	Itgb2	integrin beta 2	4.41	0	0	3.44
34	Itgb2l	integrin beta 2-like	−1.65	0	0	0
35	Jam2	junction adhesion molecule 2	1.41	0	0	0
36	Mapk11	mitogen-activated protein kinase 11 (EC:2.7.11.24)	0	0	0	−2.98
37	Mapk14	mitogen-activated protein kinase 14	2.55	−1.97	0	0
38	**Mmp2**	**matrix metallopeptidase 2**	2.84	3.33	2.47	1.84
39	Mmp9	matrix metallopeptidase 9	16.29	0	4.71	10.58
40	Msn	moesin	5.53	0	0	2.94
41	Myl9	myosin, light polypeptide 9, regulatory	2.63	0	1.62	2.29
42	Ncf1	neutrophil cytosolic factor 1	8.08	0	0	4.17
43	Ncf2	neutrophil cytosolic factor 2	1.94	0	0	1.71
44	Ncf4	neutrophil cytosolic factor 4	4.05	0	0	3.89
45	Pecam1	platelet/endothelial cell adhesion molecule 1	2.09	0	0	1.82
46	Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide	1.38	−1.45	−1.63	1.68
47	Pik3cg		2.34	0	0	2.28
48	Plcg2	phospholipase C, gamma 2	2.52	0	0	2.49
49	Pxn	paxillin	1.70	−1.43	−1.38	0

Table 3 (Continued)

No.	Gene	LTM pathway genes[2]	*Pg* + *Td* + *Tf*	*P. gingivalis*	*T. denticola*	*T. forsythia*
50	Rac1	RAS-related C3 botulinum substrate 1	1.79	0	0	0
51	Rac2	RAS-related C3 botulinum substrate 2	3.44	0	0	2.73
52	Rap1b		2.05	0	0	0
53	Rassf5	Ras association (RalGDS/AF-6) domain family member 5	2.58	0	0	2.93
54	Rhoa	ras homolog gene family, member A	1.41	0	0	0
55	Rock1	Rho-associated coiled-coil containing protein kinase 1	1.65	−1.87	0	1.67
56	Rock2	Rho-associated coiled-coil containing protein kinase 2 (EC:2.7.11.1)	0	0	0	−1.34
57	Sipa1	signal-induced proliferation associated gene 1	2.56	0	0	0
58	Vasp	vasodilator-stimulated phosphoprotein	4.63	0	0	4.30
59	Vav1	vav 1 oncogene	3.92	0	0	0
60	Vav3	vav 3 oncogene	3.37	0	2.07	3.45
61	Vcam1	vascular cell adhesion molecule 1	2.17	−1.37	0	0
62	Vcl	vinculin	4.68	0	1.65	2.81

[1]The calvarial bone and tissue gene pathways were determined by PATHWAY EXPRESS (Draghici *et al.* 2007; Khatri *et al.* 2007).
[2]Kyoto Encyclopedia of genes and genome pathways (http://www.genome.jp/kegg/).
[3]Fold change describes the ratio of mean expression in infected calvarial bone over the mean expression in uninfected control calvarial bone. Genes that are impacted to mono-infection and polymicrobial infection are indicated in bold.
Pg, *Porphyromonas gingivalis*; *Td*, *Treponema denticola*; *Tf*, *Tannerella forsythia*.

cells such as macrophages, neutrophils, epithelial cells, fibroblasts, dendritic cells after contact with a specific pathogen *in vitro* using publicly available datasets (Jenner & Young, 2005). Using microarray analysis, we now demonstrate calvarial soft tissue and calvarial bone transcriptional signature profiling to specific periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* as a distinct polymicrobial infection to compare with gene expression patterns that have been reported in human periodontitis processes (Demmer *et al.*, 2008; Papapanou *et al.*, 2009).

