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ORIGINAL ARTICLE

Periodontitis and arthritis interaction in mice involves a shared hyper-inflammatory genotype and functional immunological interferences

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Periodontitis (PD) and rheumatoid arthritis (RA) have been found to be clinically associated and to share the chronic nature of the inflammatory reaction associated with bone resorption activity. However, the mechanisms underlying such association are unknown. Therefore, we examined the basis of Actinobacillus actinomycetemcomitans- and Porphyromonas gingivalis-induced PD and pristane-induced arthritis (PIA) interaction in mice. Higher severity PD in the genetically inflammation prone acute inflammatory reactivity maximum (AIRmax) mice strain was associated with higher levels of TNF-α, IL-1β, IL-17, matrix metalloproteinase (MMP)-13, and RANKL, whereas PD/PIA co-induction resulted in even higher levels of IL-1β, IFN-γ, IL-17, RANKL, and MMP-13 levels. Conversely, PD/PIA co-induction in AIRmin strain did not alter the course of both pathologies. PIA/PD co-induction resulted in altered expression of T-cell subsets transcription factors expression, with T-bet and RORγ levels being upregulated, whereas GATA-3 levels were unaltered. Interestingly, PIA induction resulted in alveolar bone loss, such response being highly dependent on the presence of commensal oral bacteria. No differences were found in PIA severity parameters by PD co-induction. Our results show that the interaction between experimental PD and arthritis in mice involves a shared hyper-inflammatory genotype and functional interferences in innate and adaptive immune responses.
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Keywords: *periodontal diseases; arthritis; inflammatory reaction; immune response; cytokine*

Introduction

The development of regulated immune responses is essential in the host defense against harmful antigens and in the balance between tolerance and immunity to other antigens. Immune-related disorders are the result of inappropriate destruction of normal tissue by unbalanced immune inflammatory reactions, and comprise autoimmune (a failure of the immune self-tolerance mechanism) and inflammatory disorders (an excessive inflammatory reaction into exogenous antigens).[1]

Recent studies show a parallel development of inflammatory and autoimmune disorders in some patients called co-morbidity, which suggests that similar immunopathogenetic pathways operate in both types of immune-related disorders.[1–3] The possible common pathways responsible for inappropriate or excessive immune response include genetic factors, such as the human leukocyte antigen genes and single nucleotide polymorphisms (SNPs); innate and adaptive immune regulation processes; and triggering by environmental factors, such as infections or dietary components.[1–3]

Results from clinical studies point toward a co-morbidity association between chronic periodontitis (PD) and systemic rheumatic diseases, in particular rheumatoid arthritis (RA), described to increase the incidence and/or severity of PD, a chronic infectious inflammatory disease that leads to the destruction of the teeth attachment structures.[4–7] Chronic PD is the most prevalent chronic inflammatory disease in human beings, in which bacterial species that hosts periodontal biofilm trigger inflammatory and immune responses involved in tissue damage.[8,9] RA is a chronic autoimmune inflammatory disease that possibly arise because of a failure in thymic selection or a peripheral tolerance (the early mechanisms by which immune tolerance is breached and the subsequent event that triggers articular localization is poorly defined), characterized by persistent synovial inflammation and progressive destruction of cartilage and bone in the joints.[10] Recently, studies suggest that the PD and RA interaction can be bidirectional, as the control of periodontal infection reduces the severity of RA.[11,12] Besides the clinical association, PD and RA share common immunopathogenic mechanisms as the chronic inflammatory reaction associated with soft and mineralized tissue

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destruction. Indeed, pro-inflammatory (TNF-α, IL-1β) Th1 (IFN-γ)- and Th17 (IL-17A)-type cytokines are widely expressed in both diseased synovial and periodontal tissue.[10,13] Furthermore, in both diseases, these cytokines present a catabolic function over the periodontal tissues, mediated by the production of matrix metalloproteinases (MMPs) and the osteoclastogenic factor RANKL.[10,13–20] On the other hand, Th2- and anti-inflammatory cytokines such as IL-4 and IL-10 exert the reverse effect, mediating the direct downregulation of inflammatory cytokines and their signaling pathways, and also upregulating the expression of TIMPs and OPG, the endogenous inhibitors of MMPs and RANKL, respectively.[10,13,19,21,22]

