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Quantitative Gene Expression Profiling Implicates Genes for Susceptibility and Resistance to Alveolar Bone Loss

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Periodontal disease is one of the most prevalent chronic inflammatory diseases. There is a genetic component to susceptibility and resistance to this disease. Using a mouse model, we investigated the progression of alveolar bone loss by gene expression profiling of susceptible and resistant mouse strains (BALB/cByJ and A/J, respectively). We employed a novel and sensitive quantitative real-time PCR method to compare basal RNA transcription of a 48-gene set in the gingiva and the spleen and the subsequent changes in gene expression due to *Porphyromonas gingivalis* oral infection. Basal expression of interleukin-1 beta (*Il1b*) and tumor necrosis factor alpha (*Tnf*) mRNA was higher in the gingiva of the susceptible BALB/cByJ mice than in the gingiva of resistant A/J mice. Gingival *Il1b* gene expression increased further and *Stat6* gene expression was turned on after *P. gingivalis* infection in BALB/cByJ mice but not in A/J mice. The basal expression of interleukin-15 (*Il15*) in the gingiva and the basal expression of p-selectin (*Selp*) in the spleen were higher in the resistant A/J mice than in the susceptible BALB/cByJ mice. In the resistant A/J mice the expression of no genes detectably changed in the gingiva after infection. These results suggest a molecular phenotype in which discrete sets of differentially expressed genes are associated with genetically determined susceptibility (*Il1b*, *Tnf*, and *Stat6*) or resistance (*Il15* and *Selp*) to alveolar bone loss, providing insight into the genetic etiology of this complex disease.

Periodontal disease is the most common chronic inflammatory disease in humans and leads to the destruction of tooth-supporting tissues and tooth loss (37, 38). This process is characterized by elimination of the periodontal ligament, formation of periodontal pockets, and alveolar bone resorption (37). It is initiated by bacteria, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*, that accumulate as subgingival biofilms and stimulate an inflammatory response in the host gingiva (38).

An excessive or sustained response leads to chronic inflammation, which is a potent amplification system for recruiting humoral and cellular components of the immune system. Indeed, the host's own defense mechanisms contribute substantially to the etiology of periodontal disease (11, 19, 32, 33). Individuals show various levels of susceptibility or resistance to the disease, and this is at least in part genetically determined (6, 22, 24). Gingival immune responses have been associated with the pathogenesis, severity, and genetic susceptibility to human periodontal disease (11, 32, 33).

A murine model has been developed in which mice are orally infected with *P. gingivalis*, which results in alveolar bone loss (5). This model offers many benefits over human studies, such as controlled environmental conditions and infection levels, as well as the existence of a variety of genetically defined inbred strains of immunocompetent mice. Baker et al. used this system to investigate the genetic control of susceptibility to *P. gingivalis*-induced alveolar bone loss (3). The BALB/cByJ mouse strain was more susceptible, while the A/J strain was resistant to bone loss. Therefore, this model offers the means to determine how genetic variation can influence the differential host response to oral infection with *P. gingivalis*.

An understanding of gene expression levels and subsequent changes due to infection with a periodontal pathogen could provide new clues to the key host molecules that confer resistance and susceptibility to this complex disease. There are a variety of methods for quantifying gene expression, including Northern blotting, in situ hybridization, RNase protection assays, microarrays, and quantitative reverse transcriptase PCR (QPCR). Microarrays enable simultaneous analysis of a large number of genes, but samples with limited RNA can be used only after cDNA amplification, which adds a source of possible error. In contrast, QPCR has many advantages due to its high sensitivity, reproducibility, and large dynamic range, especially with limited tissue samples and immunological targets that can be expressed at low levels (12, 31, 35). Here we combined the multiple-gene analysis of microarrays with the sensitivity and accurate quantitation of QPCR in a high-throughput system using a customized ImmunoQuantArray (1, 7).

Gene expression profiles in the gingiva and the spleen could provide a broad assessment of local gene transcript availability and systemic gene transcript availability, respectively. Here we quantified the expression of a targeted set of genes associated with immunological responses and compared gingival expression to the expression in a known secondary lymphoid organ, the spleen. We compared basal levels of gene transcripts in these tissues in alveolar bone loss-susceptible and -resistant mouse strains (BALB/cByJ and A/J, respectively) and the subsequent changes in gingival gene expression due to *P. gingivalis*

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infection. This information provides insight into how differences in the expression of specific genes may give rise to disease progression and pathology in the mouse model and may suggest candidate genes whose differential expression contributes to genetic susceptibility and resistance.

MATERIALS AND METHODS

Animals. The female BALB/cByJ and A/J mice used in this study were bred and raised at The Jackson Laboratory in Bar Harbor, Maine, and were transferred to the animal colony at Bates College in Lewiston, Maine. The mice were specific pathogen free; i.e., they had a normal bacterial flora but were confirmed to be free of specific pathogens. The mice were 12 weeks old at the initiation of the studies, were kept on a 12-h light-dark cycle, and were given distilled water and food ad libitum. Once the mice were infected with *P. gingivalis*, they were kept in a separate room under the same conditions as the sham-infected mice. Bates College's Animal Care and Use Committee approved the experiments.

Bacteria. *P. gingivalis* ATCC 53977 (A7A1-28) was stored in defibrinated sheep blood at −80°C. The bacteria were maintained by weekly transfer on supplemented blood agar consisting of Trypticase soy agar base with 0.1% yeast extract, 5.0 μg of hemin per ml, 5.0 μg of menadione per ml, and 5% defibrinated sheep blood. For the experiments, the bacteria were anaerobically grown under 5% CO_2–10% H_2–85% N_2 on supplemented blood agar at 37°C for 7 days. Bacteria were suspended in phosphate-buffered saline, and the number of CFU was standardized by using the optical density at 600 nm (5).