In the present study, approximately 4476 and 1035 genes were differentially expressed in the bone and soft tissue in response to the polymicrobial infection, indicating that the polymicrobial infection-induced a more robust activation of host gene expression in calvarial bone than in overlying soft tissue. Our findings indicate that the gene expression profiles induced by polymicrobial infection in these two tissues are distinct classes of gene with very little overlap. The greatest number of these altered genes was identified as being related to biological pathways of transport, cell proliferation, cell cycle, defense and immune response, transcription, apoptosis, and inflammatory response, suggesting that the polymicrobial infection was able to induce a multitude of specific gene expression changes during infection. To our knowledge, several highly upregulated genes in bone (*Ltb4r1*, *Ceacam1*, *Evi2b*, *Cxcl7*, *Mmp9*, *Orm1*, *Hp*, *Bst1*) and soft tissue (*Defb3*, *Sprr2d*, *Sprr2i*, *Sprr2f*, *Sprr2h*) in response to the polymicrobial infection in mice have not been reported as being elicited in chronic periodontitis and aggressive periodontitis diseased gingival tissues (Demmer *et al.*, 2008; Papapanou *et al.*, 2009) and during the induction and resolution of experimental gingivitis in humans (Offenbacher *et al.*, 2009; Jonsson *et al.*, 2011) and their functional significance will need to be further evaluated to determine the biological events underlying polymicrobial periodontitis pathogenesis.

We anticipated that the combined *P. gingivalis*, *T. denticola*, and *T. forsythia* infection would induce a strong inflammatory response that would be reflected by a coordinated and controlled array of cytokines, chemokines, and oxidative burst effectors (Cohen *et al.*, 2000; Eskra *et al.*, 2003; Kesavalu *et al.*, 2002). However, we observed that the major proinflammatory cytokines interleukin-1, interleukin-6, and tumor necrosis factor that have been predicted to be crucial in the induction of chronic inflammation and bone resorption were only modestly upregulated in bone following the polymicrobial infection. However, mRNAs for defensin beta 3, chemokine ligand 7,

Table 4 Ontology analysis of extracellular matrix (ECM) –receptor interaction pathway impacted by infection with mono- and polymicrobial infection[1]

Impacted ECM-receptor interaction pathway[2]	Impact factor[3]	Input genes/no. of pathway genes[4]	*P* value
Calvarial bone			
Porphyromonas gingivalis	NSI	NSI	NSI
Treponema denticola	17.812	32/81	3.458E-7
Tannerella forsythia	23.467	35/81	1.574E-9
P. gingivalis, *T. denticola*, *T. forsythia*	11.145	45/81	1.775E-4
Calvarial tissue			
P. gingivalis	NSI[5]	NSI	NSI
T. denticola	NSI	NSI	NSI
T. forsythia	NSI	NSI	NSI
P. gingivalis, T. denticola, T. forsythia	NSI	NSI	NSI

[1]The calvarial bone and tissue gene pathways were determined by PATHWAY EXPRESS (Draghici *et al.* 2007; Khatri *et al.* 2007).
[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 soft tissue to mono-infection (*P. gingivalis* or *T. denticola* or *T. forsythia*) and polymicrobial infection with *P. gingivalis, T. denticola,* and *T. forsythia* by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these perturbations throughout the ECM pathway.
[4]Number of regulated genes in ECM–receptor interaction pathway/total number of genes currently mapped to ECM pathway.
[5]NSI, not significantly impacted pathway.

neutrophil cytosolic factor 1, lymphotoxin B, chitinase 3, chemokine ligand 9, chemokine ligand 12, and bone morphogenetic protein1 were modestly induced. Beta-defensins are believed to contribute to the host defense system by eradicating pathogens at the mucosal surface. Beta-defensins are expressed predominantly at epithelial surfaces, suggesting that these molecules are an important component of the innate immune system and their expression is inducible in response to inflammatory stimuli including both gram-positive and gram-negative bacteria (Maxwell *et al.*, 2003). The significantly highly upregulated level of *Defb3* gene (58-fold) expression during polymicrobial infection clearly demonstrates that innate immune defense antimicrobial peptides are a portion of the gene repertoire induced to overcome this gram-negative bacterial infection. The small proline-rich (Sprr) proteins are the primary constituents of the cornified layer of the epidermis, which is the major barrier against the environment, and are expressed in all squamous epithelium of the skin, scalp, and most of the epithelial lining of the digestive tract including oral epithelium (Tesfaigzi & Carlson, 1999). High levels of *Sprr* genes are detected in various diseases (inflammatory dermatoses), cancers of the skin, human papillomavirus infection, as well as being upregulated under stress (De Heller-Milev & Huber, 2000; Lehr *et al.*, 2004). 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 current finding of upregulation of expression of *Sprr2d*, *Sprr2i*, *Sprr2f*, and *Sprr2h* genes (six-fold to 24-fold) in soft tissue clearly suggested a novel view of the response to this polymicrobial infection and these genes may function *in vivo* as a major barrier against the complex bacterial challenge.