However, a major unanswered question is how a joint lesion could influence the host response at periodontal environment to affect PD outcome. Among the hypothesis raised, considering the common immune and inflammatory features of both diseases, conceptual models link both diseases by causal and noncausal pathways.[14–16,23] Noncausal hypothesis considers that environmental or host factors increase susceptibility to both RA and PD, whereas causal assumption is that RA and PD can actively interfere in the pathogenesis of each other.[14–16] Noncausal theory is supported by studies showing that the genetic factors implicated in PD, such as specific human leukocyte antigen molecules and IL-1 and FcR polymorphisms, have also been implicated as susceptibility factors for RA.[15,16] Experimental studies reinforce the noncausal shared genetic susceptibility hypothesis as mouse lines phenotype selected for the acute inflammatory reactivity maximum (AIRmax) or minimal (AIRmin) share the susceptibility or resistance phenotype to both chronic models of pristane-induced arthritis (PIA) and *Actinobacillus actinomycetemcomitans*-induced PD.[24–28] AIRmax and AIRmin mice were developed through bidirectional genetic selection, starting from a highly polymorphic population (F0) derived from the intercrossing of eight inbred mouse strains (A, DBA2, P, SWR, CBA, SJL, BALB/c, and C57BL/6), and the progressive divergence of the AIRmax and AIRmin lines during successive generations of selective breeding reached 20- and 2.5-fold differences in leukocyte infiltration and exudated protein concentrations, respectively.[27] These differences resulted from the accumulation of alleles endowed with opposite and additive effects on the inflammatory response.[27] The causal theory is supported by the finding of increased levels of pro-inflammatory and tissue destructive mediators in the periodontal tissues of RA patients or rats submitted to experimental RA induction.[7,29,30] In addition, although early studies suggested a potential link between the periodontal pathogen *A. actinomycetemcomitans* and RA,[31,32] recent studies point to a possible causal function for *Porphyromonas gingivalis* in RA pathogenesis.[33,34] However, the exact molecular mechanisms underlying such putative functional interferences remain unknown.

In this study, AIRmax and AIRmin mice were submitted to both pristane injection (PIA protocol) and subsequently infected with the periodontopathogens *A. actinomycetemcomitans* and *P. gingivalis* [experimental periodontitis (ePD) protocol] to evaluate their patterns of arthritis and PD development and to investigate the mechanisms underlying its interaction.

Results

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

We first evaluated the severity of ePD developed by AIRmax and AIRmin mice after *A. actinomycetemcomitans* infection (Figure 1). With regard to alveolar bone resorption, we found significantly less bone resorption and inflammatory cell influx into periodontal tissues post-infection in AIRmin when compared with AIRmax mice. The co-induction of PD and PIA resulted in increased alveolar bone loss and inflammatory cell migration in AIRmax strain, whereas no significant alterations were observed in AIRmin mice. When PIA development was investigated, only AIRmax presented a significant increase in foot thickness after pristane injection, which was not modulated by PD co-induction. Control mice of both strains did not present evidence of any inflammatory reaction, alveolar bone loss, and foot thickness. These results show that AIRmax mice develop a more severe PD because of the parallel induction of ePD and PIA.

