J Periodont Res 2009; 44: 443–451

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01133.x

Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection

A. P. F. Trombone[1], S. B. Ferreira Jr[2], F. M. Raimundo[2], K. C. R. de Moura[2], M. J. Avila-Campos[3], J. S. Silva[1], A. P. Campanelli[2], M. De Franco[4], G. P. Garlet[2]

[1]Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, FMRP/USP, SP, Brazil, [2]Department of Biological Sciences, Bauru School of Dentistry, FOB/USP, SP, Brazil, [3]Department of Microbiology, Institute of Biomedical Sciences, ICB/USP, SP, Brazil and [4]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Trombone APF, Ferreira Jr SB , Raimundo FM, de Moura KCR, Avila-Campos MJ, Silva JS, Campanelli AP, De Franco M, Garlet GP. Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. J Periodont Res 2009; 44: 443–451.

Background and Objective: Inflammatory immune reactions that occur in response to periodontopathogens are thought to protect the host against infection, but may trigger periodontal destruction. However, the molecular and genetic mechanisms underlying host susceptibility to periodontal infection and to periodontitis development have still not been established in detail.

Material and Methods: In this study, we examined the mechanisms that modulate the outcome of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*-induced periodontal disease in mice mouse strains selected for maximal (AIRmax) or minimal (AIRmin) inflammatory reactions.

Results: Our results showed that AIRmax mice developed a more severe periodontitis than AIRmin mice in response to *A. actinomycetemcomitans* infection, and this periodontitis was characterized by increased alveolar bone loss and inflammatory cell migration to periodontal tissues. In addition, enzyme-linked immunosorbent assays demonstrated that the levels of the cytokines interleukin-1β, tumor necrosis factor-α and interleukin-17 were higher in AIRmax mice, as were the levels of matrix metalloproteinase (MMP)-2, MMP-13 and receptor activator of nuclear

factor-κB ligand (RANKL) mRNA levels. However, the more intense inflammatory immune reaction raised by the AIRmax strain, in spite of the higher levels of antimicrobial mediators myeloperoxidase and inducible nitric oxide synthase, did not enhance the protective immunity to *A. actinomycetemcomitans* infection, because both AIRmax and AIRmin strains presented similar bacterial loads in periodontal tissues. In addition, the AIRmax strain presented a trend towards higher levels of serum C-reactive protein during the course of disease.

Conclusion: Our results demonstrate that the intensity of the inflammatory immune reaction is associated with the severity of experimental periodontitis, but not with the control of *A. actinomycetemcomitans* periodontal infection, suggesting that the occurrence of hyperinflammatory genotypes may not be an evolutionary advantage in the complex host–pathogen interaction observed in periodontal diseases.

Gustavo Pompermaier Garlet, Department of Biological Sciences, School of Dentistry of Bauru (FOB/USP), Al. Octávio Pinheiro Brisola, 9-75, CEP 17012-901, Bauru, SP, Brazil
Tel: +55 (14) 3235-8274
Fax: +55 (14) 3235-8274
e-mail: garletgp@usp.br

Key words: periodontal diseases; *Aggregatibacter actinomycetemcomitans*; immune response; cytokine; interleukin-17; receptor activator of nuclear factor-κB ligand (RANKL)

Accepted for publication May 31, 2008

Periodontal diseases are chronic inflammatory diseases of the attachment structures of the teeth. The periodontal biofilm hosts a wide diversity of potentially hazardous bacterial species that trigger inflammatory and immune responses which are believed to protect the host against infection, but also account for tissue destruction (1,2). Pro-inflammatory and T helper 1-type cytokines, such as tumor necrosis factor-α, interleukin-1β and interferon-γ, are widely expressed in diseased periodontal tissue and are present at high levels in gingival crevicular fluid (3–5). In fact, experimental periodontitis studies have demonstrated that these cytokines present a catabolic role in periodontal tissues, mediated by the production of matrix metalloproteinases (MMPs) and the osteoclastogenic factor receptor activator of nuclear factor-κB ligand (RANKL) (1,2,4,6,7). On the other hand, anti-inflammatory cytokines such as interleukin-10 exert the reverse effect, possibly mediated by the direct down-regulation of inflammatory cytokines and their signaling, and also by the up-regulation of tissue inhibitor of metalloproteinases (TIMPs) and osteoprotegerin, the endogenous inhibitors of MMPs and RANKL, respectively (2,7,8). Interestingly, a high individual variation in the levels of cytokines in diseased periodontal tissues has been reported, and therefore it is reasonable to assume that the overall balance of pro-inflammatory and anti-inflammatory signals could determine the outcome of periodontal lesions (7,8). Indeed, heritable differences in the production of cytokines can result in phenotypes prone to develop different types and degrees of immune-inflammatory reactions, and genetic variations have been associated with the resistance or susceptibility to periodontal diseases (9). However, variables such as the different compositions of periodontal biofilm, the age at onset of disease and the complex host genetic variability may hinder the interpretation of human studies. Alternatively, in the experimental periodontitis mouse model we can control microbial and disease-onset variables and employ strains with well-known distinct genetic background as a tool to mimetize genetic variations seen in humans (10). Indeed, previous studies have demonstrated a differential bone loss of distinct mouse strains in response to *Porphyromonas gingivalis* infection (10), but the molecular and genetic mechanisms underlying such differences are poorly defined. In addition, the putative differential response of distinct mouse strains may also interfere with the protective immune response raised against periodontopathogens (1,6,10,11).