Oral infection. The animals were given the antibiotics sulfamethoxazole and trimethoprim at final concentrations of 700 μg of sulfamethoxazole per ml and 400 μg of trimethoprim per ml in water bottles ad libitum for 9 days, and this was followed by 4 days without antibiotics. The experimental group was then infected. A total of 10^9 CFU of live *P. gingivalis* suspended in 100 μl of phosphate-buffered saline with 2% carboxymethyl cellulose (Sigma Chemical Co., Kalamazoo, Mich.) was given to each mouse via a feeding needle; one half of the volume was placed in the throat, and the other half was placed directly in the oral cavity. This suspension was given three times at 2-day intervals. The control group received the same pretreatment and was sham infected without the *P. gingivalis*. The mice were euthanized with CO_2 at 1, 2, 3, 4, and 6 weeks after the first administration of either the sham treatment or *P. gingivalis*, and five sham-infected and five infected mice were used at each time point.

Alveolar bone loss. The skulls were boiled for 10 to 12 min at a pressure of 15 lb/in^2 and defleshed. The skulls were then immersed overnight in 3% hydrogen peroxide and stained with 1% methylene blue. Horizontal bone loss around the maxillary molars was assessed morphometrically by measuring the distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) as described by Klausen et al. (15). Measurements were obtained at seven sites on the buccal side of the left and right maxillary molars, and a total of 14 measurements per mouse were obtained. The measurements were obtained by using a dissection microscope (magnification, ×40) equipped with a video image marker measurement system (model VIA 170; Boeckeler Instruments, Inc., Tucson, Ariz.) standardized to give measurements in millimeters. One evaluator did random and blind quality control on the measurements. The amount of change in the alveolar bone for each mouse was calculated by subtracting the CEJ-ABC distance for that mouse from the mean CEJ-ABC distance for the sham-infected group of mice of the same strain. The more bone loss, the more negative the change.

Quantification of gene expression by real-time PCR. The buccal and lingual gingiva surrounding all six maxillary molars was collected at the time of euthanasia, as was the spleen. Tissues were placed in RNAlater (Ambion, Austin, Tex.), and stored at −80°C. Tissue from each mouse was processed separately, which provided one spleen and one gingival sample from each mouse. The tissues were homogenized with a motorized pestle in Lysis/binding solution (Ambion). An RNAqueous-4PCR kit (Ambion) was used to isolate DNA-free RNA from the tissues. This RNA was made into cDNA with a RETROscript kit (Ambion). Each cDNA sample was then added to a PCR amplification mixture containing forward and reverse primers (each at a concentration of 67 nM) and SYBR Green PCR master mixture (Applied Biosystems, Foster City, Calif.). Primers were designed for the ImmunoQuantArray of immunologically relevant genes listed in Table 1 and are described in Table 2 and by Akilesh et al. (1). Primers were synthesized by MWG Biotech (High Point, N.C.) and were then arranged in a MicroAmp Optical 96-well reaction plate (Applied Biosystems). All primers are gene specific and were validated as described by Akilesh et al. (1). Primer reaction mixtures were subjected to the following DNA amplification scheme: one cycle of 50°C for 2 min (AmpErase uracil-*N*-glycosylase activation) and 95°C for 10 min (AmpliTaq Gold activation), followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension). The data were collected by using the ABI Prism 7000 sequence detection system with version 1.7 software (Applied Biosystems). The threshold cycle number (C_t) is defined as the number of PCR amplification cycles required for achieving a defined fluorescence intensity; therefore, the higher the C_t, the less of the mRNA was present originally. To validate the procedure, technical replicate analyses were performed with many samples with a very low standard deviation and high reproducibility. Consequently, for each mouse (biological replicate) only one QPCR was performed.

Statistics. For alveolar bone levels, analyses of variance (ANOVA) were performed and post hoc *t* tests were done for significant interactions by using the Bonferroni correction (STATView [SAS Institute Inc.] and Excel [Microsoft]).

In the QPCR experiments, basal levels of gene expression were compared across tissues and strains, and gingival expression data were compared with and without *P. gingivalis* infection. Differences between groups were analyzed by using a rigorous global pattern recognition (GPR) algorithm (1). GPR performs a global normalization function that compares the change in expression of each gene with the change in expression of every other gene in the ImmunoQuantArray. When control and experimental cohorts are compared, all genes whose expression is not different are used iteratively as normalizers to rank genes whose expression is significantly different in different cohorts. Comparisons thus are not dependent on the expression stability of any one normalizer gene. This analysis allows stratification of genes as a function of both the magnitude of the difference in expression and the reproducibility of the C_t values within the two comparison groups. Data are filtered to disregard any data with a raw C_t value greater than 37.5, a cycle number that approaches single-copy detection. In the more usual analysis by ANOVA, such data are necessarily included, skewing the entire data set (1). For each gene-normalizer combination, the ΔC_t values [$\Delta C_{t(\text{gene})}$ = gene C_t − normalizer C_t] for BALB/cByJ mice versus A/J mice or for uninfected groups versus infected groups are compared by an unpaired, two-tailed Student *t* test. The gene-normalizer combination is scored as a hit if the *P* value is less than 0.05. The GPR score is then derived as the fraction of normalizers that produced significant hits. A GPR score of 0.4, indicating that control and experimental cohorts were found to be statistically different compared to 40% or more of the normalizers, has been shown to reliably identify genes undergoing significant change (1). For the genes whose GPR score was greater than 0.4, the magnitude of change was then calculated as follows: $2 \hat{} [-1 (\text{mean } \Delta C_t \text{ of experimental mice} - \text{mean } \Delta C_t \text{ of control mice})]$, where ΔC_t = gene C_t − 18S rRNA C_t.