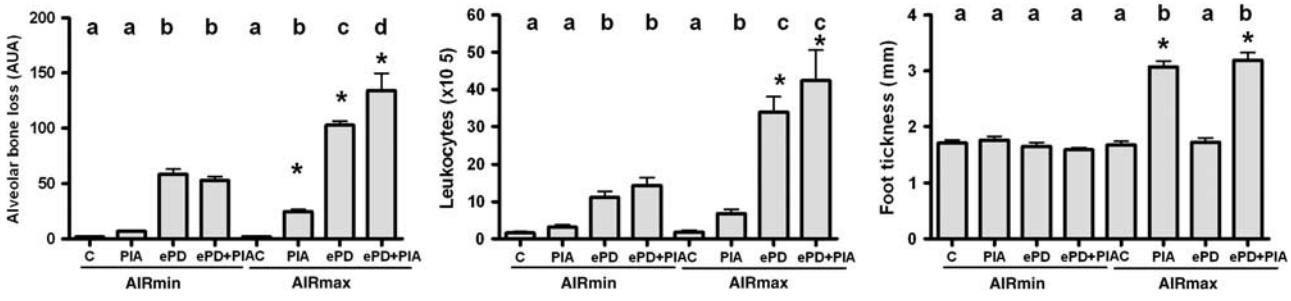

Figure 1 Interaction between pristane-induced arthritis and *A. actinomycetemcomitans*-induced periodontal disease in AIRmin and AIRmax mice strains. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated for the severity of PD through alveolar bone loss quantification (measurements of CEJ-ABC area in the palatal face of maxillary molars) and total leukocyte counts of the inflammatory infiltrate in periodontal tissues (performed in a Neubauer chamber) and for the severity of experimental arthritis (measured as the foot thickness in mm). One-way ANOVA followed by Bonferroni's test. *$P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$).

ePD/PIA interaction results in altered cytokine production in periodontal tissues

We next investigated the expression of pro- and anti-inflammatory cytokines to determine the mechanisms responsible for the differential response shown by AIR strain after ePD/PIA co-induction (Figure 2). ELISA analyses showed that IL-1β, IFN-γ, and IL-17 levels were significantly higher in ePD + PIA group, whereas only a nonstatistically significant trend toward higher TNF-α levels was observed in this group. In contrast, the levels of IL-4 and IL-10 were not modulated by PIA co-induction in AIRmax strain at the analyzed time. Interestingly, PIA induction resulted in a significant increase of IL-1β, IFN-γ, TNF-α, IL-17, and IL-10 in periodontal tissues of AIRmax mice, but in lower extent than ePD or ePD + PIA. No significant levels of cytokines were found in the tissues of control mice groups.

MMPs/TIMPs and RANKL/OPG expression

We next investigated whether the higher PD severity seen in ePD + PIA group could be due to a modulation in the balance between MMPs/TIMPs and RANKL/OPG expression (Figure 3). The quantitative analysis of MMP-13 mRNA expression in gingival tissues from ePD + PIA AIRmax mice showed a significantly higher expression, whereas the expression of TIMP-1 was found not to be modulated by PIA co-induction. Similar patterns of expression to that described in MMP-13 were observed in MMP-1 and MMP-2, whereas TIMP-2 and TIMP-3 were similarly expressed in TIMP-1 (data not shown). When the expression of osteoclast regulatory factors were analyzed, we found that RANKL expression was slightly higher in ePD + PIA, whereas OPG expression was found to be slightly decreased in this group, but when analyzed as RANKL/OPG ratio, ePD + PIA group presented a significant increase in the ratio values when compared with ePD group (data not shown). Interestingly, PIA induction resulted in a significant increase of MMP-13, RANKL, and OPG in periodontal tissues of AIRmax mice, but in lower extent than ePD or ePD + PIA.

PIA significantly alters the pattern of T-helper transcription factors expression in submandibular lymph nodes

Investigating the possible mechanism involved in the enhanced host responsiveness because of RA/PD interaction, we found that the submandibular lymph nodes (LNs) presented an altered expression pattern of the transcription factors responsible for T-cell polarization because of PIA (Figure 4). PIA induction resulted in a significant increase of Tbet, GATA-3, and RORγ in submandibular LNs of AIRmax mice, whereas ePD + PIA group presented even higher levels of Tbet and RORγ expression, while GATA-3 levels were found not to be modulated by PIA. Regarding inguinal LNs, only PIA was found to significantly upregulate tBET and RORγ expression, whereas GATA-3 levels were positively modulated by both ePD and PIA, but the effect of PIA was significantly more intense.