Mice strains that were genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory reactions, and which also present distinct chronic inflammatory responsiveness, appear to be useful models for studying the mechanisms involved in the susceptibility to inflammatory and infectious diseases (12,13). AIRmax mice are extremely susceptible to pristane-induced arthritis but are resistant to bacterial infections, whereas AIRmin mice are resistant to the development of arthritis but are susceptible to diverse infections (14,15). Therefore, the distinctiveness of AIRmax and AIRmin strains make these mice lineages extremely useful in the study of periodontal diseases.

In the present study, mice genetically selected for maximum (AIRmax) or minimum (AIRmin) inflammatory reactions were infected with the periodontopathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans* in order to evaluate their patterns of periodontitis development and control of periodontal infection, and to investigate the mechanisms underlying/involved in their differential response.

Material and methods

Experimental periodontal disease

Experimental groups comprised 8-wk-old male AIRmin and AIRmax mice (lineages generated, bred and maintained at the Butantan Institute, Sao Paulo, Brazil), maintained during the experimental period in the animal facilities of the Department of Biological Sciences of FOB/USP. Throughout the period of the study, the mice were fed with sterile standard solid mice chow (Nuvital; Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use. Periodontal infection was achieved by oral delivery of 1×10^9 colony-forming units of a

diluted culture of *A. actinomycetemcomitans* JP2 (anaerobically grown in supplemented agar medium, Tryptic Soy-Serum-Bacitracin-Vancomicin (TSBV)) in 100 μL of phosphate-buffered saline with 2% carboxymethylcellulose, placed in the oral cavity of mice with a micropipette, as previously described (16). After 48 and 96 h, this procedure was repeated. Negative controls included noninfected and sham-infected mice, which received heat-killed bacteria in 2% carboxymethylcellulose solution. After 45 d of infection, mice were killed and samples were collected for different experimental analyses.

Alveolar bone loss analysis

Evaluation of the extent of alveolar bone loss was performed as previously described (16). The maxillae were hemisected, exposed overnight in 3% hydrogen peroxide and mechanically defleshed. The palatal faces of the molars were photographed at 20× magnification using a dissecting microscope (Leica, Wetzlar, Germany) and the images were analyzed using IMAGETOOL 2.0 software (The University of Texas Health Science Center, San Antonio, TX, USA). Quantitative analysis was used to measure the area between the cemento–enamel junction and the alveolar bone crest in the three posterior teeth, in arbitrary units of area. Five animals were analyzed, and for each animal the alveolar bone loss was defined as the average cemento–enamel junction/alveolar bone crest area between the right and left arches.

Analysis of inflammatory cells

The isolation and characterization of leukocytes present in the lesion site were performed as previously described (6). The whole buccal and palatal periodontal tissues of upper molars were collected, weighed and incubated for 1 h at 37°C, dermal side down in RPMI-1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamycin and liberase blendzyme CI (Roche-F. Hoffmann-La Roche Ltd, Basel, Switzerland). The tissues of five mice, at each time-point per group, were processed in the presence of 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) using Medimachine (BD Biosciences PharMingen, San Diego, CA, USA). After processing, cell viability was assessed by Trypan Blue exclusion, and the cell count performed in a Neubauer chamber was considered as the total inflammatory cell count. Results represent the number of cells (± standard deviation) in the periodontal tissues of each mouse, normalized by the tissue weight, for two independent experiments.

Protein extraction and cytokine enzyme-linked immunosorbent assay

Measurements of cytokines and chemokines in periodontal tissues were performed as previously described (6). For protein extraction, the palatal periodontal tissue of five mice was homogenized in phosphate-buffered saline, pH 7.4, centrifuged at 220 *g* at 4°C and the supernatants were stored at −70°C. The concentrations of cytokines in periodontal extracts were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA), as follows: interleukin-1β (sensitivity > 3 pg/mL), tumor necrosis factor-α (> 3.4 pg/mL), IFN-γ (> 2 pg/mL), interleukin-17 (> 5 pg/mL) and interleukin-10 (> 4 pg/mL). The results were expressed as pg of cytokine (± standard deviation) per mg of periodontal tissue, for two independent experiments.

Real-time polymerase chain reaction amplification

The extraction of total RNA from periodontal tissues (upper molars with whole surrounding buccal and palatal periodontal tissues) was performed using Trizol reagent (Invitrogen, Rockville, MD, USA) and cDNA synthesis was accomplished as previously described (6). In order to allow quantification of the bacteria present in the biofilm and those which potentially invaded the host tissues, the extraction of bacterial DNA was performed from a sample comprising the upper molars with their alveolar bone and the whole surrounding buccal and palatal periodontal tissues, which was frozen in liquid nitrogen, mechanically fragmented and homogenized in sterile Milli-Q water with Ultra Turrax (IKA, Staufen, Germany), and subsequently processed for DNA extraction using a DNA Purification System (Promega Biosciences Inc., San Luis Obispo, CA, USA). Real-time polymerase chain reaction (PCR) quantitative mRNA or DNA analyses were performed in a MiniOpticon system (BioRad, Hercules, CA, USA), using SybrGreenMasterMix (Invitrogen), 100 nM specific primers and 2.5 ng of cDNA or 5 ng of DNA in each reaction. The primer sequences and reaction properties are depicted in Table 1. For mRNA analysis, the relative level of gene expression was calculated in reference to β-actin using the cycle threshold method. Bacterial DNA levels were determined using the cycle threshold method and normalized by the tissue weight.

