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Adaptive immune response in osteoclastic bone resorption induced by orally administered *Aggregatibacter actinomycetemcomitans* in a rat model of periodontal disease

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Keywords: B cells; bone morphogenic proteins; bone resorption; cytokine; T cells
Accepted 5 March 2010

SUMMARY

There is mounting evidence that innate and adaptive immunity are critical for periodontal disease-mediated bone resorption. These studies examined the role of B and CD4 T cells in adaptive immunity of rats infected with *Aggregatibacter actinomycetemcomitans* (*Aa*). Sprague-Dawley male rats were fed *Aa*-containing mash or control-mash for 2 weeks. B and CD4 T cells were obtained from draining lymph nodes at 2, 4 and 12 weeks, postinoculation. Quantitative polymerase chain reaction-based messenger RNA expression was conducted for 89 cytokine family genes. Disease-relevance of the differentially expressed genes was assessed using a biological interaction pathway analysis software. B and CD4 T cells of *Aa*-infected rats increased and were activated, resulting in enhanced isotype-switched serum immunoglobulin G by 2 weeks postinoculation. Bone resorption was evident 12 weeks after *Aa*-feeding. In B cells, interleukin-2 (IL-2), macrophage-inhibiting factor, IL-19, IL-21, tumor necrosis factor (TNF), CD40 ligand (CD40L), CD70, bone morphogenetic protein 2 (BMP2), BMP3, and BMP10 were upregulated early; while IL-7, Fas ligand (FasL), small inducible cytokine subfamily E1, and growth differentiation factor 11 (GDF11; BMP11) were upregulated late (12 weeks). BMP10 was sustained throughout. In CD4 T cells, IL-10, IL-16, TNF, lymphotoxin-beta (LTβ), APRIL, CD40L, FasL, RANKL and osteoprotegerin were upregulated early, whereas IL-1β, IL-1RN, IL-1F8, IL-24, interferon-α1, GDF11 (BMP11), and GDF15 were upregulated late (12 weeks). Adaptive immunity appears crucial for bone resorption. Several of the deregulated genes are, for the first time, shown to be associated with bone resorption, and the results indicate that activated B cells produce BMP10. The study provides a rationale for a link between periodontal disease and other systemic diseases.

INTRODUCTION

Periodontal disease is a pandemic and costly disease that is characterized as an inflammatory reaction to bacterial infection, which involves both the innate and adaptive arms of the immune system. The disease can lead to bone resorption, and has also been

conjectured to increase the risk for heart and other systemic diseases (Albandar, 2002; Offenbacher *et al.*, 2007). The spectrum of clinical conditions that characterize the disease correlate with host responses to oral bacteria including *Aggregatibacter actinomycetemcomitans* (*Aa*) (Ebersole *et al.*, 1983) and *Porphyromonas gingivalis* (Ebersole *et al.*, 1986). It has been determined that *Aa*, a gram-negative facultative capnophilic rod, is the causative agent in localized juvenile periodontitis (LJP) (Zambon, 1985). This agent is also believed to be a key pathogen for localized progressing and severe forms of adult periodontitis (Dzink *et al.*, 1985; Zambon *et al.*, 1988). *Aa* releases several virulence factors, including endotoxin and leukotoxin (Fives-Taylor *et al.*, 1999). The infection is accompanied by local and systemic humoral immune responses (Ebersole & Taubman, 1994). In earlier studies, altered CD4/CD8 T-cell ratios and autologous mixed lymphocyte reaction in LJP suggested a potential regulatory role of T cells in periodontitis (Kinane *et al.*, 1989).

A relationship between bacterial infection and periodontal bone resorption has been well established, and the roles played by inflammatory mediators in the bone resorption process have been studied at various levels. Inhibition of interleukin-1 (IL-1) and tumor necrosis factor (TNF) was found to inhibit bone loss in experimental periodontitis (Assuma *et al.*, 1998; Graves *et al.*, 1998). A longitudinal study on subjects susceptible to localized aggressive periodontitis showed a 50-fold increase in macrophage inflammatory protein 1α (MIP-1α), as well as elevation of IL-1β (Fine *et al.*, 2009). Employing an experimental rat adoptive transfer/gingival challenge periodontal disease model, it was demonstrated that antigen-specific T lymphocytes and gingival instillation of antigen and lipopolysaccharide were required for bone resorption (Taubman *et al.*, 2005). T helper 17 cytokine (IL-17) has been identified as a proinflammatory cytokine that induces other cytokines and tissue-degrading enzymes, including IL-6 and matrix metalloproteinases. It has been shown to be involved in osteoclastogenesis by inducing receptor activator of nuclear factor-κB ligand (RANKL) expression on osteoblasts (Kotake *et al.*, 1999); and expression of IL-17 has been observed in gingiva from patients with periodontitis (Cardoso *et al.*, 2009).

A role for RANKL in T-cell-mediated bone resorption was demonstrated in this rat model. Interference with RANKL by systemic administration of osteoprotegerin (OPG), the decoy receptor for (and inhibitor of) RANKL, was found to result in abrogation of periodontal bone resorption in this rat model (Taubman *et al.*, 2005). Recent studies on humans have demonstrated that RANKL levels in gingival crevicular fluid were low in healthy or gingivitis groups, but increased in periodontitis. On the other hand, OPG levels were higher in healthy groups than in periodontitis or gingivitis groups (Bostanci *et al.*, 2007). Hence, RANKL and OPG levels in gingival crevicular fluid were oppositely regulated in periodontitis, but not gingivitis, resulting in an enhanced RANKL/OPG ratio.

Irrespective of which inflammatory mediators trigger bone loss in periodontal disease, it has been observed that the uncoupling of the process of bone formation from that of bone loss, which is likely to contribute to a net bone loss, is an important predisposition factor in periodontal bone resorption (Graves, 2008). A rat model that closely mimics human periodontal disease was developed from *Aa* strain CU1010, which binds avidly to surfaces. The model provided convincing evidence that, unlike laboratory variants, the clinical isolates colonize, persist and integrate into an already established econiche (Fine *et al.*, 2001). This model was used to develop a reproducible method of evaluating bone loss in a rodent model of periodontal disease (Fine *et al.*, 2009). We have used the *Aa* rat model to address the question of how cytokines and related proteins of draining lymph node B and CD4 T cells contribute to periodontal disease and bone resorption.

METHODS

Bacterial strains, culture conditions and maintenance

Wild-type *Aa* CU1000N Rif, was originally isolated from a patient with aggressive periodontitis and maintained in its rough, adherent form on *Aa* growth medium (AAGM) agar (Fine *et al.*, 1999). Bacteria were grown in 100 ml AAGM containing 35 μg/ml rifampicin (Rif) antibiotic in tissue culture flasks in an atmosphere of 10% CO_2/90% air for 2–3 days at 37°C. *Aa* cells that adhered to the wall of the flask were removed by scraping. Cells were resuspended in phosphate-buffered saline (PBS) containing 3% sucrose, vortexed and resuspended to an approximate

optical density of 0.8, equivalent to 10^8 cells/ml, as previously described (Fine *et al.*, 2009).

Animals

Management of the rat model has been previously described (Schreiner *et al.*, 2003). Twenty-five specific-pathogen-free, Sprague-Dawley male rats (5–10 weeks of age) weighing 150–250 g were purchased from Taconic farms (Hudson, NY), housed in separate cages and fed powdered food (Laboratory Rodent Meal Diet 5001, Purina Mills Feeds, St Louis, MO). To depress the 'natural' resident flora, rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days. During the last 2 days of antibiotic treatment, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex, Procter and Gamble, Cincinnati, OH). After a subsequent period of 3 days without antibiotic treatment, the 24 rats were divided into two groups of 12 each. Group 1 was fed wild-type strain CU1000N Rif; group 2 was fed carrier food without *Aa*, and served as uninoculated control. To insure that rats consume the inocula, they were fasted for 3–4 h before feeding. The study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey: Protocol approval number 06040A2.