Control of experimental A. actinomycetemcomitans *infection is unaltered by PIA*

In view of the differential strength of the inflammatory immune response seen in ePD + PIA group, we next investigated the control of *A. actinomycetemcomitans* infection (Figure 5). Our results show that PIA co-induction neither result in changes in the bacterial load levels in periodontal tissues, nor in the levels of the antimicrobial mediators myeloperoxydase and iNOS, as well as in serum C reactive protein. Throughout ePD,

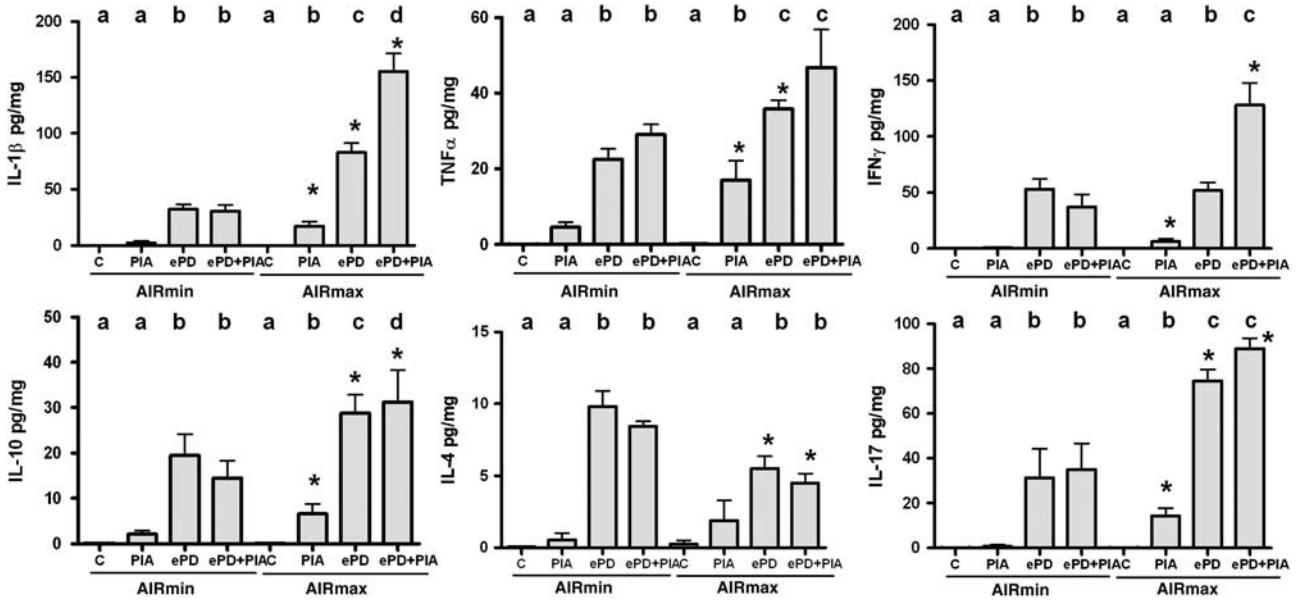

Figure 2 Cytokine response in periodontal tissues of AIRmin and AIRmax mice after the induction of experimental periodontal disease and arthritis. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated regarding the levels of IL-1β, TNF-α, IFNγ, IL-17, IL-4, and IL-10 protein in periodontal tissues by enzyme-linked immunosorbent assay (ELISA). The results are presented as picograms of cytokine per milligram of tissue, mean ± s.d. One-way ANOVA followed by Bonferroni's test. *$P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$).