A major difference between the calvarial bone and soft tissue sample responses was the impact of polymicrobial infection on the LTM pathway in bone resulting in the activation of actin (regulation of cytoskeleton) and α-cadherin. Leukocytes have a number of functions, including activation of endothelial cell signals, production of reactive oxygen species with subsequent activation of integrin beta 2 (ITGB2), platelet endothelial cell adhesion molecule-1 (PECAM1), matrix metalloproteinases (MMPs), cadherin 5 (CDH5), and CAMs. Many of the LTM pathway genes such as integrins, focal adhesion molecules, and cadherins have been reported to be upregulated during induction of experimental gingivitis in humans, which is consistent with the activation of the LTM pathway (Offenbacher *et al.*, 2009). PECAM-1 is one of the most abundant proteins on the endothelial cell surfaces, is expressed on the surface of platelets and leukocytes, and its expression increases significantly with increasing size of inflammatory infiltrates in the lesions of gingivitis and periodontitis (Gemmell *et al.*, 1994). A similar robust impact on LTM pathway genes was observed after *T. forsythia* mono-infection (Bakthavatchalu *et al.*, 2010b) and to a lesser extent following a *T. denticola* infection (Bakthavatchalu *et al.*, 2010a). In contrast, this pathway was unaffected in bone and soft tissue after *P. gingivalis* infection (Meka *et al.*, 2010). The *P. gingivalis* induced greater differential regulation of genes in a mixed infection

Table 5 ECM-receptor interaction pathway genes impacted by polymicrobial and mono-infection in calvarial bone[1]

No.	Gene	ECM-receptor pathway genes[2]	*Pg + Td + Tf*	*P. gingivalis*	*T. denticola*	*T. forsythia*
1	Cd36	CD36 antigen	1.87[3]	0	0	0
2	Cd44	CD44 antigen	6.71	0	1.93	3.72
3	Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal)	2.56	0	0	1.86
4	Chad	chondroadherin	2.83	0	2.32	1.80
5	Col11a1	collagen, type XI, alpha 1	2.82	0	3.58	2.25
6	Col11a2	collagen, type XI, alpha 2	0	0	1.37	1.51
7	Col1a1	collagen, type I, alpha 1	4.44	0	4.55	3.34
8	Col1a2	collagen, type I, alpha 2	5.06	0	6.24	3.76
9	**Col3a1**	**collagen, type III, alpha 1**	5.66	6.27	7.57	4.36
10	Col4a1	collagen, type IV, alpha 1	1.52	0	0	0
11	Col5a1	collagen, type V, alpha 1	5.47	0	4.68	3.28
12	Col5a2	collagen, type V, alpha 2	4.44	0	4.51	2.95
13	Col6a1	collagen, type VI, alpha 1	1.72	0	1.87	0
14	Col6a2	collagen, type VI, alpha 2	2.66	0	2.83	1.70
15	Comp	cartilage oligomeric matrix protein	3.16	0	2.27	3.35
16	Dag1	dystroglycan 1	0	−1.44	−1.22	−1.56
17	Fn1	fibronectin 1	5.12	0	4.82	4.35
18	Gp1ba	glycoprotein 1b, alpha polypeptide	1.46	0	0	1.93
19	Gp1bb	glycoprotein Ib, beta polypeptide	4.95	0	0	4.36
20	Gp5	glycoprotein 5 (platelet)	2.36	0	1.37	2.27
21	Ibsp	integrin binding sialoprotein	21.94	0	11.91	7.13
22	Itga2b	integrin alpha 2b	2.34	0	0	2.58
23	Itga4	integrin alpha 4	−1.58	−1.57	0	−1.86
24	Itga5	integrin alpha 5 (fibronectin receptor alpha)	3.07	0	0	2.09
25	Itga6	integrin alpha 6	1.49	0	0	2.32
26	Itga7	integrin alpha 7	0	0	−1.38	0
27	Itga9	integrin alpha 9	2.30	1.84	−1.38	0
28	**Itgav**	**integrin alpha V**	3.43	1.62	2.11	1.87
29	Itgb1	integrin beta 1 (fibronectin receptor beta)	2.00	0	0	0
30	Itgb3	integrin beta 3	0	0	0	1.52
31	Itgb4	integrin beta 4	2.18	0	0	2.29
32	Itgb5	integrin beta 5	1.61	0	0	0
33	Itgb6	integrin beta 6	−2.07	−1.99	0	0
34	Lama3	laminin, alpha 3	0	0	1.33	1.75
35	Lama4	laminin, alpha 4	2.19	1.84	1.63	0
36	Lama5	laminin, alpha 5	−1.50	0	0	0
37	Lamb1-1	laminin B1 subunit 1	3.82	1.82	2.08	0
38	Lamc1	laminin, gamma 1	2.85	0	0	0
39	Lamc2	laminin, gamma 2	−1.62	0	0	−1.77
40	Npnt		2.15	4.95	0	0
41	**Sdc1**	**syndecan 1**	9.68	5.99	3.68	5.88
42	Sdc2	syndecan 2	2.01	0	1.95	0
43	Sdc3	syndecan 3	0	−1.50	0	1.66
44	Sdc4	syndecan 4	1.55	0	0	0
45	Spp1	secreted phosphoprotein 1	5.64	0	4.01	4.14
46	**Thbs1**	**thrombospondin 1**	6.38	5.79	1.88	3.76
47	Thbs2	thrombospondin 2	2.18	7.03	2.66	0
48	Thbs3	thrombospondin 3	0	2.39	1.92	0
49	**Thbs4**	**thrombospondin 4**	5.90	3.99	3.24	4.94
50	**Tnc**	**tenascin C**	5.14	4.27	6.08	4.02