Feeding of animals

After fasting for 3 h, animals in Group 1 were provided with 1 g powdered food placed in special feeder trays containing 10^8 *Aa* cells in 3% sucrose in PBS. Group 2 received food containing 3% sucrose in PBS but devoid of any bacterial inoculum. After 1 h the inoculated food was removed and replaced with regular powdered food. The inoculation-feeding regimen was repeated for 8 days (Schreiner *et al.*, 2003).

Antibody reagents

Anti-CD32 (clone D34-485), for blocking FcγII receptor, was purchased from BD Biosciences (San Jose, CA). The following immunomagnetic microbead-labeled antibodies were obtained from Miltenyi Biotec, Inc. (Auburn, CA): anti-CD4 (clone OX-38) and CD45RA (clone OX-33). Anti-IA [major histocompatibility complex (MHC) class II, clone 14-4-4S] antibody obtained from American Type Culture Collection (Manassas, VA) was fluorescein isothiocyanate-conjugated by us. Alkaline phosphatase (AP) -conjugated goat anti-rat immunoglobulin G (IgG) was purchased from Sigma-Aldrich (St Louis, MO); and AP-conjugated goat anti-rat IgG2a, IgG2b and IgG2c antibodies were obtained from Bethyl Laboratories, Inc. (Montgomery, TX). FASTTM p-nitrophenyl phosphate substrate tablets were purchased from Sigma-Aldrich.

Sampling of rat oral microflora

All samples were obtained after rats were anesthetized with isofluorine gas. Following the last inoculation, rats were placed on a diet of regular powdered food. Two weeks later the rats were anaesthetized and their oral microflora was sampled with a cotton tip swab for soft tissue sampling, and with a balsa wood toothpick (Johnson & Johnson, Piscataway, NJ) for hard tissue sampling. Both samples were placed in individual tubes containing 1 ml PBS.

Determination of *Aa* colonization

To determine that *Aa* indeed colonized the oral cavities of the rats, toothpick and cotton tip swab samples were obtained from oral cavities of the *Aa*-fed and control rats. Samples (0.1 ml) obtained from soft and hard tissue surfaces in individual rats were plated on AAGM and AAGM containing rifampicin at a concentration of 35 μg/ml for recovery of *Aa* used in the feeding experiment. The plates were incubated for 2 days in an atmosphere of 10% CO_2 at 37°C. To further identify *Aa* in the recovered bacteria, the catalase test was used. To finally confirm that *Aa* was recovered, polymerase chain reaction (PCR) was performed using leukotoxin-specific primers. The forward and reverse primers (5′-GGAATTCCTAGGTATTGCGAAACAATTTGATC-3′ and 5′-GGAATTCCTGAAATTAAGCTGGTAATC-3′, respectively) amplified a 262-base-pair (bp) PCR product from the *Aa leukotoxin* gene, as previously described (Goncharoff *et al.*, 1993). Negative controls using *Escherichia coli* template DNA and reaction mix minus template DNA were included. To ensure that the reactions not showing the leukotriene-specific bands had adequate template DNA and could support a PCR, a positive PCR control was run using primers for bacterial ribosomal DNA (RRN primers).

These forward and reverse primers (5′-CAGGATTAGATACCCTGGTAGTCCACGC-3′ and 5′-GACGGGCGGTGTGTACAAGGCCCGGGAACG-3′ amplified a 625-bp product, also as described by Goncharoff *et al.* (Goncharoff *et al.*, 1993).

Assays for *Aa* and leukotoxin-specific antibody production

The IgG antibody, reactive with *Aa*, was assessed by enzyme-linked immunosorbent assay (Engvall & Perlmann, 1971; Smith, 1995). For this purpose, preinoculation tail bleeds were taken from rats in each experimental group. Blood was collected from all rats by cardiac puncture after their sacrifice. The wells of microtiter dishes were coated with the primary antigen (50 μl of a 1 : 50 dilution of *Aa* cell lysate) and left overnight at 4°C. After washing and blocking steps, 50 μl of 1 : 200 dilutions of serum samples were added to the wells coated with bacterial lysate. After incubation, washings and blocking, 50 μl of appropriately diluted secondary antibodies were added, followed by appropriate dilutions of AP-conjugated goat anti-rat IgG2a, IgG2b and IgG2c antibodies. After two hour incubation period, 1 mg/ml substrate solution of *p*-nitrophenyl phosphate was added and the amount of product was measured with a Bio-Rad Benchmark microplate reader at 405 nm (Bio-Rad Laboratories, Hercules, CA), using Microplate Manager III Macintosh data-analysis software. To identify the level of background reaction to the antigen, antigen from each inoculated bacterial strain used for a particular group was compared with control rat sera. All assays were performed in triplicate. *Aa*-specific antibody production induced by *Aa* (corrected for background) was expressed as absorbance units for serum from *Aa*-fed rats in comparison to serum from control rats.

For assay for leukotoxin-specific antibody production, a similar procedure employed in the determination of *Aa*-specific antibody production was used. *Aa* leukotoxin at 0.2 μg/ml (provided by Dr Nataliya Balashova of the NJDS Oral Biology Department) was used at 1 : 200 dilution to coat microtiter plate wells. Sera and secondary antibodies were added as described above. Leukotoxin-specific antibody production induced by *Aa* (corrected for background) was expressed as absorbance units for serum from *Aa*-fed rats in comparison to serum from control rats.

Cell isolation and analysis

At the ends of 2-, 4- and 12-week periods, single-cell suspensions were obtained from the submandibular and cervical lymph nodes of the rats. Lymphocyte populations were isolated by Ficoll–Hypaque density gradient centrifugation. $CD4^+$ T cells were positively isolated using immunomagnetic bead-labeled anti-rat CD4. B cells were positively isolated using microbead-labeled anti-rat CD45RA. Flow cytometry was conducted on unfractionated and purified B-cell and CD4 T-cell fractions. FlowJo software (Tree Star, Inc., Ashland, OR) was used to analyse the fluorescence-activated cell sorting (FACS) data.

Real-time quantitative SuperArray gene expression profile of B and CD4 T cells

Total RNA was extracted from the purified B and $CD4^+$ T cells, using Stat-60 (Tel-Test, Inc., Friendswood, TX), followed by treatment with DNaseI (Applied Biosystems/Ambion, Inc., Austin, TX). Complementary DNA was synthesized using Superscript III reagents (Invitrogen Life Technologies, Carlsbad, CA). For real-time quantitative PCR, a rat Common Cytokines RT^2 Profiler™ PCR Array was employed (SABiosciences, Frederick, MD). The list of genes is shown in Table 1. For data analysis, adjusted Ct value of each set of genes, within the experimental sample, was acquired by normalizing the raw Ct value to the average of a set of five housekeeping genes on each superarray plate: Ribosomal protein, large, P1 (*RPLP1*); hypoxanthine phosphoribosyltransferase 1 (*HPRT1*); ribosomal protein L13A (*RPL13a*); lactate dehydrogenase A (*LDHa*); and beta actin (*ACTb*). ΔCt was calculated as ΔCt = normalized Ct value of *Aa*-treated rat sample–normalized Ct value of control rat sample. The expression value plotted in the tables is $2^{\Delta Ct}$ ($\log_2$ fold-change).