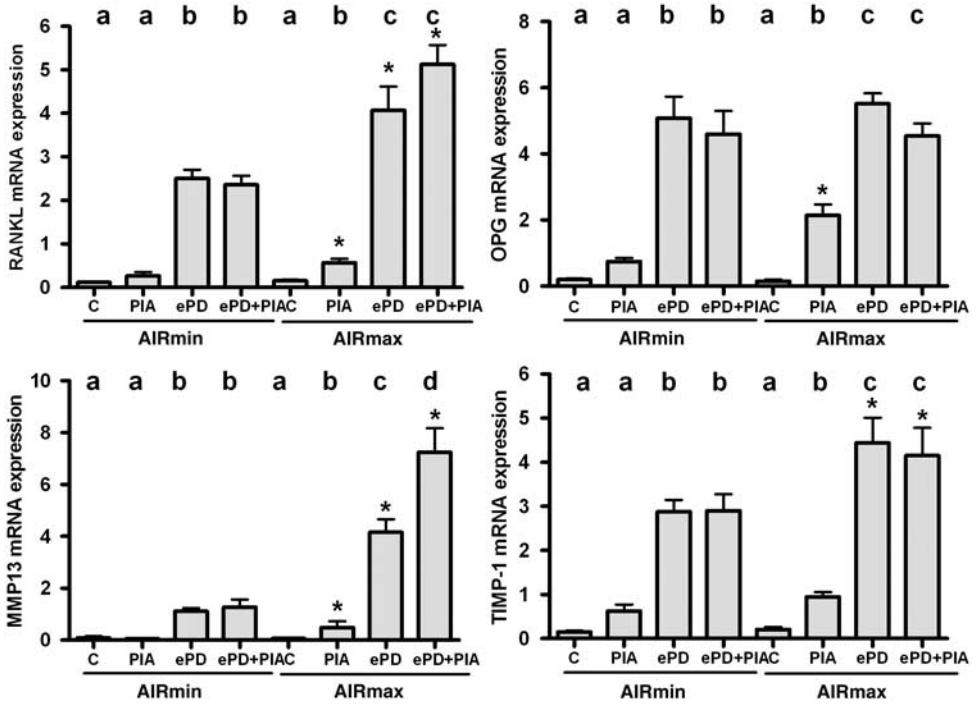

Figure 3 MMP-13, TIMP-1, RANKL, and OPG expression in periodontal tissues of AIRmin and AIRmax. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated for the levels of RANKL, OPG, MMP-13, and TIMP-1 mRNA in periodontal tissues, quantified by real-time PCR, using the SybrGreen System and the Ct method. The results are presented as the expressions of the target mRNAs with normalization to β-actin, mean ± s.d. All infected groups are statistically different from noninfected controls. One-way ANOVA followed by Bonferroni's test. *$P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$). #Indicate that AA DNA not detected.