Quantification of antimicrobial mediators

Quantification of antimicrobial mediators was performed as described previously (1). The levels of serum C-reactive protein were determined in serum samples using a commercially available agglutination kit (Labtest Diagnóstica, São Paulo, Brazil). Myeloperoxidase activity in homogenized periodontal tissue was measured by enzymatic reactivity, as the absorbance at 450 nm. The serum titer of immunoglobulin G specific to *A. actinomycetemcomitans* was measured using ELISA.

Statistical analysis

Data are presented as mean ± standard deviation, and the statistical significance between the infected and control mice of both strains was analyzed by analysis of variance, followed by the Bonferroni post test, performed using GRAPHPAD PRISM 3.0 software (GraphPad Software Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Table 1. Primer sequences and reaction properties

Target	Sense and antisense sequences	At (°C)	Mt (°C)	bp
MMP-2	CTGATGGCACCCATTTACACCT GATCTGAGCGATGCCATCAAA	60	82	186
MMP-13	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
TIMP-1	ACTGCAGGATGGACTCTTGCA TTTCAGAGCCTTGGAGGAGCT	30	82	206
TIMP-3	TTCTCAGCGAGGATGGCACTT AAACACGGTTCAGGATGCTGG	60	81	200
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
iNOS	CGTCATTTCTGTCCGTCTCT TTGCTGGCTGATGGCTGGCG	56	82	390
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495
Aggregatibacter actinomycetemcomitans	ATGCCAACTTGACGTTAAAT AAACCCATCTCTGAGTTCTTCTTC	60	78	557

At, annealing temperature; bp, base pairs of amplicon size; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; Mt, melting temperature; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TIMP, tissue inhibitor of metalloproteinase.

Chemokines, chemokine receptors and cytokines in periodontal tissues

We next investigated the expression of pro-inflammatory and anti-inflammatory cytokines, in order to determine the mechanisms responsible for the differential response demonstrated by AIR strains (Fig. 2). ELISA analyses demonstrated that interleukin-1β, tumor necrosis factor-α and interleukin-17 levels were significantly higher in AIRmax mice than in AIRmin mice at 45 d postinfection. In contrast, the levels of interleukin-10 were only slightly higher in the AIRmax strain, and no significant differences were found in the levels of interferon-γ in the periodontal tissues of AIRmin- and AIRmax-infected mice at the time-point of analysis. No significant levels of cytokines were found in the tissues of control mice groups.

Results

A. actinomycetemcomitans infection triggers differential alveolar bone loss and inflammatory reaction in AIRmax and AIRmin mice

We first evaluated the severity of experimental periodontitis developed by AIRmax and AIRmin mice after *A. actinomycetemcomitans* infection (Fig. 1). With regard to alveolar bone resorption, we found significantly less bone resorption postinfection in AIRmin mice when compared with AIRmax mice (Fig. 1A). Similarly, quantitative analysis of the inflammatory cells extracted from the gingival tissue of *A. actinomycetemcomitans*-infected AI Rmin mice also showed significantly fewer leukocytes compared with AIRmax-infected mice (Fig. 1B), findings that were confirmed by the histological analyses (Fig. 1C,D). Control mice of both strains did not present evidence of any inflammatory reaction or alveolar bone loss (Fig. 1). These results demonstrate that AIRmax mice develop a more severe periodontitis after *A. actinomycetemcomitans* infection when compared with the AIRmin strain.

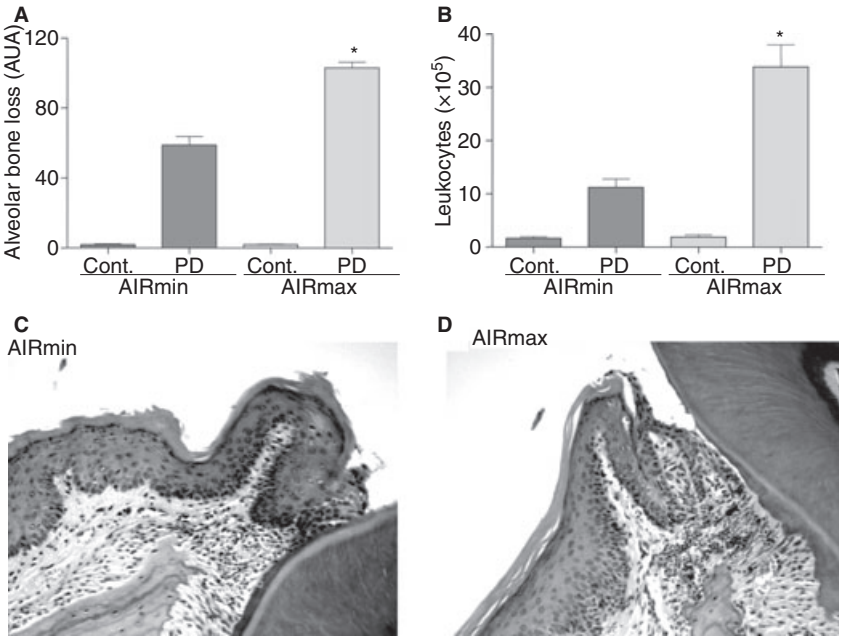

Fig. 1. Alveolar bone loss and inflammatory cell migration after oral inoculation of AIRmin and AIRmax mice strains with *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* (PD) and control uninfected mice (Cont.) were evaluated for: (A) alveolar bone loss quantification, performed through measuring the cemento–enamel junction and the alveolar bone crest area in the palatal face of maxillary molars and in arbitrary units of area (AUA) (B) total leukocyte counts of the inflammatory infiltrate (performed in a Neubauer chamber), as described in the Material and methods. Histotological sections were obtained of periodontal tissues of AIRmin (C) and AIRmax (D) infected mice (hematoxylin and eosin staining; magnification, ×400). All infected groups were statically different from noninfected controls. $*p < 0.05$ AIRmin vs. AIRmax.