RESULTS

Alveolar bone response to *P. gingivalis* infection over time. The effect of a *P. gingivalis* oral infection on alveolar bone levels was assayed over time. An ANOVA revealed significant interactions among the variables mouse strain, *P. gingivalis*, infection, bone level, and bone measurement site ($P = 0.05$). The bone loss in infected BALB/cByJ mice reached significant levels compared to that in sham-infected BALB/cByJ mice 6 weeks postinfection ($P = 0.01$) (Fig. 1). In BALB/cByJ mice 6 weeks postinfection, post hoc *t* tests with data from individual sites showed that most of the alveolar bone loss was restricted to certain sites. BALB/cByJ mice had significant bone loss ($P < 0.05$) at 6 of 14 sites (data not shown). The A/J mice did not show any significant bone loss at any time (Fig. 1), despite the fact that infection was confirmed by the development of an anti-*P. gingivalis* immunoglobulin G (IgG) antibody. The titers in infected A/J mice were comparable to the titers in infected BALB/cByJ mice and followed a similar time course over the 6 weeks postinfection, reaching maximal levels at 3 weeks in both strains (data not shown).

Basal expression profiling of the mouse gingiva and the mouse spleen. To determine the basal gene expression profiles in the gingiva and the spleen from individual sham-infected BALB/cByJ and A/J mice, we performed QPCR for the ImmunoQuantArray of 48 genes listed in Table 1. These genes

TABLE 1. Targeted gene ImmunoQuantArray used for all QPCR assays

Gene	Mouse genome ID[a]	GenBank accession no.	Gene product name and synonyms
Costimulatory/activation cell surface ligands			
Cd44	88338	AJ251594	CD44 antigen
Cd80	101775	NM_009855	CD80 antigen
Leukocyte cell surface differentiation markers			
Cd4	88335	NM_013488	CD4 antigen
Cd8a	88346	AJ131778	CD8, alpha chain
Chemokines and chemokine receptors			
Ccrl2	192094	NM_017466	Chemokine (C-C motif) receptor-like 2 (CmkbrIL2)
Ccr7	103011	NM_007719	Chemokine (C-C motif) receptor 7 (Cmkbr 7.2)
Cxcr3	1277207	NM_009910	Chemokine (C-X-C motif) receptor 3
Cxcr4	109563	NM_009911	Chemokine (C-X-C motif) receptor 4 (Cmkar4; fusin)
Cxcl10	1352450	NM_021274	Chemokine (C-X-C motif) ligand 10 (1P-10)
Fc receptors			
Fcer1g	95496	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide
Fcgr3	95500	NM_010188	Fc receptor, IgG, low affinity III
Stress response			
Hspa1b	99517	AF109906	Heat shock protein 1B (Hsp70.1)
Cytokines and cytokine receptors			
Ifng	107656	AKC89574	gamma interferon
Ifngr2	107654	NM_008338	gamma interferon receptor 2
Illb	96543	NM_008361	IL-1β
Illr1	96545	NM_008362	IL-1 receptor, type 1 (Illr alpha chain)
Il2	96548	NM_008366	IL-2
Il2rg	96551	NM_013563	IL-2 receptor, gamma chain
I14	96556	NM_021283	IL-4
I16	96559	NM_031168	IL-6
Il10	96537	NM_010548	IL-10
Il15	103014	NM_008357	IL-15
Il17	107364	NM_010552	IL-17 (Ctla-8)
Il18	107936	NM_008360	IL-18 (Igif)
Opg (Tnfrsf11b)	109587	NM_008764	TNF receptor superfamily, member 11b (OPG; soluble Trance ligand; osteoclastogenesis inhibitor)
Tnf	104798	NM_013693	TNF
Leukocyte adhesion			
Itgax	96609	NM_021334	Integrin alpha X (Cd11c)
Sell	98279	NM_011346	Selectin, lymphocyte
Selp	98280	NM_011347	Selectin, platelet (p-selectin)
Innate immune response			
Defb1	1096878	NM_007843	Defensin beta 1
Tlr2	1346060	NM_011905	Toll-like receptor 2
Tlr4	96824	NM_021297	Toll-like receptor 4
Tlr9	1932389	NM_031178	Toll-like receptor 9
Immune activation/signal transduction			
C2ta	108445	NM_007575	Class II transactivator
Irak1	107420	NM_008363	IL-1 receptor-associated kinase 1 (Illrak)
Jak1	96628	NM_146145	Janus kinase 1
Jak2	96629	NM_008413	Janus kinase 2
Nfkbib	104752	NM_010908	Nuclear factor of kappa light chain gene enhancer in B-cell inhibitor, beta (1kB)
Notch3	99460	NM_008716	Notch gene homolog 3 (*Drosophila*)
Plcd	97614	NM_019676	Phospholipase C, delta
Stat1	103063	NM_009283	Signal transducer and activator of transcription 1
Stat3	103038	NM_011486	Signal transducer and activator of transcription 3
Stat6	103034	NM_009284	Signal transducer and activator of transcription 6
Tnfrsf17	1343050	NM_011608	TNF receptor superfamily, member 17 (BCMA)
Other			
Rn18s	97943	X00686	rRNA
Gapd	95640	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase
Gpil	95797	NM_008155	Glucose phosphate isomerase 1
Hprl	96217	NM_013556	Hypoxanthine guanine phosphoribosyl transferase

[a] Mouse genome informatics (www.informatics.jax.org).