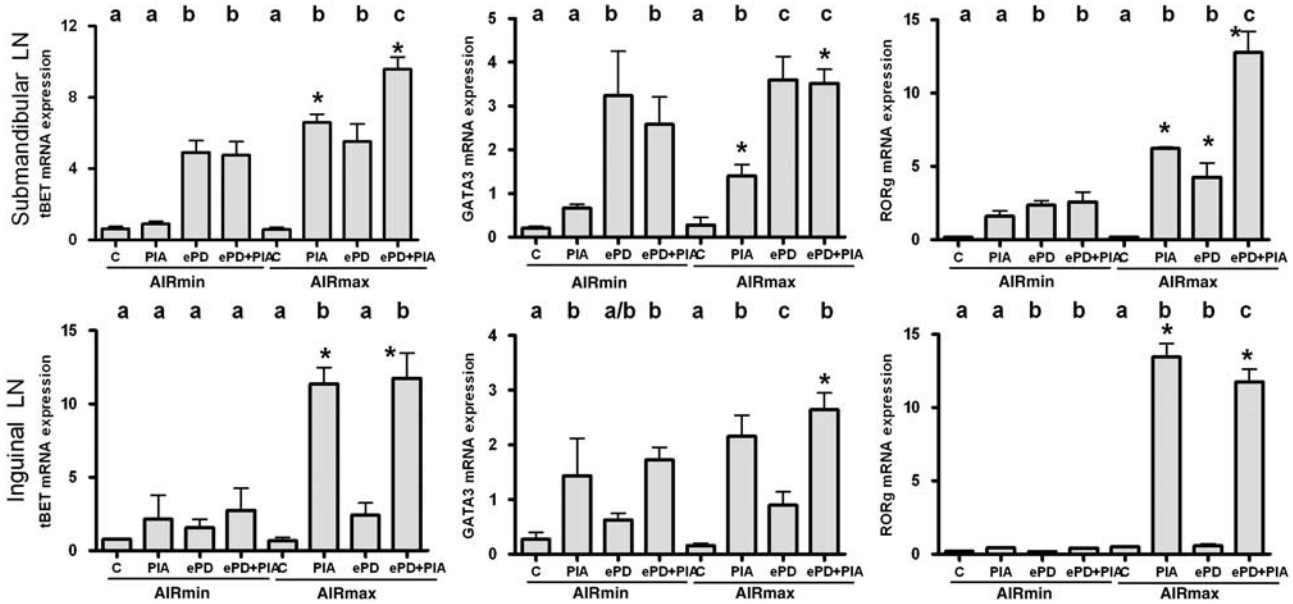

Figure 4 Expression of T-helper subsets transcription factors in the lymph nodes of AIRmin and AIRmax mice after the induction of experimental periodontal disease and arthritis. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with Pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated for the levels of tBET, GATA-3, and RORγ mRNA in the submandibular and inguinal lymph nodes, quantified by real-time PCR, using the SybrGreen System and the Ct method. The results are presented as the expressions of the target mRNAs with normalization to β-actin, mean ± s.d. One-way ANOVA followed by Bonferroni's test. *$P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$).

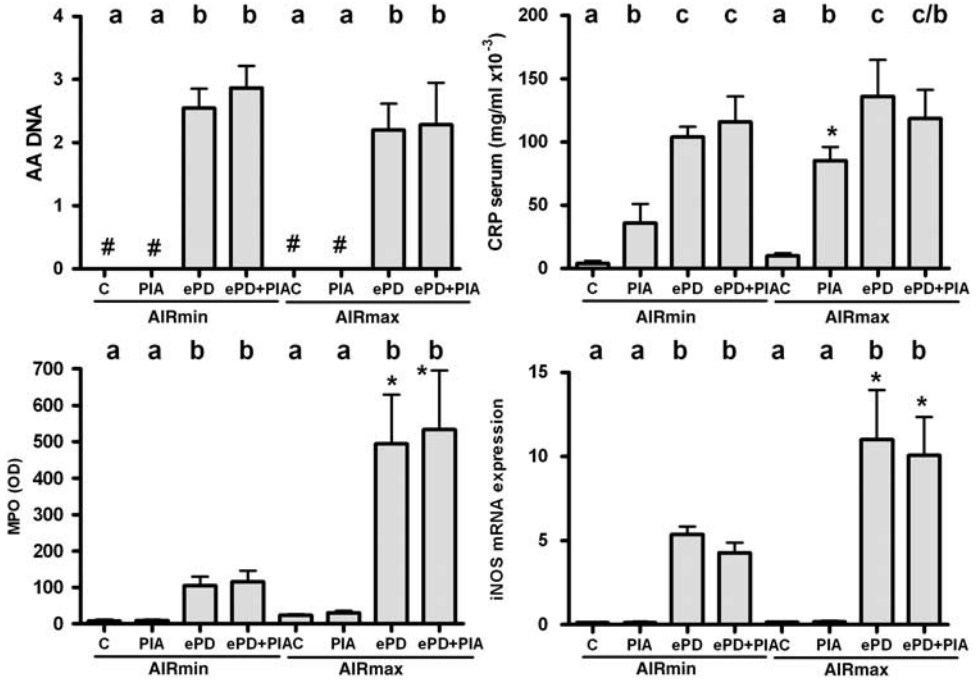

Figure 5 The control of *A. actinomycetemcomitans* infection after the induction of experimental arthritis. AIRmin and AIRmax mice infected orally with *A. actinomycetemcomitans* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated for *A. actinomycetemcomitans* load in periodontal tissues quantified by real-time PCR, using the SybrGreen system and normalized by tissue weight; serum levels of C reactive protein, presented as mg ml^{-1} $\times 10^{-3}$; levels of iNOS expression in periodontal tissues, quantified by real-time PCR, using the SybrGreen System and the Ct method; levels of myeloperoxidase in periodontal tissues, presented as OD. One-way ANOVA followed by Bonferroni's test. *$P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$). #Indicate that AA DNA not detected.