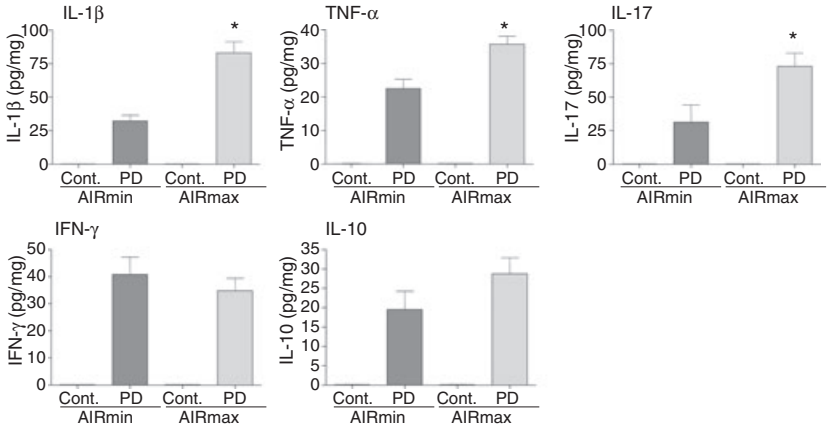

Fig. 2. AIRmin and AIRmax mice present a differential cytokine response during the course of experimental periodontal disease. Periodontal tissues of AIRmin and AIRmax mice infected orally with *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (PD) and control uninfected mice (Cont.) were harvested at 45 d of infection. The levels of interleukin-1β, tumor necrosis factor-α, interferon-γ, interleukin-17 and interleukin-10 protein in periodontal tissues were determined at the indicated time-points by enzyme-linked immunosorbent assay, as described in the Material and methods. The results are presented as pg of cytokine per mg of tissue (mean ± standard deviation). The results of all infected groups were statistically different from those of noninfected controls. $*p < 0.05$ AIRmin vs. AIRmax.

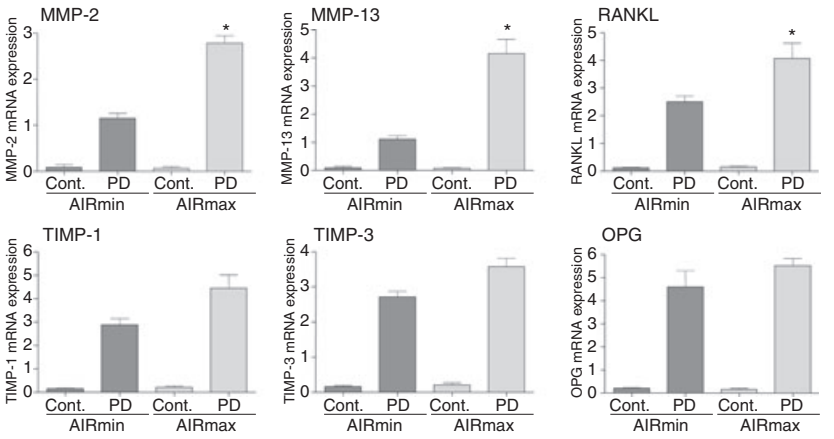

Fig. 3. Matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) expression in periodontal tissues of AIRmin and AIRmax mice. Periodontal tissues of AIRmin and AIRmax mice infected orally with *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (PD) and control uninfected mice (Cont.) were harvested at 45 d of infection. The levels of MMPs, TIMPs, RANKL and osteoprotegerin mRNA were quantified by real-time polymerase chain reaction, using the SybrGreen System and the cycle threshold method. The results are presented as the expression of target mRNAs with normalization to β-actin (mean ± standard deviation). All infected groups were statistically different from non-infected controls. $*p < 0.05$ AIRmin vs. AIRmax.

MMPs/TIMPs and RANKL/osteoprotegerin expression

We next investigated whether the higher periodontitis severity seen in AIRmax mice could be caused by a modulation in the balance between MMPs/TIMPs and RANKL/osteoprotegerin expression (Fig. 3). Quantitative analysis of the mRNA expression of MMP (MMP-2 and MMP-13) in gingival tissues from infected AIRmax mice showed a significantly higher expression by comparison with infected AIRmin mice. Conversely, the expression of TIMPs (TIMP-1 and TIMP-3) was found to be only slightly increased in AIRmax mice. Investigating the expression of osteoclast regulatory factors, we found that RANKL expression was significantly higher in AIRmax mice, whereas osteoprotegerin expression was similar in AIRmin and AIRmax mice. When the MMPs/TIMPs and RANKL/osteoprotegerin ratios were analyzed, we found that both ratios were higher in AIRmax mice than in AIRmin mice (data not shown).