TABLE 2. Partial list of primers for the ImmunoQuantArray[a]

Gene	Primer	Sequence
Ccrl2	CCR1L2.1F	TGATGGTTGTGTTGATCCTCATAAA
	CCR1L2.1R	TCGCTGTACAAGGCCAGGTAA
Ccr7	Cmkbr7.2F	TCATTGCCGTGGTGGTAGTC
	Cmkbr7.2R	TGACGCCGATGAAGGCATA
Cxcr3	Cxcr3.1F	AGAGGCGTTTTCGAGCTATGAG
	Cxcr3.1R	GGATTGAGGCAGCAGTGCAT
Cmkar4	Cmkar4.1F	TGGCTGACCTCCTCTTTGTCA
	Cmkar4.1R	GCAGTTTCCTTGGCCTTTGA
Cxcl10	IP-10F	TGGCCTCTGTTGTCAAGTTTTG
	IP-10R	AACAGGGTCAAGGATGAAAGTGA
Fcgr3	Fcgr3.1F	GACACGGGCCTTTATTTCTACGT
	Fcgr3.1R	CGGCCTGCTTGTAAGTTGCT
Il17	IL17rF	GCTGGAAAGTTTCTCCGACTCA
	IL17rR	CACAGCGTGTCTCAAACAGTCAT
Opg (*Tnfrsf11b*)	Tranceligand.1F	GAAGGGCGTTACCTGGAGATC
	Tranceligand 1R	CTGAATTAGCAGGAGGCCAAAT
Selp	P-SelectinF	CAACACCACCTGGGAAGCTTT
	P-SelectinR	CCAGGGATTGGAACAGTTCATT
Defb1	Defb1F	GAGCCAGGTGTTGGCATTCT
Defb1	Defb1R	TTACAATCCATCGCTCGTCCTT
Tlr9	Tlr9.1F	GGTGACTATCAAGCCAGAGATGTTT
	Tlr9.1R	GGCCTGCAACTGTGGTAGCT
Jak1	Jak1F	ACTGCAGATGCCCACCATTAC
	Jak1R	AAGCAGGTGACAGTCATAAGAATGTT
Jak2	Jak2F	ATTCGTGTCATTAATTGACGGGTATT
	Jak2R	TAGGGCTGCATCGTAGCACATATA
Nfkbib	IKB.1F	CACCCAAGAGATGCCTCAGATAC
	IKB.1R	TTTGTGGATGACAGCTACATGGA
Notch3	Notch3.1F	CTGCCATGCAGCGCATACT
	Notch3.1R	CAGAATGGCGGGACACAGT
Plcd	PLC-delta1.2F	AAATAGTTTTGTCCGCCATAACG
	PLC-delta1.2R	AGCACATACCCACAACCTCCAT
Stat1	STAT1F	TCCTTCTGGCCTTGGATTGA
	STAT1R	ACCGTTCCACCCATGTGAA
Stat3	STAT3.1F	TCTCCTTCTGGGTCTGGCTAGA
	STAT3.1R	TGTCCTTTTCCACCCAAGTGA
Stat6	STAT6F	AGATGAGGCTTTCCGGAGTCA
	STAT6R	CCCATATCTGAGCTGAGTTGCA
Tnfrsf17	BCMAF	GCCGACACCGAGCTGACTAG
	BCMAR	CTTGCCGTAGTCACCCGTTT
Gpi1	GPI-F	GGGTAGGTGGCCGCTATTC
	GPI-R	GGTCTCACAGCCGTAGCAGTT

[a] For the remaining primer sequences, see reference 1.

included genes whose expression is associated with various adaptive and innate immunological processes (1). The relative expression of these genes is shown in Table 3. Considerable differences in the gene expression pattern were observed between the gingiva, a tissue in close apposition to the infection site and to the alveolar bone, and the spleen, a tissue with a known primary immunological function. In keeping with the known function of the spleen, the majority of the 48 genes were expressed at appreciable levels in the spleens of both strains of mice.

About one-half of these genes were also robustly expressed in the gingiva. The basal expression of *Hspa1b* (*Hsp 70.1*) mRNA in both mouse strains and of *Opg* mRNA in BALB/cByJ mice was, in fact, significantly higher in the gingiva than in the spleen ($P \leq 0.00001$ for *Hspa1b* and $P = 0.02$ for *Opg*, as determined by the *t* test). There was not detectable chemokine mRNA or mRNA for most of the interleukins, with the exception of interleukin-18 (IL-18) (*Il18*) in both mouse strains and of IL-1β (*Il1b*) in BALB/cByJ mice. An RNA message was present for the receptors for IgG, IgE, and gamma interferon. *Tlr4* mRNA was present, but *Tlr2* or *Tlr9* mRNA was not present. *Stat1* and *Stat3* mRNA were present, but *Stat6* mRNA was not present. Messages for both p-selectin (*Selp*) and l-selectin (*Sell*) were present. These results indicate that prior to specific infection the gingiva expresses mRNA for many immunologically important proteins.