Assay for bone loss

The rat maxillae were cleaned and defleshed by dermestid beetles (Carolina Biological Supply Company, Burlington, NC). Fine cleaning was performed with micro-tools under a dissecting microscope. The maxillae were stained with 1% methylene blue so that the cemento–enamel junction could be distinguished. Photographs were taken of the stained jaws using a

Table 1 List of genes employed in SuperArray gene expression of B and CD4 T cells

Gene families	Genes					
Interferons	TNFα1	IFNα2	IFNα4	IFNβ1	IFNγ	IFNα5
Interleukins	IL-10	IL-11	IL-12a	IL-12b	IL-13	IL-15
	IL-16	IL-17b	IL-17f	IL-18	IL-19	IL-1a
	IL-1b	IL-1f10	IL-1f5	IL-1f6	IL-1f8	IL-1rn
	IL-2	IL-21	IL-24	IL-3	IL-4	IL-5
	IL-6	IL-7	IL-9	IL-17a	IL-20	IL-22
	IL-27	IL-1f9				
Bone morphogenic	BMP1	BMP10	BMP2	BMP3	BMP4	BMP5
proteins (BMPs) and	BMP6	BMP7	GDF1	GDF10	GDF11	GDF15
Transforming growth	GDF5	GDF8	GDF9	INHA	INHBa	GDF2
factor-β family	GDF3					
Tumor necrosis	TNFSF7	TNFSF13b	LTα	LTβ	TNF	TNSRSF11b
factor superfamily	TNFSF10	TNFSF11	TNFSF12	TNFSF13	TNFSF15	TNFSF18
	TNFSF4	TNFSF5	TNFSF6	TNFSF9		
Other growth factors/cytokines	CSF1	CSF2	CTF1	CTF2	FGF10	LIF
	FBS1	MIF	SCGB3a1	SCYE1		

DP12 microscope digital camera system (Olympus) at a 9.2 × magnification. The jaws were positioned using soft wax with the lingual side facing up with the occlusal plane of the molars perpendicular to the microscope stage. To measure horizontal bone loss, the photographs were printed on an Epson Stylus Photo R320 (Epson America Inc., Long Beach, CA) on 4 × 6 glossy paper (Kodak Ultima Picture Paper, Eastman Kodak Co., Rochester, NY). The images were coded so that the examiner did not know the treatment group of the jaws measured. The vertical distance between the cemento–enamel junction and the alveolar bone crest was measured at 10 sites on the three maxillary molars on each sides of the jaw (sites A to J) in duplicate by two examiners, as previously reported (Fine *et al.*, 2009). Both right and left maxillary jaws were measured. The distance was measured in millimeters by putting a pencil-mark dot on the anatomical landmarks on the photo and using an electronic digital caliper (Marathon Watch Co. Ltd, Richmond Hill, Ontario, Canada) to measure the distance between the dots.

The raw bone loss data were decoded and sent to an independent statistician who used SAS 9.1 software (SAS Institute Inc., Cary, NC) for the analysis. The total bone loss for each rat and each group was compiled using the mean data for the two readings for each of the two examiners. The repeat readings of each examiner and the comparison of readings between examiners showed good correlation when tested by Pearson's correlation coefficient. The means of the total bone loss were compared between the *Aa*-fed group and its non-inoculated control group at the different time points by unpaired *t*-test. To determine disease, the mean and standard deviation of total bone loss at each site were calculated for the control (non-bacteria-fed) group at each time point. The means of the right and left sides were calculated separately. A diseased site was defined as a site where the measurement of bone loss (as defined as the distance between the cemento–enamel junction and the bone crest) was greater than two standard deviations above the mean of the bone loss measurement for that site in the control group. If a rat had two or more diseased sites per jaw side, it was considered a diseased rat. To compare the associations between groups (*Aa*-fed versus control rats) and disease incidence, the treatment groups within the treatment groups were compared using Fisher's Exact Test. The results were considered significant if $P < 0.05$. The results of the determination of diseased rats between the two readings of each examiner and between the examiners were compared using a measure of agreement (kappa) score (Fine *et al.*, 2009).

Examination of biological interaction pathways of differentially expressed genes

The data obtained from the SuperArray gene expression experiment were imported into biological relationship-finding software (PATHWAY STUDIO v7, Ariadne

Genomics, Inc., Rockville, MD). This versatile data-mining algorithm (Nikitin *et al.*, 2003), which employs MedScan, a natural language processing engine for MEDLINE abstracts (Novichkova *et al.*, 2003), was used to query published literature using the RESNET 7 database (Yuryev *et al.*, 2006). Genes with at least four-fold expression change (i.e. $\log_2$-fold >2) were imported into PATHWAY STUDIO for analyses. The following rules were applied to generate biological association networks: (i) the algorithm type was common targets; (ii) the entity types were protein and disease; (iii) the filter was regulation. The literature evidence for each relation in the final pathway was manually inspected for accuracy and relevance. This approach has proved valuable as the next generation tool for drug discovery (Suderman & Hallett, 2007).

RESULTS

Recovery of *Aa* from oral cavities of *Aa*-fed rats

To ascertain that the rats fed *Aa* were infected by the inoculated bacteria, soft and hard tissue samples obtained from the oral cavities of the *Aa*-fed and control rats were examined 2 weeks after the last feeding. Bacterial colonies were discernible within 2 days of culture of samples from all the *Aa*-fed rats, grown on AAGM containing rifampicin, but not from the control rats. Additionally, the bacterial colonies harvested from the samples obtained from the *Aa*-fed rats all tested positive in the catalase test, further indicating that the *Aa* was recovered from the oral cavities of the *Aa*-fed mice. Finally, leukotoxin-specific PCR conducted on samples obtained from the oral cavities, amplified a 262-bp PCR product in all the samples obtained from *Aa*-fed, but not in those from the control rats. Samples from all rats showed the amplified 625-bp RRN primer product positive control, confirming the presence of bacteria. A representative gel of the PCR products is show in Fig. 1.

Lymphocyte activation after *Aa* feeding

Lymphocytes, isolated over Ficoll–Hypaque density gradient, were enumerated at 2, 4 and 12 weeks postfeeding of *Aa*. Lymphocyte counts were significantly ($P < 0.05$) elevated in the *Aa*-fed rats than in control rats at the early time periods of 2 and 4 weeks post feeding (Fig. 2A). The B and CD4

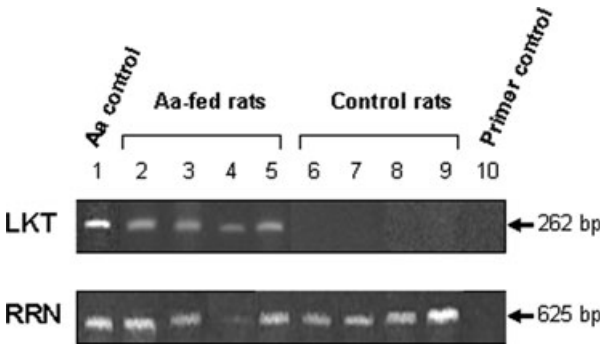

Figure 1 Result of polymerase chain reaction (PCR) conducted with cultures from *Aggregatibacter actinomycetemcomitans* (*Aa*) - fed and control rats. Lane 1 represents a positive control obtained from a culture plate of the original *Aa* used in the experiment. Lanes 2–5 represent samples from *Aa*-fed rats, and lanes 6–9 represent samples from control rats. Lane 10 represents PCR primer control (without template). As there were no detectable bacterial colonies on the *Aa* growth medium + rifampicin agar plates for the control rats, the broth was used to conduct the PCR. Samples from control rats did not show detectable *Aa*, as indicated by the absences of the *Aa*-specific 262-base-pair (bp) leukotoxin (LKT) PCR product (top panel). The bottom panel shows amplification of a 625-bp RRN PRC product for the *Aa*-fed and control rats, confirming the presence of bacteria in all the samples.

T-cell numbers were also elevated in the cervical and submandibular lymph nodes of *Aa*-fed rats compared with the same nodes from control rats. The difference was only statistically significant ($P < 0.05$) for B cells at 12 weeks of sampling. FACS analysis of the lymphocytes from the rats indicated that B cells from *Aa*-fed rats upregulated their MHC class II (I-A). The fluorescence intensity for MHC class II expression, determined at 12 weeks postfeeding, was significantly enhanced ($P < 0.05$) for B cells from *Aa*-fed rats, compared with B cells from control rats (Fig. 3).