The control of experimental *A. actinomycetemcomitans* infection

In view of the differential strength of the inflammatory immune response seen in AIRmin and AIRmax mice, we next investigated the control of *A. actinomycetemcomitans* infection in these strains (Fig. 4). Our results demonstrated that both AIRmin and AIRmax mice presented similar bacterial load levels in periodontal tissues, in spite of the higher levels of the antimicrobial mediators myeloperoxidase and inducible nitric oxide synthase found in the tissues of AIRmax mice. We also found that *A. actinomycetemcomitans*-infected AIRmax mice presented a nonstatistically significant trend towards a higher acute-phase response (i.e. higher levels of C-reactive protein in the serum) and to a lower weight gain throughout the course of disease than AIRmin mice. Finally, no significant differences were found between the levels of *A. actinomycetemcomitans*-specific immunoglobulin G in the serum of AIRmin- and AIRmax-infected mice. Throughout experimental periodontitis, different organs were evaluated for possible bacterial dissemination (by means of real-time PCR) but no alterations were found (data not shown).

Discussion

Studies demonstrate a high individual variation in the levels of cytokines in diseased human periodontal tissues,

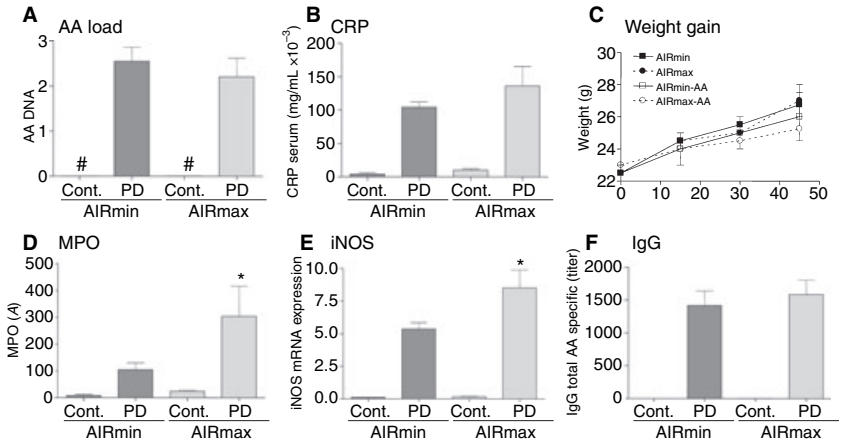

Fig. 4. The control of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* infection in AIRmin and AIRmax mice. Periodontal tissues of AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* (PD) and control uninfected mice (Cont.) were evaluated for: (A) *A. actinomycetemcomitans* (AA) load in periodontal tissues, quantified by the real-time polymerase chain reaction using the SybrGreen system and normalized by tissue weight; (B) serum levels of C-reactive protein (CRP), presented as mg/mL $\times 10^{-3}$; (C) weight gain of mice during the course of experimental periodontal disease; (D) levels of myeloperoxidase in periodontal tissues, presented as absorbance (*A*); (E) levels of inducible nitric oxide synthase in periodontal tissues, quantified by real-time polymerase chain reaction, using the Sybr-Green System and the cycle threshold method; and (F) the titers of *A. actinomycetemcomitans*-specific immunoglobulin G (IgG) in the serum, quantified by enzyme-linked immunosorbent assay; all were performed as described in the Material and methods. All infected groups were statistically different from noninfected controls, except for weight gain analysis. , undetected; *$p < 0.05$ AIRmin vs. AIRmax.

and the overall balance between pro-inflammatory and anti-inflammatory mediators is believed to determine the disease outcome (7–9). In accordance, knockout mice models demonstrate a cause-and-effect relationship between different pro-inflammatory and anti-inflammatory cytokines and the symptoms of experimental periodontitis (1,11,17). However, the use of knockout mice, albeit being a very interesting tool, represents an extreme situation where a given cytokine is completely absent and therefore does not properly mimetize the putative effect of genetic variations that could account for the individual variations seen in human disease.

Therefore, in this study we employed two mice strains genetically selected for maximum (AIRmax) or minimum (AIRmin) inflammatory reactions as a model to study the effect of their well-known distinct genotypes and phenotypes, regarding acute and chronic inflammatory immune reactions (12–15,18), on the development of experimental periodontitis and in the control of periodontal infection. Our results demonstrated that AIRmax mice infected with *A. actinomycetemcomitans* exhibit significantly increased inflammatory cell migration and alveolar bone resorption when compared with the AIRmin strain. Accordingly, these mice strains were selected by means of bidirectional selective breeding in order to develop maximal or minimal inflammatory responsiveness (12). In fact, further studies demonstrated the dichotomous behavior of these strains in response to a wide a range of microbial and inflammatory stimuli (13–15,18,19).