Basal gene expression profiles of susceptible and resistant mouse strains. Differential expression of genes in the tissues of the bone loss-susceptible BALB/cByJ mice and the bone loss-resistant A/J mice could suggest genes associated with susceptibility or resistance. Few strain-specific differences were detected. The genes whose basal expression differed significantly (GPR score, >0.4) in the BALB/cByJ and A/J strains are indicated in Table 3.

The differences were then further quantified. Gingival *Il1b*, *Opg*, and *Tnf* gene expression was significantly higher (3.4, 3.8, and 2.9 times higher, respectively) in the bone loss-susceptible BALB/cByJ mice than in the bone loss-resistant A/J mice,

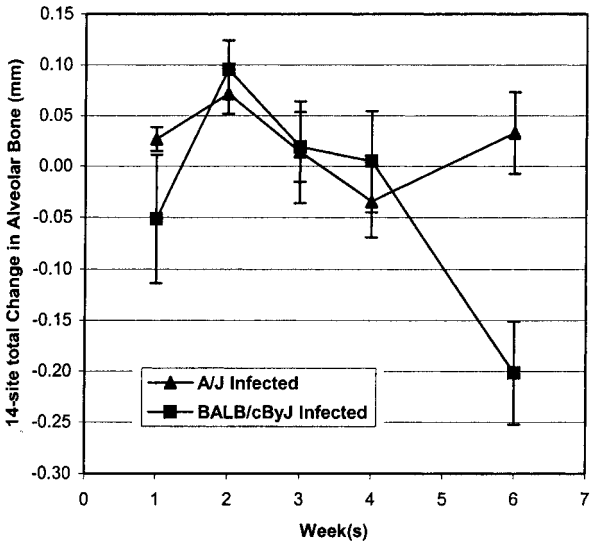

FIG. 1. Alveolar bone change in response to *P. gingivalis* infection in A/J and BALB/cByJ mice over time. The *y* axis shows the total difference in CEJ-ABC bone levels at 14 sites in infected mice and sham-infected mice. Bone loss is indicated by negative values. *P. gingivalis*-infected BALB/cByJ mice had significant bone loss at 6 weeks ($P = 0.01$, as determined by a *t* test with Bonferroni correction,). Infected A/J mice did not show bone loss. The data are means ± standard errors of the means ($n = 5$ mice per group).

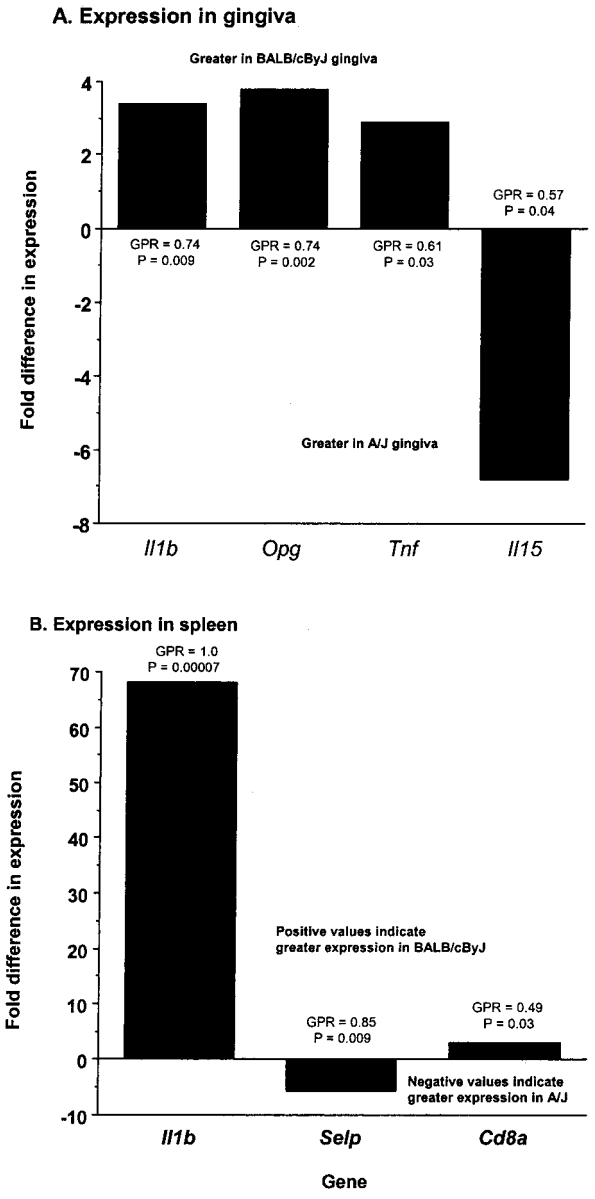

FIG. 2. Genes with significantly different basal expression levels in the BALB/cByJ and A/J strains of mice (GPR, >0.4). Genes are arranged in order of their GPR scores. The ΔC_t for the genes in each mouse was calculated compared with the value for 18S rRNA, and the ΔC_t data grouped by mouse strain was subjected to a *t* test to derive the *P* value. The fold changes quantify the differences in the mean ΔC_t values between the two mouse strains. Each group contained four to eight mice, and one QPCR was performed per tissue from each mouse. (A) In the gingiva, *Il1b*, *Opg*, and *Tnf* mRNA were more highly expressed in the BALB/cByJ mice than in the A/J mice, while expression of *Il15* was higher in A/J mice. (B) In the spleen, *Il1b* expression was 68-fold higher in BALB/cByJ mice than in A/J mice, while *Selp* expression was 5.6-fold higher in A/J mice than in BALB/cByJ mice.

while *Il15* expression was 6.8 times higher in the A/J gingiva than in the BALB/cByJ gingiva (Fig. 2A). In the spleen, the basal expression of *Il1b* was 68 times higher and the basal expression of *Cd8a* was 2.8 times higher in BALB/cByJ mice than in A/J mice (Fig. 2B). In contrast, the basal expression of *Selp* in the spleen was 5.6 times higher in A/J mice. These data suggest that allelic variation between BALB/cByJ and A/J mice results in differential expression of these genes.