Enhanced *Aa*-specific antibody production postfeeding

In the early phase of the feeding experiment (weeks 2 and 4 combined), *Aa*-specific IgG and IgG2a responses were significantly ($P < 0.05$) enhanced in rats fed *Aa* compared with control rats. Although a subtle increase in production of IgG2b *Aa*-specific antibodies was observed, this increase was not statistically significant (Fig. 4A). The differences, however, disappeared by week 12 (Fig. 4B). Leukotoxin-specific serum IgG antibody production was also significantly ($P < 0.05$) increased in rats fed *Aa*,

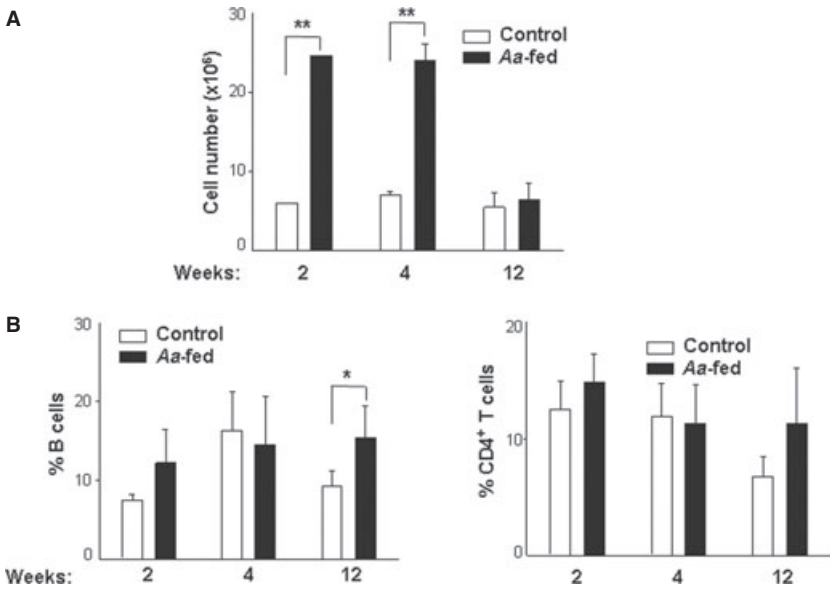

Figure 2 Analysis of lymphocyte populations obtained from draining cervical and submandibular lymph nodes of *Aggregatibacter actinomycetemcomitans* (*Aa*) -fed rats (*Aa*), compared with control rats (Control). (A) Number of viable lymphocytes recovered from lymph nodes of *Aa*-fed rats (*Aa*), compared with control rats (Control). (B) Percentage of viable B and CD4 T cells recovered from positive isolation over microbead-labeled anti-CD45RA and anti-CD4 columns, respectively. The number of rats assayed for each of *Aa*-fed and control rats, were: three for *Aa*-fed, and two for control rats (at 2 weeks postfeeding); three rats each for *Aa*-fed and control rats (at 4 weeks postfeeding); and, seven rats each for *Aa*-fed and control rats (at 7 weeks postfeeding). The data are represented as mean ± SEM. Statistics were calculated by two-tailed *t*-test; $^{*}P < 0.05$; $^{**}P < 0.001$.

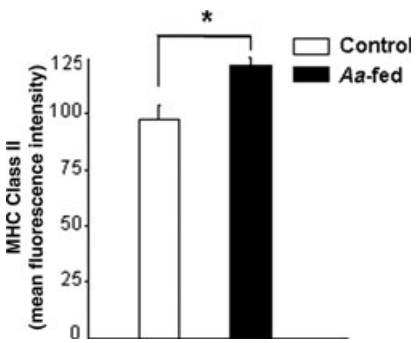

Figure 3 Examination of activation status of B lymphocytes obtained from cervical and submandibular lymph nodes, from *Aggregatibacter actinomycetemcomitans* (*Aa*) -fed rats compared with control rats at 12 weeks postfeeding. Histogram represents mean fluorescence intensity of major histocompatibility complex (MHC) class II expression in lymphocytes obtained from lymph nodes of three *Aa*-fed rats, compared with those from four control rats. The data are represented as mean ± SEM. Statistics were calculated by two-tailed *t*-test; $^{*}P < 0.05$.

compared with control rats, by weeks 2 and 4 combined (Fig. 4C), but not by week 12 (Fig. 4D). No leukotoxin-specific IgG2c antibody production was detectable at any of the time points.

Differential gene expression in lymph node B and CD4 T cells of *Aa*-fed rats

The B and CD4 T-cell activation, coupled with *Aa*-specific immunoglobulin isotype switching, observed after feeding of *Aa* to rats would be expected to drive cytokine production programs that could explain the bone resorption that accompanies *Aa*-induced periodontal disease. We, therefore, examined total RNA extracted from cervical and submandibular lymph nodes draining the oral cavity, for cytokine gene expression. Multiplex real-time quantitative SuperArray analysis was conducted on purified (positively selected) B cells and CD4 T cells obtained from the lymph nodes of *Aa*-fed and control rats, at 2, 4 and 12 weeks postfeeding. A total of 89 cytokine family genes, including those for inflammatory cytokines and bone morphogenic proteins were examined (Table 1). A stepwise examination of gene expressions of B and CD4 T cells at specific time points after infection with *Aa* would shed light on the biological processes leading to inflammatory responses that ultimately result in bone resorption, after bacterial infection. The approach would also help to clarify the

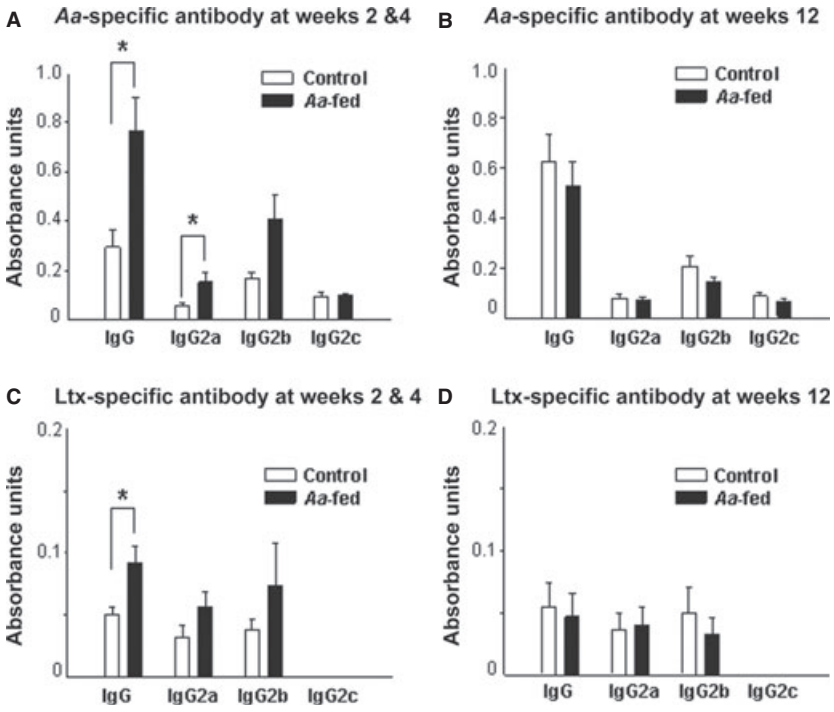

Figure 4 Induction of antibody production (shown as absorbance units) for *Aggregatibacter actinomycetemcomitans* (*Aa*) -fed rats compared with control rats. (A) *Aa*-specific antibody production for serum collected from *Aa*-fed and control rats at 2 and 4 weeks (five *Aa*-fed rats and five control rats), after initiation of feeding. (B) *Aa*-specific antibody production for serum collected from *Aa*-fed and control rats at 12 weeks (seven *Aa*-fed rats and seven control rats), after initiation of feeding. (C) Leukotoxin-specific antibody production for serum collected from *Aa*-fed and control rats at 2 and 4 weeks (five *Aa*-fed rats and five control rats), after initiation of feeding. (D) Leukotoxin-specific antibody production for serum collected from *Aa*-fed and control rats at 12 weeks (seven *Aa*-fed rats and seven control rats), after initiation of feeding. The data are represented as mean ± SEM. Significant differences in antibody production in *Aa*-fed rats, compared with control rats, are indicated by $*P < 0.05$.

basis of other systemic diseases that accompany *Aa* infection.