In order to clarify the mechanisms by which the AIR strains of mice develop distinct patterns of experimental periodontitis severity, we next evaluated the levels of different cytokines thought to be associated with periodontitis development. Our results demonstrated increased levels of tumor necrosis factor-α, interleukin-1β and interleukin-17 in the periodontal tissues of AIRmax mice compared with AIRmin mice. In accordance, previous studies demonstrated increased production of these inflammatory cytokines by AIRmax mice in pristane-induced arthritis and dextran sodium sulfate-induced colitis models (15,19). Regarding periodontal diseases, both tumor necrosis factor-α and interleukin-1β have been classically associated with periodontal disease pathogenesis, where they are are described as inducers of cell migration through inducing the expression of chemokines (1,4). Interestingly, the levels of interferon-γ, also a pro-inflammatory cytokine that can contribute to sustain inflammatory reactions through the up-regulation of inflammatory cytokine and chemokine production (6), were found to be similar in AIRmin and AIRmax strains of mice. In accordance, previous studies demonstrated that the levels of interferon-γ can vary between the AIRmin and AIRmax strains of mice, depending on the nature of the inflammatory stimulus (15,18). On the other hand, anti-inflammatory cytokines such as interleukin-10 are thought to attenuate periodontal disease progression, restraining the inflammatory signaling and their deleterious effects to the periodontal tissues (7,20). Interestingly, the interleukin-10 levels were found to be slightly increased in the periodontal tissues of AIRmax mice, probably in an attempt to restrain the intense inflammatory reaction. However, the ratios of pro-inflammatory cytokines/interleukin-10 were significantly higher in AIRmax mice than in AIRmin mice, suggesting the predominance of the inflammatory activity reaction in AIRmax mice, even with the discrete up-regulation of interleukin-10. In accordance, in the course of experimental periodontitis in mice, the initial phase characterized by the intense increase of inflammatory reaction and alveolar bone loss is associated with the expression of interleukin-1β, tumor necrosis factor-α and interferon-γ, while the latter increase of interleukin-10 expression is linked with reduced rates of increased cellular infiltration in periodontal tissues and alveolar bone loss (7). Accordingly, the modulation of inflammatory cell migration (i.e. the MMPs/TIMPs and RANKL/osteoprotegerin balance) by

T helper 1-type and T helper 2-type cytokines is thought to be an important factor in the determination of human periodontitis severity (1,7).

Beyond the T helper 1/T helper 2 archetype, another cytokine that could contribute to the increased responsiveness of the AIRmax strain is interleukin-17, which was also found to be up-regulated in AIRmax mice. Interleukin-17, a product of T helper 17 cells, has recently emerged as a positive regulator of pro-inflammatory cytokines, MMPs and RANKL expression by several cell types (21). Indeed, interleukin-17 was detected in diseased gingival tissue and gingival crevicular fluid, and has been demonstrated to have an important role in the progression of inflammatory bone loss (22,23). In addition, the overexpression of interleukin-17 is also implicated in the development of autoimmune diseases, such as rheumatoid arthritis (21). Interestingly, AIRmax mice were found to be similarly susceptible to the development of both experimental arthritis (15) and periodontitis, pathologies that share several characteristics such as the chronic nature of the inflammatory reaction associated with bone resorption activity (21,24). Indeed, periodontitis and arthritis have been found to be clinically associated (24), suggesting that these diseases could share genetic susceptibility/resistance patterns. Therefore, the broad-spectrum up-regulation of expression of inflammatory cytokines could explain the increased inflammatory cell migration presented by AIRmax mice.

The sustained chronic inflammatory reaction is thought to trigger periodontal tissue destruction as a consequence of an imbalance in the expression of MMPs (which regulate the turnover of extracellular matrix degradation), of RANKL (which controls osteoclast differentiation and activation) and of their respective inhibitor (TIMPs and osteoprotegerin) (2,25). In accordance, our data show that the expression of both MMPs (MMP-2 and MMP-13) and RANKL were higher in AIRmax mice than in AIRmin mice. While MMP-2 is believed to be involved in both extracellular matrix (ECM) and bone degradation, MMP-13 seems to be specifically found in bone resorption sites (25,26). In fact, tumor necrosis factor-α, interleukin-1β and interleukin-17, found to be up-regulated in AIRmax mice, are involved in tissue damage driven by RANKL and MMPs (1,4,22). Interestingly, interleukin-17 seems to be less potent as a direct MMP inducer than interleukin-1β and tumor necrosis factor-α, but interleukin-17-induced interleukin-1β and tumor necrosis factor-α generate an inflammation amplification loop, which consequently increases the expression of MMPs and RANKL (22). On the other hand, only a slight increase in the expression of TIMPs and osteoprotegerin was found in the periodontal tissues of AIRmax mice, resulting in higher ratios of MMPs/TIMPs and RANKL/osteoprotegerin in AIRmax mice than in AIRmin mice. Therefore, the predominance of a tissue catabolic activity is associated with the increased tissue destruction seen in AIRmax mice.