Table 5 (Continued)

No.	Gene	ECM-receptor pathway genes[2]	*Pg* + *Td* + *Tf*	*P. gingivalis*	*T. denticola*	*T. forsythia*
51	Tnxb	tenascin XB	−1.23	0	0	0
52	Vtn	vitronectin	0	0	−1.33	−1.93
53	Vwf	Von Willebrand factor homolog	4.06	0	1.48	3.12

[1]The calvarial bone and tissue gene pathways were determined by Pathway Express (Draghici *et al.* 2007; Khatri *et al.* 2007).
[2]Kyoto Encyclopedia of genes and genome pathways (http://www.genome.jp/kegg/).
[3]Fold change describes the ratio of mean expression in infected calvarial bone over the mean expression in uninfected control calvarial bone. Genes that are impacted to mono- and polymicrobial infection are indicated in bold.

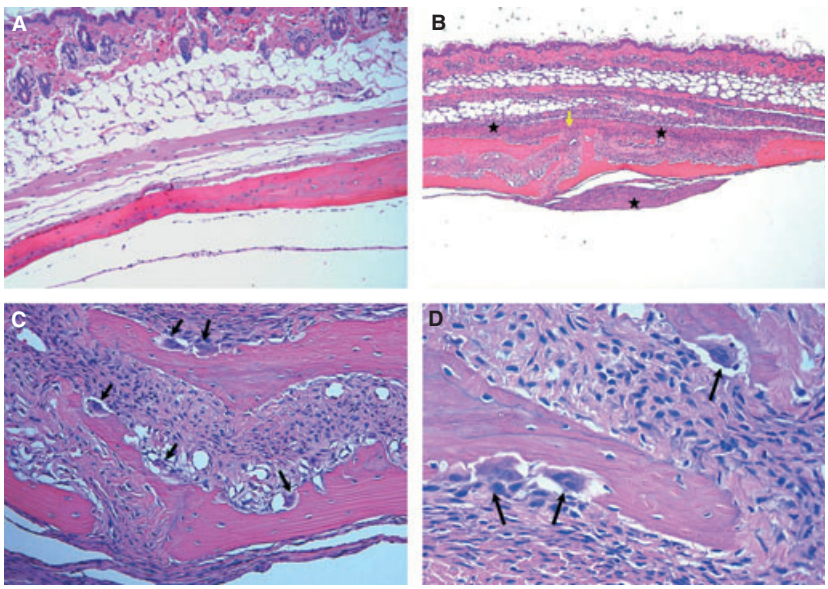

Figure 4 Effects of polymicrobial infections with *Porphyromonas gingivalis*/*Treponema denticola*/*Tannerella forsythia* on mouse calvaria. Live *P. gingivalis* + *T. denticola* + *T. forsythia* (5×10^8 cells each) bacteria as polymicrobial consortium were injected once daily for 3 days into the subcutaneous tissues overlying the calvaria of mice. All photomicrographs are of slides stained with hematoxylin and eosin. (A) Lack of edema and inflammation in the calvarial soft tissue of the sham-infected control mouse (magnification ×10). (B) A section from a polymicrobially infected animal demonstrates significant disruption of the suture area (yellow arrow) with an intense mixed inflammatory infiltrate consisting primarily of neutrophils, lymphocytes and macrophages along with areas of edema and increased vascularity (magnification ×5). Inflammation is noted on both the dermal and supraosteal areas (asterisk). (C) Numerous osteoclasts (black arrows) are seen throughout the inner aspects of the calvarial bone mainly in the suture area. (magnification ×20). (D) Activated osteoclasts within resorption lacunae at higher magnification (×40).

compared with a mono-infection, consistent with its characterization as a stealth pathogen (Hajishengallis, 2009). In addition, the phenotypic properties of *P. gingivalis* change dramatically when the organism is in a community with other oral bacteria (Kuboniwa *et al.*, 2009). These findings suggested that the complex infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* impacted the LTM pathway genes in bone and to a lesser extent in soft tissues with the host recognizing the challenge as a summation of the three individual bacteria.