The gene expression program in the early phase (2 and 4 weeks) of the response was different, in many ways, from the genes differentially induced at 12 weeks. By 2 weeks of feeding, five genes [those for bone morphogenetic protein 10 (BMP10); IL-2, IL-19; IL-21 and macrophage migration inhibitory Factor (MIF)] were upregulated by more than four-fold in B cells from *Aa*-fed rats (Table 2). Another set of 26 genes were downregulated in B cells by more than four-fold by 2 weeks. By 4 weeks, a similar set, plus additional genes [BMP2, BMP3, BMP10, TNF, IL-2, IL-19, MIF, CD40 ligand (CD40L), and CD70] were upregulated in B cells in mice fed *Aa* (Table 2). Almost the same set of genes were downregulated in B cells by 4 weeks of feeding *Aa*. By 12 weeks of feeding BMP10, B-cell activating factor (BAFF, TNFSF13B), TNFSF6 (FasL), IL-7, small inducible cytokine subfamily E (SCYE1), and growth differentiation factor 11 (GDF11) were upregulated by at least four-fold in B cells from *Aa*-fed rats. A comparatively smaller set of 12 genes were downregulated in B cells by 12 weeks (compared with 2 or 4 weeks).

With respect to lymph node CD4 T cells, seven genes were differentially upregulated by at least four-fold in *Aa*-fed rats at 2 weeks (IL-10, IL-16, lymphotoxin β (LTβ), TNF, FasL, APRIL, TNFSF13 and CD40L). Nineteen genes were downregulated in CD4 T cells from *Aa*-fed rats at 2 weeks (Table 3). By 4 weeks a similar set of seven genes were differentially upregulated in lymph node CD4 T cells from *Aa*-fed rats [IL-10, IL-16, LTβ, APRIL, TNFSF13, CD40L, RANKL, TNFSF11 and OPG (TNFSF11b)]. As with B cells, a larger number of 22 genes were downregulated in lymph node CD4 T cells in *Aa*-fed rats (Table 3). By 12 weeks of feeding, five genes were differentially upregulated in lymph node CD4

Table 2 Differentially expressed genes in B cells from *Aggregatibacter actinomycetemcomitans*-infected rats

Gene	Common Name	Fold Change (Log_2)		
		2 weeks	4 weeks	12 weeks
Upregulated genes				
BMP10	Bone morphogenetic protein 10	7.3	7.7	3.0
IL-19	Interleukin-19	10.3	10.3	–
IL-2	Interleukin-2	8.5	8.5	–
MIF	Macrophage migration inhibitory factor	3.8	11.9	–
IL-21	Interleukin 21	8.6	–	–
BMP2	Bone morphogenetic protein 2	–	5.6	–
BMP3	Bone morphogenetic protein 3	–	6.0	–
TNF	Tumor necrosis factor-alpha	–	3.0	–
TNFSF5	CD40 ligand	–	3.3	–
TNFSF7	CD70 (TNF superfamily member 7)	–	7.1	–
TNFSF13B	BAFF (TNF superfamily member 13B)	–	–	2.4
TNFSF6	Fas ligand (TNF superfamily member 6)	–	–	2.9
IL-7	Interleukin 7	–	–	3.4
SCYE1	Small inducible cytokine subfamily E, member 1	–	–	3.6
GDF11	Growth differentiation factor 11 (BMP11)	–	–	2.9
Downregulated genes				
BMP1	Bone morphogenetic protein 1	−9.0	−11.4	−2.7
CTF1	Cardiotrophin 1	−8.8	−13.6	−4.4
GDF10	Growth differentiation factor 10	−8.3	−18.1	−4.5
IL-11	Interleukin 11	−8.1	−12.8	−4.1
IL-12a	Interleukin 12a	−14.2	−15.3	−2.3
IL-18	Interleukin 18	−10.9	−9.3	4.6
INHBa	Inhibin beta-A	−9.0	−14.6	−4.3
BMP4	Bone morphogenetic protein 4	−17.9	−11.2	–
GDF11	Growth differentiation factor 11	−7.0	−17.7	–
GDF15	Growth differentiation factor 15	−10.3	−11.5	–
GDF5	Growth differentiation factor 5	−8.0	−9.2	–
IFNa2	Interferon alpha family, gene 2	−6.6	−17.6	–
IL-13	Interleukin 13	−9.3	−12.0	–
IL-15	Interleukin 15	−11.3	−11.3	–
IL-17b	Interleukin 17B	−9.8	−12.8	–
IL-1α	Interleukin 1 alpha	−14.9	−11.6	–
IL-1β	Interleukin 1 beta	−14.8	−12.9	–
IL-1F10	Interleukin 1 family, member 10	−11.2	−14.4	–
IL-1F8	Interleukin 1 family, member 8	−9.0	−11.4	–
IL-1rn	Interleukin 1 receptor antagonist	−6.7	−10.3	–
IL-6	Interleukin 6	–	−11.7	−3.7
IL-7	Interleukin 7	−17.0	−20.2	–
LIF	Leukemia inhibitory factor	−16.3	−10.2	–
TNFSF4	OX40L (TNF superfamily member 4)	−13.0	−16.9	–
TNFSF13B	BAFF (TNF superfamily member 13B)	−13.2	–	–
BMP2	Bone morphogenetic protein 2	–	–	−4.3
TNFRSF11b	OPG	–	–	−8.0
CTF1	Cardiotrophin 1	−8.8	−13.6	−4.4

T cells (IL-1β, IL-24, OPG, growth differentiation factor 11, GDF11 and GDF15), whereas eight genes were differentially downregulated in lymph node CD4 T cells at 12 weeks.

Bone loss induced by *Aa* feeding

At the end of 2-, 4- and 12-week periods after feeding *Aa*, cleaned and defleshed maxillae of a group of

Table 3 Differentially expressed genes in CD4 T cells from *Aggregatibacter actinomycetemcomitans* infected rats

		Fold change (Log_2)		
Gene	Common name	2 weeks	4 weeks	12 weeks
Upregulated genes				
IL-10	Interleukin 10	2.9	8.3	–
IL-16	Interleukin 16	6.1	5.3	–
LTβ	Lymphotoxin B	4.2	4.5	–
TNFSF13	APRIL	3.3	6.4	–
CD40L	CD40 Ligand	5.2	5.5	–
FasL	Fas ligand	4.8	–	–
TNF	Tumor necrosis factor (TNF superfamily, member 2)	2.6	–	–
IFNα1	Interferon alpha 1	–	–	3.7
GDF11	Growth differentiation factor 11 (BMP11)	–	–	3.5
GDF15	Growth differentiation factor 15	–	–	2.8
IL-1β	Interleukin 1 beta	–	–	3.4
IL-1RN	Interleukin 1 receptor antagonist	–	–	3.5
IL-1F8	Interleukin 1 family, member 8	–	–	3.0
IL-24	Interleukin 24	–	–	3.5
TNFSF11	RANKL	–	7.8	–
TNFRSF11b	OPG	–	15.2	15.3
Downregulated genes				
BMP1	Bone morphogenetic protein 1	−10.5	−4.4	−2.3
BMP3	Bone morphogenetic protein 3	−7.1	−7.6	−0.5
IL-17b	Interleukin 17B	−12.4	−5.5	−3.9
IL-9	Interleukin 9	−5.4	−2.9	−3.2
BMP2	Bone morphogenetic protein 2	−10.8	−4.2	–
CTF1	Cardiotrophin 1	−11.7	−3.6	–
GDF15	Growth differentiation factor 15	−5.3	−4.7	–
GDF5	Growth differentiation factor 5	−6.7	−3.6	–
GDF8	Growth differentiation factor 8	−6.7	−5.9	–
IL-11	Interleukin 11	−9.6	−4.0	–
IL-18	Interleukin 18	−7.7	−7.8	–
IL-1α	Interleukin 1 alpha	−8.9	−5.5	–
IL-1β	Interleukin 1 beta	−9.4	−0.2	–
IL-1F8	Interleukin 1 family, member 8	−9.3	−5.4	–
IL-21	Interleukin 21	−4.7	−7.2	–
INHBa	Inhibin beta-A	−11.0	−3.8	–
LIF	Leukemia inhibitory factor	−6.7	−3.6	–
TNFSF15	Tumor necrosis factor Superfamily, member 15	−5.5	−5.9	–
IL-2	Interleukin 2	−4.4	–	–
IL-6	Interleukin 6	–	−3.6	–
BMP4	Bone morphogenetic protein 4	–	−4.8	–
SCYE1	Small inducible cytokine subfamily E, member 1	–	−8.5	–
INHa	Inhibin alpha	–	−7.9	−3.3
CSF1	Colony stimulating factor 1 (macrophage)	–	–	−3.2
TNFSF9	Tumor necrosis factor superfamily, member 9	–	–	−2.7
IL-19	Interleukin 19	–	–	−5.1