Gingival gene expression profiles in response to oral infection. Changes in gene expression in the gingiva due to *P. gingivalis* infection were investigated at 1, 2, 3, 4, and 6 weeks for each of the 48 genes. One week postinfection in the bone loss-susceptible BALB/cByJ mice, *Il1b* and *Opg* mRNA were more highly expressed in the gingiva of *P. gingivalis*-infected mice than in the gingiva of sham-infected mice (the values were 6.5- and 12.1-fold higher, respectively) (Fig. 3). In addition, *Stat6* expression increased 3.4-fold (Fig. 3), going from being undetectable in the gingiva of sham-infected mice to exhibiting medium expression in the gingiva of infected mice. There was greater variability from mouse to mouse in the gingiva of *P. gingivalis*-infected BALB/cByJ mice at later times, such that no genes were found to be significantly differently expressed by our rigorous GPR criteria at any later time. In the bone loss-resistant A/J mice, no genes showed differential expression at any time after *P. gingivalis* infection.

DISCUSSION

The studies described here are the first studies to comprehensively analyze the immunological gene expression profiles of the mouse gingiva. A number of immunological genes were basally expressed in the gingiva without exposure to *P. gingivalis* infection (Table 3). Because of the expression of these immunological genes, their encoded proteins are likely to be readily available to support an immune response. The gingiva thus is an immunologically competent tissue even prior to specific infection with a periodontal pathogen.

TABLE 3. Basal gene expression levels in the gingiva and the spleen of the bone loss-susceptible BALB/cByJ mice and the bone loss-resistant A/J mice[a]

Gene	Gingiva		Spleen	
	BALB/cByJ (n = 8)	A/J (n = 9)	BALB/cByJ (n = 4)	A/J (n = 4)
Costimulatory/activation cell surface ligands				
Cd44	High[b]	High[b]	Very high	Very high
Cd80	Medium	Medium	High	High
Leukocyte cell surface differentiation markers				
Cd4			High	High
Cd8a			**High**	High
Chemokines and chemokine receptors				
Ccrl2				
Ccr7			High	Medium
Cxcr3				
Cxcr4			Medium	High
Cxcl10		—[b]	Medium	
Fc receptors				
Fcer1g	Medium[b]	Medium	High	High
Fcgr3	High	High	Very high	Very high
Stress response				
Hspa1b	High[c]	High[c]		
Cytokines and cytokine receptors				
Ifng			Medium	Medium
Ifngr2[d]	Medium	Medium	High	Medium
Il1b[d]	**Medium**	—[b]	**High**	Medium
Il1rl				
Il2		—[b]		Medium
Il2rg			Very high	Very high
Il4				Medium
Il6	—[b]	—[b]	Medium	Medium
Il10			Medium	Medium
Il15		**Medium**	High	High
Il17				
Il18	High[b]	High[b]	High	High
Opg (*Tnfrsf11b*)[d]	**High**[c]	Medium[b]	Medium	Medium
Tnf[d]	Medium	Medium	High	High
Leukocyte adhesion				
Itgax[d]	Medium	Medium	Very high	Very high
Sell	Medium	Medium	Very high	Very high
Selp	High[b]	High	High	**Very high**
Innate immune response				
Defb1	—[b]	—[b]		
Tlr2			Medium	Medium
Tlr4	Medium[b]	Medium	High	High
Tlr9			High	High
Immune activation/signal transduction				
C2ta			High	Medium
Irak1			Medium	Medium
Jak1	High	High	Very high	Very high
Jak2	Medium[b]	Medium[b]	Medium	Medium
Nfkbib	Medium	Medium	High	High
Notch3	Medium[b]	Medium[b]		
Plcd	—[b]	—[b]		
Stat1	High	High	Very high	Very high
Stat3[d]	Medium	Medium	High	High
Stat6			Medium	Medium
Tnfrsf17			Medium	Medium
Other				
Gapd	Medium	Medium	High	High
Gpi1	Very High	Very high[b]	Very high	Very high
Hprt	High[b]	Medium	High	High

[a] Within a tissue, when there were significant differences in expression between the two strains (GPR score, >0.4; $P < 0.05$, as determined by the 18S rRNA t test), the results for the strain with higher expression are in boldface type. All data were normalized (ΔC_t) to 18S rRNA, consistently the most highly expressed gene, and placed into categories based on their relative expression. The categories were designated as follows: very high expression (ΔC_t of <11), high (ΔC_t of 11 to 17.5), and medium (ΔC_t of 17.5 to 22). Blank spaces indicate low expression or not detectable with a ΔC_t of >22. Except where indicated otherwise, expression in the spleen was significantly higher than expression in the gingiva of the same mouse strain ($P < 0.05$, as determined by the t test).

[b] Not significantly different in the gingiva than in the spleen of the same mouse strain ($P > 0.05$, as determined by the t test).

[c] Significantly higher expression in the gingiva than in the spleen ($P < 0.05$, as determined by the t test).

[d] The gene is known to be expressed in human gingivae based on EST libraries (9) or QPCR studies of healthy human gingival biopsies (20, 30).