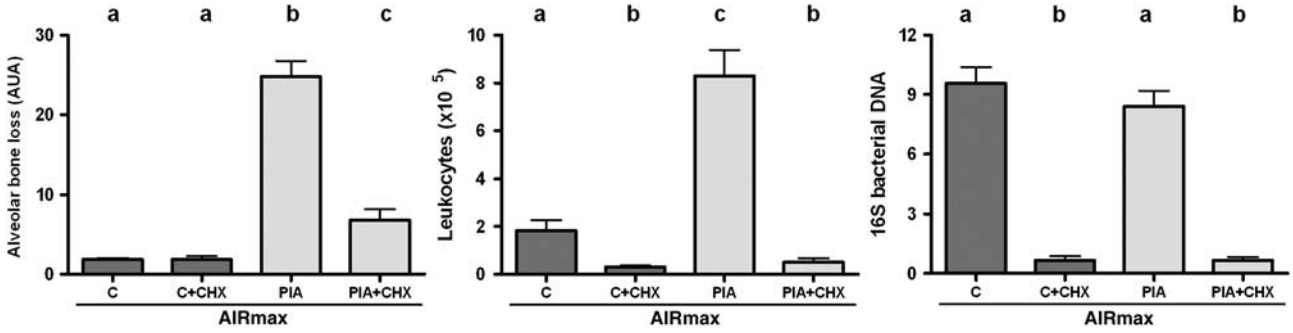

Figure 6 Arthritis-induced bone loss is highly dependent on oral microbiota. AIRmax mice intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were treated with chlorhexidine (CHX, 1-chlorhexidine gluconate 0.12% plus carboxymethylcellulose 2% topically applied in oral cavity each 2 days) and were evaluated for the alveolar bone loss (measurements of CEJ-ABC area in the palatal face of maxillary molars), total leukocyte counts of the inflammatory infiltrate in periodontal tissues (performed in a Neubauer chamber); bacterial load (16S) in periodontal tissues, quantified by real-time PCR, using the SybrGreen system and normalized by tissue weight. One-way ANOVA followed by Bonferroni's test. different letters indicate statistical differences within the experimental groups ($P<0.05$).

different organs were evaluated for possible bacterial dissemination (by means of real-time PCR), but no alterations were found (data not shown).

Control of normal oral microbiota inhibits the PIA-induced alveolar bone loss in AIRmax mice

Interestingly, PIA-induced bone loss was found to be dependent on the oral normal flora, as the control of oral bacteria with topical chlorhexidine (CHX), performed to mimic the clinical use of these anti-microbial agent in PD treatment, significantly abrogated alveolar bone loss (Figure 6). We also observed that the CHX treatment abrogated the migration of leukocytes to the periodontal tissues when compared with the mice presenting the normal oral flora. Finally, topical CHX treatment was found to be indeed effective in the view of the reduced bacterial 16S DNA load in both groups (control and PIA).

Co-induction of PIA and P. gingivalis*-induced PD results in increased severity of periodontal disease without effects in the severity of experimental arthritis*

To investigate whether a distinct periodontopathogen infection would result in distinct RA/PD interaction than *A. actinomycetemcomitans*, we next evaluated the severity of ePD developed by AIRmax and AIRmin mice after *P. gingivalis* infection (Figure 7). Similar to *A. actinomy-*

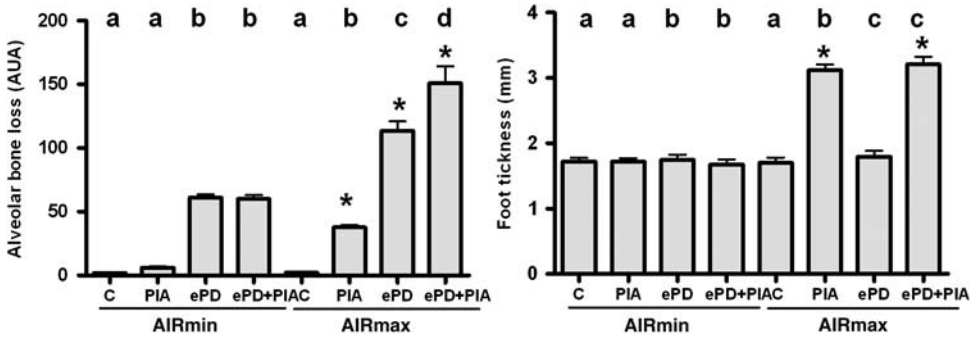

Figure 7 *P. gingivalis*-induced periodontitis severity is increased by pristane-induced arthritis co-induction, but severity of experimental arthritis is not altered. AIRmin and AIRmax mice infected orally with *P. gingivalis* [experimental periodontitis (ePD)-induction protocol] and intraperitoneally injected with pristane [experimental-induced arthritis (PIA) protocol], as well control mice (C) were evaluated for the severity of PD through alveolar bone loss quantification (measurements of CEJ-ABC area in the palatal face of maxillary molars) and for the severity of experimental arthritis (measured as the foot thickness in mm). One-way ANOVA followed by Tukey's test. $^{*}P<0.05$ vs respective group in AIRmin strain; different letters indicate statistical differences within the experimental groups in each strain ($P<0.05$).