A robust transcriptional change was also observed with ECM proteins ($n = 14$) in calvarial bone. Surprisingly, almost all the components of the ECM–receptor interaction pathway in calvarial bone (but not in soft tissue) including collagen, laminin, chondroadherein, fibronectin, osteopontin, tenascin, bone sialoprotein, Von Willebrand factor, and thrombospondin were upregulated following the polymicrobial infection. However, these ECM receptor interaction pathway components were not altered with *P. gingivalis* infection (Meka *et al.*, 2010), were modestly affected

following *T. forsythia* challenge (Bakthavatchalu *et al.*, 2010b), and significantly increased with *T. denticola* mono-infection (Bakthavatchalu *et al.*, 2010a). Collectively, collagen, fibronectin, osteopontin, and bone sialoprotein are the major constituents of periodontal tissues. They are critical for their regeneration after injury and seem to have a unique distribution within the periodontium and accumulate predominantly at the hard tissue interfaces. The MMPs 2, 3, 9, 12, 13, 14, and 23 were upregulated in both bone and soft tissue after the polymicrobial challenge. Probe sets representing tissue inhibitor of metalloproteinases (TIMPs) 1, 2 and 3 also demonstrated a modest upregulation during the polymicrobial infection. This murine infection model outcomes for the MMPs and TIMPs are similar to recent microarray data showing that several MMPs (1, 2, 3, 7, 9, 13, 14, 28) and TIMPs (2, 3) were significantly upregulated in human periodontitis gingival tissues (Demmer *et al.*, 2008; Kubota *et al.*, 2008). Our results with ECM protein regulation are also in agreement with *Escherichia coli* lipopolysaccharide stimulation of canine transcriptional changes (Higgins *et al.*, 2003). As *P. gingivalis*, *T. denticola*, and *T. forsythia* levels are quantitatively higher in subgingival plaque samples from deep periodontal pockets of patients with adult periodontitis (Socransky *et al.*, 1998) they may synergistically induce expression of several MMPs and cathepsins, which collectively can degrade ECM proteins in the periodontium and may contribute to the bone resorption and ligament attachment loss observed in periodontal disease (Demmer *et al.*, 2008; Kubota *et al.*, 2008). These observations are consistent with the 'red complex' microorganisms – functionally critical members of the pathogenic biofilms eliciting the hard tissue destruction observed in periodontitis.