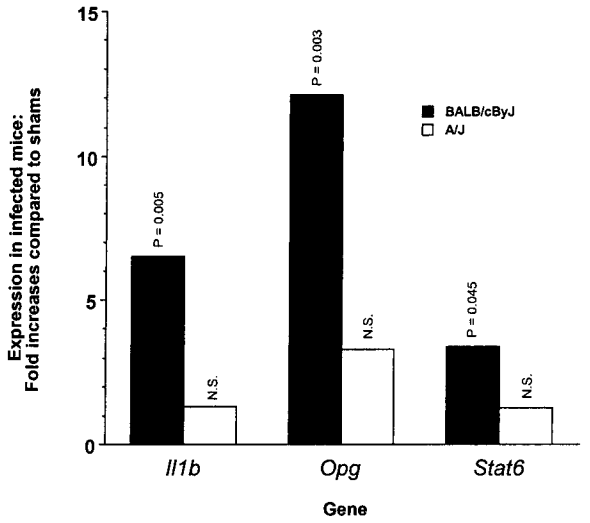

FIG. 3. Gingival gene expression in *P. gingivalis*-infected BALB/cByJ and A/J mice relative to the expression in sham-infected mice 1 week postinfection (GPR, >0.4). Genes are arranged in order of their GPR scores. The ΔC_t for the genes in each mouse were then calculated by comparison with the value for 18S rRNA, and the ΔC_t data for sham-infected mice were compared with the data for infected mice with a *t* test to derive the *P* value. The fold changes indicate the differences in the mean ΔC_t values between the sham-infected mice and the infected mice. Each group contained three mice, and one QPCR was performed per mouse. In BALB/cByJ mice, expression of *Il1b*, *Opg*, and *Stat6* was significantly increased in infected mice. In A/J mice, gene expression did not change significantly with infection. N.S., not significant.

No such comprehensive survey of gene expression has been done with healthy human gingivae to our knowledge. A few studies have examined the expression of a small number of genes in healthy gingivae. Few of the genes included here have been investigated previously, but for those that have been, expression in humans correlated with expression in mice (20, 27, 30). Expressed sequence tags (ESTs) isolated from human gingival tissue provide an approximation of the genes expressed in that tissue. Human gingival EST libraries are not well represented in the public dbEST resource. This database comprises 5.2 million human ESTs, yet it includes only 919 ESTs from gingival tissue. These gingival ESTs represent 766 known genes, many which are structural or housekeeping genes (data not shown). Of the genes used in our ImmunoQuantArray, four (*Ifngr2*, *Il1b*, *Itgax*, and *Stat3*) overlapped with genes represented in the gingival EST libraries (9), indicating that they were expressed sufficiently in the human gingival EST libraries to be represented despite the limited library size. Our QPCR analysis revealed 16 additional immunological genes expressed basally in the gingiva (Table 3) that have not been previously catalogued in this tissue in humans. Because our murine results duplicate results from humans where the two data sets overlap, it is likely that these data are predictive of basal gene expression in the human gingiva.

To gain an understanding of immunity-related genes that distinguish the susceptible and resistant strains, we compared the basal gene expression profiles in uninfected mice. The great majority of the 48 genes analyzed failed to show expression differences between the two mouse strains. However, *Tnf* was more highly expressed in the gingiva of the susceptible strain (Fig. 2A). Tumor necrosis factor (TNF) is important in bone remodeling, and excess stimulation is associated with bone thinning (21). QPCR has shown that TNF is more highly expressed in gingivae from patients with chronic periodontitis than in healthy human gingivae (30).

In contrast, *Il15* showed greater gingival basal expression in resistant mice (Fig. 2A). IL-15 preferentially stimulates the development of CD8 T cells, as well as elements of innate immunity, including NK cells (23). CD4 T cells are associated with alveolar bone loss after *P. gingivalis* infection, while CD8 cells have no effect (2). If higher levels of IL-15 in A/J mice lead to development of more CD8 cells and fewer CD4 T cells, this could contribute to A/J bone loss resistance.

Abnormal expression of adhesion molecules on neutrophils and macrophages is implicated in the pathogenesis and susceptibility of some forms of periodontal disease (19). Several families of adhesion molecules are involved in the extravasation process and in chemotaxis. For example, neutrophil and macrophage rolling is the first step in crossing the blood vessel wall, and adhesion molecules are upregulated by endothelial cells in response to signals such as IL-1β, C5a, and TNF to aid this process. Cysteine protease and serine protease families produced by *P. gingivalis* have been found to degrade adhesion molecules (18), thus increasing the virulence of the bacteria. p-selectin (*Selp*) mRNA was found to be basally expressed at higher levels in the spleens of A/J mice than in the spleens of BALB/cByJ mice (Fig. 2B), suggesting that high levels of adhesion molecules may contribute to disease resistance. p-selectin-deficient mice lose larger amounts of alveolar bone than normal mice lose in response to *P. gingivalis* (4).