rats were examined for bone loss and incidence of disease. The direct visual method (DVM) was used for the measurement of bone loss as previously described previously (Fine *et al.*, 2009). The sites used for bone loss measurement are described in Fig. 5. Even though evidence for bone loss as determined by DVM was beginning to develop as early as 2 weeks after *Aa* feeding, a statistically significant ($P < 0.05$ by unpaired *t*-test) difference in mean total bone loss between the *Aa*-fed and control groups was only seen at 12 weeks (Fig. 6). Using our disease analysis method (Fine *et al.*, 2009), a significant

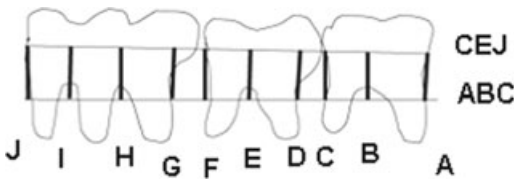

Figure 5 Sites for bone loss. The figure depicts the direct visual method (DVM) of measurement of bone loss using digital photographs taken by a camera attached to a dissecting microscope set at low magnification. Distance from the cemento–enamel junction (CEJ) to alveolar bone crest (ABC) was measured for 10 points indicated on the back three molars, (A–J). A diseased rat has two or more diseased sites where bone loss measurement is greater than the control mean (bone loss) plus two standard deviations on the same side (Fine *et al.*, 2009).

difference in disease was seen between the *Aa*-fed and control groups at 12 weeks ($P < 0.05$) by Fisher's exact test (Fig. 6).

Examination of biological interaction pathways of differentially expressed genes

To examine the mechanisms by which the differentially expressed genes in lymph nodes from *Aa*-fed rats might impact on bone resorption, the genes that were upregulated by at least four-fold in B and CD4 T cells of *Aa*-fed rats were imported into PATHWAY STUDIO software, to build Biological Interaction Networks based on published literature derived from the RESNET 7 database. Gene expression signatures for B and CD4 T cells for the early phase of disease were evident by 2 weeks, and were similar by 4 weeks (Tables 2 and 3). Dramatic differences in differential gene expression were evident at 12 weeks, at which time bone resorption was clearly observed (Fig. 6).

Upregulated genes that featured prominently, with disease relationships, by 12 weeks postinfection, include IL-1 receptor antagonist (IL-1RN), IL-1 family member 8 (IL-1F8), IL-1F10, IL-7, IL-24, BAFF (TNFSF13B), GDF11 (or BMP11), GDF15, BMP10, and SCYE1. At 12 weeks postinfection, there is strong evidence of bone resorption (Fig. 6). This is accompanied by activation of an elaborate disease-related network (Fig. 7). Such diseases include periodontitis, inflammation, arthritis, bone loss, cardiovascular disease, lymphoma, leukemia, diabetes and autoimmune disease.

DISCUSSION

There is no doubt that inflammatory cytokines play an important role in the etiology of bone resorption that develops from periodontal disease. However, the contribution of lymphocyte subpopulations to the disease has not been well defined. Our studies provide more insight into the contribution of B and CD4 T cells to the disease, and address the biological networks that could be used by the differentially

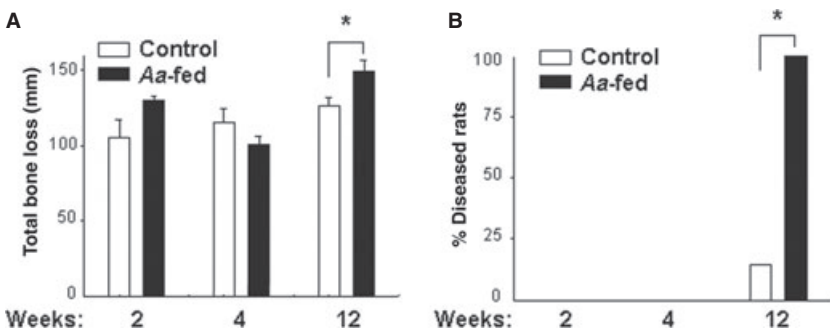

Figure 6 Bone loss and disease incidence in *Aggregatibacter actinomycetemcomitans* (*Aa*) -fed rats compared with control rats at 2 weeks (three *Aa*-fed rats and two control rats), 4 weeks (three *Aa*-fed rats and three control rats) and 12 weeks (seven *Aa*-fed rats and seven control rats postfeeding. (A) Total bone loss in right and left maxillary jaws determined by direct visual measurement (Fine *et al.*, 2009). Microscopic measurements (in mm) were determined at 9.2 × magnification. The data are represented as mean ± SEM The average total bone loss between control and *Aa*-fed animals was compared by unpaired *t*-tests at each time point. The groups were significantly different at 12 weeks $P < 0.05$. (B) Percentage of rats determined to be diseased by virtue of exhibiting significant bone loss (Fine *et al.*, 2009). Numbers of diseased rats in the *Aa*-fed and groups at each time point were compared by Fisher's Exact Test. Only the 12-week time point showed significance $P < 0.05$.