However, increased levels of the inflammatory and antimicrobial mediators myeloperoxidase and inducible nitric oxide synthase produced by AIRmax mice did not improved the protective immunity to *A. actinomycetemcomitans* infection, as demonstrated by the similar bacterial load presented by AIRmin and AIRmax mice. Conversely, both myeloperoxidase and inducible nitric oxide synthase are believed to be active factors in the control of periodontopathogens (1,6,27), and AIRmax mice characteristically present an improved clearance of *Salmonella typhimurium* and *Listeria monocytogenes* infections (14,28). *Salmonella typhimurium* and *L. monocytogenes* are intracellular bacteria whose effective control requires both innate and adaptive immune responses, in well-known mechanisms dependent on interferon-γ, tumor necrosis factor-α and antimicrobial mediators, such as reactive oxygen and nitrogen intermediates (14). Conversely, the mechanisms involved in the control of *A. actinomycetemcomitans*, a periodontopathogen capable of invading epithelial and endothelial cells, are poorly known, but seem to involve similar mechanisms (1,6,20,25). However, the ability of *A. actinomycetemcomitans* to attach to and colonize the subgingival biofilm, an environment that may confer efficient protection to periodontopathogens (29,30), might impair its clearance by the immune system, despite the development of robust responses. In addition, AIRmax mice also presented a trend towards a higher acute-phase response after *A. actinomycetemcomitans* infection. As increased levels of C-reactive protein in serum are an important risk factor for atherosclerosis, myocardial infarction and ischemic stroke, this increased systemic responsiveness could potentiate the occurrence of the detrimental systemic effects attributed to periodontal diseases (31–33). Interestingly, the C57BL/6 strain of mice usually employed in our studies, develop an intermediary response between AIRmin and AIRmax strains, and also present a bacterial load similar to that seen in the AIR strains (1,7). Therefore, it is reasonable to suggest that the interaction of hosts presenting hyperinflammatory genotypes/phenotypes with periodontopathogens leads to excessive tissue damage without enhancing the control of infection, and also could even increase the risk of systemic complications associated with periodontal diseases. However, the impaired development of an inflammatory immune response against periodontopathogens (i.e. in tumor necrosis factor p55 and interferon-γ-deficient mice) results in an increased bacterial load and more intense systemic effects of periodontal infection (1,7), reinforcing that a highly complex host–pathogen interaction takes place in the periodontal environment and that very tight immunoregulation is required to maintain periodontal health.

Some known genetic characteristics of AIRmin and AIRmax strains of mice, such as variations in the *SLC11A1* (i.e. solute carrier family 11a member 1, formerly named NRAMP1 – natural resistance-associated macrophage protein 1) gene, also suggest a possible role for this gene in the immunopathogenesis of periodontitis. Slc11a1 protein is pleiotropic, interfering with macrophage activation,

oxidative burst, inflammatory cytokine production and expression of major histocompatibility complex molecules. While in mice *Slc11a1* has been described as a major modulator of susceptibility to infectious diseases, in humans *SLC11A1* has been implicated in rheumatoid arthritis susceptibility (14,28,34,35), characteristics that reinforce a possible role in the development of periodontitis. However, *SLC11A1* has never been investigated in human periodontits. In addition, it is important to consider that closely linked genes, in linkage disequilibrium with *SLC11A1* as the result of selection processes, may be implicated in the differential outcome of experimental periodontitis seen in AIRmin and AIRmax mice strains.

Taken together, our results demonstrate some molecular mechanisms underlying the distinct outcome of periodontal disease in mice strains genetically selected for maximal or minimal inflammatory reactions. In addition, we demonstrated that the increased inflammatory immune reaction presented by the AIRmax strain did not confer additional protection against *A. actinomycetemcomitans*, suggesting that the hyperinflammatory genotypes may not be an evolutionary advantage in the complex host–pathogen interaction that comprises periodontal disease. However, it is important to consider that periodontitis is a disease of complex traits, in which several immunoregulatory mechanisms and cofactors are involved, and experimental periodontitis in mice may fail to reproduce some characteristics observed in their human counterpart. Therefore, further studies are required to investigate the existence of a putative optimum level of host response, which could restrain the infection with minimum damage to the host tissues, and if genetic variations would be associated with the infection patterns in patients with periodontitis. This knowledge may allow us to direct the development of strategies to prevent and treat periodontitis, aimed to modulate the host immune response in order to maximize the control of periodontal infection and minimize the local and systemic consequences of periodontitis.

Acknowledgements

We would like to thank Daniele Santi Ceolin and Tania Cestari for their excellent technical assistance. This study was supported by FAPESP and CNPq.