Many acute-phase proteins were upregulated in soft tissue and bone during the polymicrobial infection including serum amyloid A1, orosomucoid 1, aquaporin 7, aquaporin 9, haptoglobin, and complement protein 1q and tumor necrosis factor related protein 1. During bacterial infection innate and adaptive immunity co-regulate through soluble factors, such as cytokines, complement proteins and by their specific receptors expressed on various cells. The complement protein 1q and tumor necrosis factor related protein 1 (*C1qtnf1*) gene were overexpressed in calvarial bone following infection. This member of the C1q and tumor necrosis factor superfamily represents a group of proteins involved in host defense, inflammation, apoptosis, autoimmunity, cell differentiation, and insulin-resistant obesity (*C1qtnf1* is an adiponectin paralogue in mice). C1q triggers the production of interleukin-8, interleukin-6 and monocyte chemoattractant protein-1 by endothelial cells may also contribute to the acute-phase response. These data clearly indicate that during the polymicrobial infection the host initiates a strong acute-phase response by expressing *C1qtnf1*, Saa1, and *Orm1* in calvarial bone and soft tissue (Higgins *et al.*, 2003).

The leukotriene B_4 receptor 1 (*Ltb4r1*; 18-fold) was highly expressed in calvarial bone following the polymicrobial infection. Leukotriene B_4, a product of 5-lipoxygenase, is a potent mediator of inflammation expressed in several inflammatory diseases including periodontitis, bronchial asthma, and atherogenesis. Additionally, it plays a role in the regulation of innate and adaptive immunity, since the LTB_4–BLT1 (leukotriene B_4 receptor 1) axis is required for the development of a T helper type 2 immune response in bronchial asthma (Terawaki *et al.*, 2005). Moreover, LTB_4 was reported to be significantly elevated in gingival tissue and gingival crevicular fluid from patients with chronic periodontitis and generalized aggressive periodontitis (Emingil *et al.*, 2001). The example of *Ltb4r1* gene expression suggested that the periodontal pathogens induced potent lipid inflammatory mediators that could contribute to the role in the pathophysiology of periodontitis.

The characteristic inflammation and osteoclastic full thickness calvarial bone resorption defects observed in response to polymicrobial infection were similar to the effects that we observed in the calvariae of mice following infection with *P. gingivalis* (Meka *et al.*, 2010), *T. denticola* (Bakthavatchalu *et al.*, 2010a), *T. forsythia* (Bakthavatchalu *et al.*, 2010b), *Campylobacter rectus* and *Fusobacterium nucleatum* (Zubery *et al.*, 1998), suggesting that there is not a synergistic action of these micro-organisms to induce genes that regulate osteoclastogenesis or that the response to individual organisms is maximal.

In conclusion, the present study provided a comprehensive gene expression profile of mouse calvarial soft tissue and calvarial bone that accompanied a localized, acute infection with a polybacterial challenge with *P. gingivalis*, *T. denticola*, and *T. forsythia*. In addition, this study demonstrated that

although the polymicrobial infection resulted in a multitude of specific gene expression changes, there appeared to be some targeted biological pathways that were more selectively altered and could provide some guidance for further understanding the polymicrobial challenge leading to the pathogenesis of periodontitis. Lastly, by depositing our raw data in the GEO repository (http://www.ncbi.nlm.nih.gov/projects/geo/), we enable prospective researchers to conduct more targeted analyses focusing on specific pathways and genes of their interest related to the pathogenesis, diagnosis, and/or therapeutic targets in periodontal disease.

DISCLOSURE

The authors have no financial conflict of interest.

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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. Leukocyte transendothelial migration (LTM) pathway genes impacted by polymicrobial and mono-infection in calvarial tissue.

Table S4. Extracellular matrix (ECM) receptor-interaction pathway genes impacted by polymicrobial and mono-infection in calvarial tissue.

Table S5. Comparison of expression of selected genes in calvarial tissue and calvariae by microarray and real-time quantitative polymerase chain reaction.

Figure S1. Cell adhesion molecules (immune system) pathway.

Figure S2. Tight adherens junction in calvarial bone.

Figure S3. Antigen processing and presentation in calvarial bone.

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