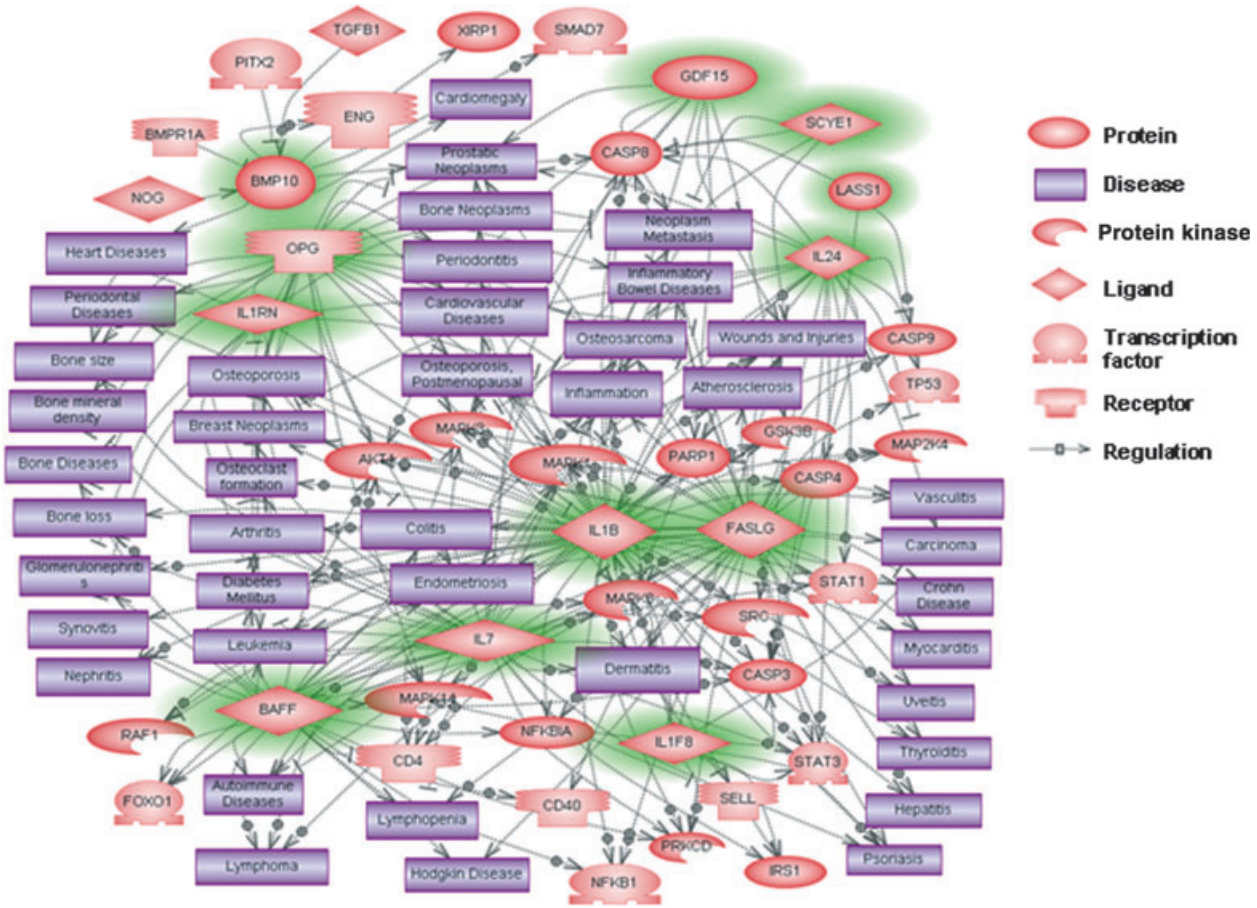

Figure 7 Proposed biological interaction network of differentially expressed genes from B and CD4 T cells of *Aggregatibacter actinomycetemcomitans* (*Aa*) -fed rats at 12 weeks postinfection by *Aa*, and their relationship to disease. Genes upregulated by at least four-fold (i.e. Log_2-fold >2) in B and CD4 T cells derived from cervical and submandibular lymph nodes of *Aa*-fed rats, in comparison to B and CD4 T cells from control rats, were imported into Pathway Studio for analyses. The picture shows interactions between upregulated genes in the expression data (shown as green highlights) and their interactions with related genes and diseases. The biological relationships revealed by the network are depicted in the pallets at the right of the figure. The relevance of the expression data to various diseases, as determined by the mining of the published Resnet 7 database in Pathway Studio, is indicated in the network.

expressed genes, in contributing to other systemic diseases. Our studies on the *Aa* rat model for periodontal disease demonstrate that *Aa* infection induces a robust adaptive immune response in B and CD4 T cells derived from local draining lymph nodes of the oral cavity. The B and CD4 T-cell numbers increased in *Aa*-inoculated rats compared with control rats. It should be noted that there was a dramatic increase in the absolute number of total lymphocytes recovered from lymph nodes (Fig. 2A), as compared with the percentages of B and CD4 T cells (Fig. 2B) recovered. This could be because we did not examine CD8 T cells in our studies. Furthermore, there could also be a minor proportion of natural killer T cells that were also not examined.

Activated CD4 T cells and their cytokine products would induce isotype-switching in *Aa*-specific antibodies. As early as 2–4 weeks postinfection, *Aa*-specific IgG and IgG2a antibodies were significantly increased in *Aa*-fed rats compared with control rats. It is of interest that T helper type 1 (Th1) cytokines TNF and LTβ) (Abbas *et al.*, 1996) were upregulated early (2–4 weeks) in the inflammatory response, which could explain the significant switch in *Aa*-specific antibody production to IgG2a. This is consistent with the observation that Th1 cytokines drive isotype switch to IgG2a in inflammatory responses of atherosclerosis (Schulte *et al.*, 2008). In this respect, the early upregulation of CD70 (TNFSF7) in B cells is of interest. CD70 has been shown to be expressed on a

subpopulation of germinal center B cells (Hintzen *et al.*, 1994). Interactions between CD70 and its ligand CD27 have been implicated in clonal expansion of B cells. Furthermore, CD70 has recently been shown to promote the development of $CD4^+$ T cells that produce a variety of effector cytokines including Th1 and Th2 types, hence contributing to the enhancement of a broad spectrum of immune responses (Hashimoto-Okada *et al.*, 2009). This activation of the humoral arm of the immune response, in collaboration with the induction of various cytokines by the activated B and CD4 T cells initiates a cascade of biological programs that lead to the induction of bone resorption.

While the innate immune responses to bacterial infection contribute to the production of inflammatory cytokines implicated in bone resorption, the adaptive immune response has been shown to have a major impact on the pathology of bone resorption. Bacterial invasion of gingival tissue is reported to result in the production of inflammatory cytokines which stimulate bone resorption (Rowe & Bradley, 1981; Graves, 2008). Our studies indicate a role for cytokines, bone morphogenic and other related proteins in a stepwise induction of osteoclastic bone resorption.

The role of cytokines in *Aa*-induced bone resorption

The early phase of the immune response to *Aa* (2–4 weeks postinfection) was characterized by upregulation of a set of inflammatory cytokines that were generally distinct from those upregulated in the late phase of the response (12 weeks postinfection). These include cytokines known to be present in saliva or gingival crevicular fluids, as well as several previously undescribed ones. However, the present study goes further in describing the cellular sources of the cytokines, as well as the time course of their induction. During the early phase of the immune response to *Aa*, IL-2 production by B cells is induced. IL-2 is a potent inducer of T-cell proliferation and Th1 and Th2 effector T-cell differentiation and provides T cells with a long-lasting competitive advantage, hence contributing to both the induction and the termination of inflammatory immune responses (Hoyer *et al.*, 2008). In view of the importance of the newly defined T helper 17 cytokine (IL-17), in periodontal disease, it is of interest that there was upregulation in IL-17 in $CD4^+$ T cells (2.8-fold) and B cells (twofold), in lymph nodes from *Aa*-infected rats, compared with control rats. This level of expression is below our stringent criterion of four fold differential gene expression. This finding is in conformity with the finding that IL-17 might be involved in the inflammatory response and bone resorption in animal models of periodontal disease (Oseko *et al.*, 2009; Xiong *et al.*, 2009).

Whereas the bone resorption protein RANKL (TNFSF11) was induced in the CD4 T cells, its soluble decoy receptor OPG (TNFSF11b) was also induced in the CD4 T cells. Recent developments in the field of osteoimmunology, which examines the crosstalk of immune cells and bone, have uncovered a novel role for the RANKL–RANK–OPG system in other processes such as in controlling autoimmunity or immune responses in the skin (Leibbrandt & Penninger, 2010). Despite the sustained upregulation of OPG, bone resorption still occurred.

The critical balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption has been described as 'coupling' of bone formation to bone resorption (Parfitt, 1982). This process is controlled by a large number of biological pathways that have not yet been clearly elucidated. The importance of the inflammatory cytokines IL-1 and TNF in periodontal disease has been demonstrated in several studies. However, the stepwise induction of these cytokines in response to infection has not been addressed in the same as way we used in our studies. In our studies, TNF-α was upregulated early in the response: by 2 weeks in CD4 T cells (Table 3) and by 4 weeks in B cells (Table 1). Both IL-1α and IL-1β were downregulated in B and CD4 T cells early in the response (2 and 4 weeks postinfection). However, both B and CD4 T cells responding to *Aa*, upregulated the inflammatory cytokine TNF-α and IL-1β in CD4 T cells by 12 weeks after infection. It is of interest that, at 12 weeks postinfection, two other IL-1 family member genes IL1F10 and IL1RN, were also upregulated. IL1RN has been reported to act as an antagonist of IL-1 (Towne *et al.*, 2004), and selectively inhibits the effects of IL1 by competing for the IL1 receptor on all surfaces of the synovium when injected into patients with rheumatoid arthritis (Schiff, 2000). Hence, at the time when osteoclastic bone resorption becomes prominent, the inflammation could be antagonized by IL1RN.