References

1. Garlet GP, Cardoso CR, Campanelli AP *et al*. The dual role of p55 tumour necrosis factor-alpha receptor in Actinobacillus actinomycetemcomitans-induced experimental periodontitis: host protection and tissue destruction. *Clin Exp Immunol* 2007;**147:**128–138.
2. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. *J Periodontol* 2005;**76:**2033–2041.
3. Cutler CW, Stanford TW, Abraham C, Cederberg RA, Boardman TJ, Ross C. Clinical benefits of oral irrigation for periodontitis are related to reduction of pro-inflammatory cytokine levels and plaque. *J Clin Periodontol* 2000;**27:**134–143.
4. Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol* 2003;**74:**391–401.
5. Honda T, Domon H, Okui T, Kajita K, Amanuma R, Yamazaki K. Balance of inflammatory response in stable gingivitis and progressive periodontitis lesions. *Clin Exp Immunol* 2006;**144:**35–40.
6. Garlet GP, Cardoso CR, Campanelli AP *et al*. The essential role of IFN-gamma in the control of lethal *Aggregatibacter actinomycetemcomitans* infection in mice. *Microbes Infect* 2008;**10:**489–496.
7. Garlet GP, Cardoso CR, Silva TA *et al*. Cytokine pattern determines the progression of experimental periodontal disease induced by Actinobacillus actinomycetemcomitans through the modulation of MMPs, RANKL, and their physiological inhibitors. *Oral Microbiol Immunol* 2006;**21:**12–20.
8. Yamazaki K, Yoshie H, Seymour GJ. T cell regulation of the immune response to infection in periodontal diseases. *Histol Histopathol* 2003;**18:**889–896.
9. Yoshie H, Kobayashi T, Tai H, Galicia JC. The role of genetic polymorphisms in periodontitis. *Periodontology 2000* 2007;**43:**102–132.
10. Baker PJ. Genetic control of the immune response in pathogenesis. *J Periodontol* 2005;**76:**2042–2046.
11. Burns E, Bachrach G, Shapira L, Nussbaum G. Cutting Edge: TLR2 is required for the innate response to Porphyromonas gingivalis: activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone resorption. *J Immunol* 2006;**177:**8296–8300.
12. Ibanez OM, Stiffel C, Ribeiro OG *et al*. Genetics of nonspecific immunity: I. Bidirectional selective breeding of lines of mice endowed with maximal or minimal inflammatory responsiveness. *Eur J Immunol* 1992;**22:**2555–2563.
13. Peters LC, Jensen JR, Borrego A *et al*. Slc11a1 (formerly NRAMP1) gene modulates both acute inflammatory reactions and pristane-induced arthritis in mice. *Genes Immun* 2007;**8:**51–56.
14. Araujo LM, Ribeiro OG, Siqueira M *et al*. Innate resistance to infection by intracellular bacterial pathogens differs in mice selected for maximal or minimal acute inflammatory response. *Eur J Immunol* 1998;**28:**2913–2920.
15. Vigar ND, Cabrera WH, Araujo LM *et al*. Pristane-induced arthritis in mice selected for maximal or minimal acute inflammatory reaction. *Eur J Immunol* 2000;**30:**431–437.
16. Garlet GP, Avila-Campos MJ, Milanezi CM, Ferreira BR, Silva JS. Actinobacillus actinomycetemcomitans-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration. Microbes Infect 2005;**7:**738–747.
17. Alayan J, Ivanovski S, Farah CS. Alveolar bone loss in T helper 1/T helper 2 cytokine-deficient mice. *J Periodontal Res* 2007;**42:**97–103.
18. Carneiro AS, Ribeiro OG, De Franco M *et al*. Local inflammatory reaction induced by Bothrops jararaca venom differs in mice selected for acute inflammatory response. *Toxicon* 2002;**40:**1571–1579.
19. Di Pace RF, Massa S, Ribeiro OG *et al*. Inverse genetic predisposition to colon versus lung carcinogenesis in mouse lines selected based on acute inflammatory responsiveness. *Carcinogenesis* 2006;**27:**1517–1525.
20. Sasaki H, Okamatsu Y, Kawai T, Kent R, Taubman M, Stashenko P. The interleukin-10 knockout mouse is highly susceptible to Porphyromonas gingivalis-induced alveolar bone loss. *J Periodontal Res* 2004;**39:**432–441.
21. Kramer JM, Gaffen SL. Interleukin-17: a new paradigm in inflammation, autoimmunity, and therapy. *J Periodontol* 2007;**78:**1083–1093.
22. Beklen A, Ainola M, Hukkanen M, Gurgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res* 2007;**86:**347–351.
23. Yu JJ, Ruddy MJ, Wong GC *et al*. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed

bone requires IL-17 receptor-dependent signals. *Blood* 2007;**109:**3794–3802.

24. Al-Katma MK, Bissada NF, Bordeaux JM, Sue J, Askari AD. Control of periodontal infection reduces the severity of active rheumatoid arthritis. *J Clin Rheumatol* 2007;**13:**134–137.
25. Hannas AR, Pereira JC, Granjeiro JM, Tjaderhane L. The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand* 2007;**65:**1–13.
26. Andersen TL, del Carmen Ovejero M, Kirkegaard T, Lenhard T, Foged NT, Delaisse JM. A scrutiny of matrix metalloproteinases in osteoclasts: evidence for heterogeneity and for the presence of MMPs synthesized by other cells. *Bone* 2004;**35:**1107–1119.
27. Miyasaki KT, Wilson ME, Brunetti AJ, Genco RJ. Oxidative and nonoxidative killing of Actinobacillus actinomycetemcomitans by human neutrophils. *Infect Immun* 1986;**53:**154–160.
28. Borrego A, Peters LC, Jensen JR *et al*. Genetic determinants of acute inflammation regulate Salmonella infection and modulate Slc11a1 gene (formerly Nramp1) effects in selected mouse lines. *Microbes Infect* 2006;**8:**2766–2771.
29. Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. *Trends Microbiol* 2005;**13:**589–595.
30. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC. How we got attached to Actinobacillus actinomycetemcomitans: A model for infectious diseases. *Periodontology 2000* 2006;**42:**114–157.
31. Mombelli A. Periodontitis as an infectious disease: specific features and their implications. *Oral Dis* 2003;**9**(suppl 1)**:**6–10.
32. Offenbacher S, Beck JD. A perspective on the potential cardioprotective benefits of periodontal therapy. *Am Heart J* 2005; **149:**950–954.
33. Scannapieco FA. Systemic effects of perio dontal diseases. *Dent Clin North Am* 2005;**49:**533–550, vi.
34. Shaw MA, Clayton D, Blackwell JM. Analysis of the candidate gene NRAMP1 in the first 61 ARC National Repository families for rheumatoid arthritis. *J Rheumatol* 1997;**24:**212–214.
35. Awomoyi AA. The human solute carrier family 11 member 1 protein (SLC11A1): linking infections, autoimmunity and cancer? *FEMS Immunol Med Microbiol* 2007;**49:**324–329.