Il1b was also expressed differentially in the two strains of mice. Proinflammatory cytokines, like IL-1β, are important factors in the initiation and development of the inflammatory cascade to eliminate the bacteria. However, IL-1β can also play a role in the destruction of local tissues by stimulating bone resorption and collagenase production by fibroblasts (8, 26, 28, 36). Basal expression of *Il1b* was higher in BALB/cByJ mice than in A/J mice in both the gingiva and the spleen. Importantly, *Il1b* expression was also significantly increased in the gingiva of infected BALB/cByJ mice early in the infection process. In contrast, there were no significant changes in gene expression in A/J mice after infection. The higher BALB/cByJ basal *Il1b* expression and the elevated response to infection may result in excessive stimulation of the inflammatory cascade or bone remodeling cells and tip the balance away from homeostasis toward destruction. Similar results have been reported by Kornman et al. in humans (16, 17). A specific genotype of the polymorphic *IL-1* gene cluster is associated with severity of periodontitis in nonsmoking humans (16, 17). This *IL-1* genotype comprises of a variant of *IL-1*β that is associated with a two- to fourfold increase in IL-1β production, the range of difference reported here for *Il1b* expression in mice (Fig. 2A). A genetic propensity for increased IL-1 secretion may be a significant mechanism associated with susceptibility to the disease.

Moreover, STAT6, a key signaling molecule by which IL-4 pushes the differentiation of antigen-activated CD4 T cells toward the Th2 phenotype (39), was upregulated by infection in BALB/cByJ mice but not in A/J mice. Th2 cells secrete IL-6,

an important mediator in bone loss, and CD4 T cells and IL-6 have both been shown to be important in susceptibility to bone loss in this model (2).

The basal expression of *Opg* mRNA in the gingiva was higher in the BALB/cByJ mice than in the A/J mice. This finding was unexpected because osteoprotegerin (OPG) is an inhibitor of osteoclastogenesis. Osteoclast precursors carry the receptor activator of NF-κB (RANK) on their surfaces. When RANK binds RANKL, its ligand on osteoblasts, these precursors are stimulated to differentiate into osteoclasts (14). OPG is a soluble decoy receptor for RANKL. OPG binding to RANKL prevents its binding to RANK, inhibiting osteoclast differentiation (14). Thus, the stimulus for osteoclastogenesis depends on an imbalance between OPG and RANKL, with RANKL predominating. In our mice, it may be that both *Opg* and *Rankl* are upregulated, with *Rankl* expression being greater, or it may be that *Rankl* remains upregulated longer than *Opg*, pushing the host tissue away from homeostasis and toward osteoclastogenesis. *Opg* expression was enhanced in all mice 1 week postinfection (Fig. 3) but had returned to basal levels in all mice by 3 weeks (data not shown). In addition to expression on osteoblasts, RANKL is expressed on T cells activated by some, but not all, bacterial species (13). *A. actinomycetemcomitans* infection induces RANKL expression on CD4 T cells and leads to alveolar bone loss (34). *P. gingivalis* outer membrane proteins, however, do not induce RANKL (29). *Rankl* was not in our gene array, so we are unable to say whether *P. gingivalis* infection induces its expression in vivo or describe its relative levels compared with those of *Opg*.

It may also be that bone loss is triggered by RANK-independent pathways, so that *Opg* mRNA expression is not indicative of resistance or susceptibility. Results from clinical studies do not currently provide a clear answer. In one study the levels of RANKL protein were higher in gingival biopsies from periodontitis patients, and the levels of OPG were higher in tissue from periodontally healthy patients (10). However, in another study the workers found OPG mRNA expressed in 80% of periodontitis lesions (with gingival fibroblasts as the source), while only 25% of lesions expressed RANKL mRNA (27).

Both TNF alpha and IL-1β can stimulate osteoclastogenesis independent of RANK, as can lipopolysaccharide (14). TNF mRNA levels were significantly higher in the bone loss-susceptible BALB/cByJ mice than in the resistant A/J mice. *P. gingivalis* lipopolysaccharide induces IL-1β and bone resorption (25). *Il1b* was upregulated the first week after oral infection with *P. gingivalis* in the susceptible BALB/cByJ mice but not in the resistant A/J mice (Fig. 3). While the differences in *Il1b* expression achieved significance only during the first week as determined by our demanding statistical criteria, its expression remained elevated in some of the infected BALB/cByJ mice and did not return to basal levels in all mice until 6 weeks (data not shown). Indeed, *Opg* upregulation could be a compensatory mechanism attempting to control the osteoclastogenesis stimulated by IL-1β and TNF. The *IL1b* and *Tnf* results combined with the expression pattern of *Opg* suggest the importance of RANK-independent pathways for bone loss after *P. gingivalis* infection.

Upregulation by infection was transient, and expression returned to basal levels by the time that bone loss was macroscopically visible at 6 weeks after *P. gingivalis* infection. These results are consistent with a model in which the levels of a small subset of genes integral to osteoclastogenesis are elevated soon after infection yet the genes trigger a chronic periodontal erosive process.

These experiments implicate *Il1b* by two criteria and *Tnf* and *Stat6* by one criterion as key genes involved in susceptibility to alveolar bone loss. One of the roles of the immune system is to maintain homeostasis when it is confronted with challenges. The relatively higher basal expression levels of *Il1b* and *Tnf* in susceptible mice may predispose them to a tip in the balance away from homeostasis and toward destructive mechanisms in response to an infectious challenge. The finding that *Il1b* and *Stat6* are upregulated in infected gingivae is consistent with involvement of these genes in disruption of the homeostasis of the bone remodeling process leading to osteoclastogenesis.

Lower basal levels of *Il1b* and *Tnf* and lower responses of *Il1b* and *Stat6* to infection may contribute to the bone loss resistance of A/J mice. Higher basal levels of Il-15 mRNA in the gingivae and of p-selectin mRNA in the spleen implicate these genes as genes that are possibly associated with resistance. Our experiments associate key differentially expressed genes with alveolar bone loss, thus providing insight into the genetics of the disease pathoetiology.

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