The apoptosis protein FasL (TNFSF6) was upregulated in CD4 T cells by 2 weeks and in B cells by 12 weeks postinfection. Earlier studies showed that IL-1β was responsible for most, but not all, bone resorption activity in peripheral blood mononuclear cell culture supernatants (Stashenko *et al.*, 1987). In other studies, activation of the acquired immune response of mice to infection with *Porphyromonas gingivalis* resulted in induction of IL-1β, TNF-α and apoptosis genes, among others, (Behl *et al.*, 2008). In our studies, CD4 T cells were found to upregulate LTβ early in the response. Gingival crevicular fluid of patients with periodontal disease was also found to contain increased amounts of LTβ (Pradeep *et al.*, 2007).

Although it was concluded from *in vitro* studies that MIF was an inhibitor of osteoclastogenesis (Jacquin *et al.*, 2009), other studies suggest that MIF may regulate rheumatoid arthritis synovial hyperplasia by acting directly and via involvement in the effects of IL-1β and TNF-α (Jacquin *et al.*, 2009). We found a progressive upregulation of MIF in B cells responding to *Aa* at 2 and 4 weeks postinfection. It is of interest that we have found upregulation in IL-7 in B cells responding to *Aa*, suggesting a possible role for this cytokine in bone resorption. Studies conducted on bone marrow transplantation (BMT) patients revealed an important role for IL-7 in post-BMT bone loss (Baek *et al.*, 2006).

Other cytokines that featured prominently in the *Aa*-induced disease include IL-16, which was upregulated in CD4 T cells in the early phase of the response. IL-16 has recently been shown to be involved in the selective migration of CD4 T cells, participates in inflammatory diseases (Akiyama *et al.*, 2009) and was detected in gingival crevicular fluid (Sakai *et al.*, 2006). IL-19, a novel cytokine of the IL-10 family, was also upregulated in CD4 T cells in response to *Aa*. IL-19 was found to be produced by synovial cells in rheumatoid arthritis patients and promotes joint inflammation (Sakurai *et al.*, 2008). IL-21, which has recently been shown to induce RANKL and was implicated in arthritis (Jang *et al.*, 2009), was upregulated in B cells responding to *Aa*. There was also an induction of IL-24 by 12 weeks in CD4 T cells responding to *Aa*. Studies conducted on rheumatoid arthritis showed an increase in IL-24 in the synovium of patients with rheumatoid arthritis, and this cytokine was implicated in recruitment of neutrophil granulocytes (Kragstrup *et al.*, 2008). B-cell-activating-factor (BAFF, or TNFSF13B) and a proliferation-inducing ligand (APRIL), members of the TNF family, were upregulated in B cells and CD4 T cells, respectively, in response to *Aa* infection. Both of these factors were recently found to be upregulated in children with atopic dermatitis (Jee *et al.*, 2009), and so would represent factors that characterize *Aa*-induced periodontal disease. SCYE1, also known as endothelial monocyte-activating polypeptide II (EMAP-II), is an inflammatory cytokine with chemotactic activity, that has been suggested to play a role in tooth eruption (Liu & Wise, 2008). It was upregulated by 12 weeks in B cells responding to *Aa* infection. Hence, SCYE1 may play a role in bone resorption-related diseases.

The role of BMPs and GDFs in *Aa*-induced bone resorption

The BMPs and GDFs are members of the transforming growth factor-β (TGF-β) superfamily. These proteins play a critical role during development and organogenesis in delivering positional information in both vertebrates and invertebrates. They are also involved in the development of hard as well as soft tissue (Herpin *et al.*, 2004). BMPs are also known to act locally on target tissues to affect proliferation and survival (Rosen, 2006). BMP2, even though dispensable for bone formation, is a necessary component of the signaling cascade that governs fracture repair (Tsuji *et al.*, 2006). In our studies, BMP2 was induced in B cells early during week 4 of the inflammatory process, at the same time that RANKL was induced in CD4 T cells. This suggests that bone repair mechanisms were induced early, well ahead of impending bone resorption. However, by 12 weeks of infection by *Aa*, BMP2 was shut down, as bone resorption proceeded. BMP3 was also upregulated at week 4 in B cells responding to *Aa*. BMP3 has been identified as a negative regulator in the skeleton, because mice lacking BMP3 have increased bone mass. Transgenic mice overexpressing BMP3 had altered endochondral bone formation, resulting in spontaneous rib fractures (Gamer *et al.*, 2009). On the other hand, it has been suggested that BMP2 and BMP3 might be coregulated. BMP2 was found to enhance BMP3 and BMP4 mRNA expressions in primary cultures of fetal rat calvarial osteoblasts. The enhancement of BMP3 and BMP4 mRNA expressions by BMP2 was associated with an increased

expression of bone cell differentiation marker genes (Chen *et al.*, 1997). It is of interest that BMP2 and BMP3 were upregulated in B cells at the same time (4 weeks postinfection), and were shut down at 12 weeks, at which time bone resorption was evident.

B cells responding to *Aa* upregulated BMP10 at all time points, in B cells. BMP10 has been shown to regulate myocardial hypertrophic growth (Chen *et al.*, 2006), and may function as a tumor suppressor and apoptosis regulator for prostate cancer (Ye *et al.*, 2009). To our knowledge, our work is the first report on the production of BMP10 by B cells responding to infection. The expression pattern of BMP10 in our studies suggests that it might be involved in inflammation, as well as in bone resorption. Furthermore, the involvement of BMP10 in cardiac hypertrophy and cancer, suggests that it might represent one of the 'missing links' between periodontal disease and other systemic diseases like heart disease and cancer. Evidence for this is provided in the modeled biological interaction pathway depicted in Fig. 7.

GDF11, also known as BMP11, has been reported to have an important role in establishing embryonic axial skeletal patterns (McPherron *et al.*, 1999). Transfection of the GDF11 gene was found to stimulate a large amount of reparative dentin formation in amputated dental pulp of canine teeth *in vivo* (Nakashima *et al.*, 2003). In our studies, GDF11 (BMP11) was upregulated at 12 weeks postinfection, in both B and CD4 T cells, at the time of bone resorption. This suggests that GDF11 may have a novel role in bone resorption. The fact that GDF11 activation has been observed in cancer (Yokoe *et al.*, 2007), may also provide another link between periodontal disease and cancer.

GDF15 was upregulated in both B and CD4 T cells of *Aa*-infected rats at 12 weeks, coinciding with the time of bone resorption. However, there are conflicting reports on the role of GDF15 in bone resorption and other systemic diseases. In a recent study, pure GDF15 and the GDF15-containing growth medium of $1,25(OH)_2$-vitamin D_3-treated prostate adenocarcinoma LNCaP cells were found to suppress osteoclast differentiation (Vanhara *et al.*, 2009). Elevation in GDF15 has been associated with cardiovascular disease (Kempf & Wollert, 2009), and colorectal cancer metastasis (Xue *et al.*, 2009) so GDF15 may also contribute to one of the links between periodontal disease and cancer.

Concluding remarks

Studies on purified B and CD4 T cells derived from lymph nodes draining the oral cavity of rats infected with *Actinobacillus actinomycetemcomitans* bacteria, demonstrated that inflammatory processes are initially activated early (2–4 weeks) postinfection. This, ultimately, culminates in activation of bone resorption pathways that result in overt bone resorption by 12 weeks postinfection. Apart from induction of known inflammatory cytokines (such as TNF-α, IL-1β, and LTβ), other cytokines and TGF-β superfamily member genes, not previously associated with bone resorption, were found to be upregulated in B and/or CD4 T cells. Some of these genes have known effects on systemic diseases such as cancer, autoimmune disease and diabetes. Our studies suggest a potential link between periodontal disease and other systemic diseases.

ACKNOWLEDGEMENTS

We thank the staff of the Comparative Medicine Facility of the New Jersey Medical School for their help with the procedures involving rats. We are also grateful to Shuying Jiang and Dr. Barbara Greenberg of the New Jersey Dental School Department of Research for help with statistical analysis of the bone loss and disease data. This work was supported by grants from the Foundation of University of Medicine and Dentistry of New Jersey (grant #36-08 and PC31-10).

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