cetemcomitans infection, we observed significantly less bone resorption in AIRmin when compared with AIRmax mice. The co-induction of PD and PIA also resulted in increased alveolar bone loss in AIRmax strain, whereas no significant alterations were observed in AIRmin mice. Regarding PIA development, only AIRmax presented a significant increase in foot thickness after pristane injection, which was not modulated by *P. gingivalis*-induced ePD co-induction.

Discussion

Clinical studies show the co-morbidity between RA and PD,[11,14–16,35] pathologies that share similar pathogenetic effector pathways such as the chronic inflammatory reaction associated with soft and mineralized tissue destruction. Indeed, RA can increase the incidence and severity of PD (independently or only partially dependent) of oral hygiene status or modifying factors.[4–7,15,16,35] Accordingly, our results show that PIA increase the severity of ePD in AIRmax mice strain, whereas in AIRmin strain (resistant to PIA development),[25–28] the severity of ePD was not altered by pristane injection, showing that the development of PIA, and not the pristane injection, is involved in the modulation of ePD severity. Similarly, adjuvant-induced arthritis in rats also results in signs of periodontal destruction.[29] However, the mechanisms underlying such co-morbidity interaction remain unknown. Among the hypothesis raised, conceptual models link both diseases by causal and noncausal pathways.[14–16,23]

Noncausal hypothesis state that environmental or host factors could concomitantly increase susceptibility to both RA and PD.[4–7] Accordingly, our data show that AIRmin and AIRmax mice strains share the susceptibility/resistance phenotype to both PIA and ePD.[24–28] This finding is reinforced in the view of the absence of environmental factors potentially involved in such interaction (namely socio-economic status and tobacco smoking)[16] in the experimental models used in this study. Nevertheless, noncausal theory is supported by studies that independently implicated specific polymorphisms and human leukocyte antigen molecules as susceptibility factors for both RA and PD.[15,16] A recent study shows that the distinct inflammatory and immunological responsiveness of AIRmin and AIRmax strains involves a broad variation in the expression of genes co-localized with earlier mapped regions for inflammation-related phenotypes in chromosomes 1, 3, 6, and 11, and that codify genes involved in inflammatory response, signal transduction, cell proliferation, and immune cell chemotaxis.[36] Indeed, genome-wide association studies confirm the clinical observations regarding the co-morbidity of immune-related diseases, and describe the existence of shared genetic susceptibility clusters, involving general immune pathways of T-cell differentiation, T-cell signaling, and the innate immune response.[1] However, in spite of RA being included in this study, PD was not considered.[1] Although the exact SNPs underlying such differential responsiveness are under investigation, one genetic variation involved in the control of immune inflammatory reactions between AIRmin and AIRmax strains is well known and involves the gene SLC11A1 (NRAMP1).[28,37] Interestingly, SNPs in SLC11A1 have been associated with the resistance/susceptibility to human RA development,[38–40] but unfortunately no data regarding SLC11A1 association with human PD is available in the literature. Accordingly, with the shared genetic susceptibility hypothesis, preliminary data from our group[41] point to a significant higher frequency of the 3/3 genotype of SLC11A1 rs34448891 SNP (earlier associated to RA)[38] in chronic PD patients. However, broader studies are required to replicate this finding, as well to confirm the hypothesis in patients affected with both RA and PD simultaneously. In this context, it is also very important to consider that, conversely to that happens with RA, SNPs and human leukocyte antigen molecules have been inconsistently associated with PD. This discrepancy is unsurprising in the view of the complex and multifactorial etiology of PD and to some limitations of the design of the studies that have been investigated in PD genetics (that is a control group that routinely comprises periodontally healthy subjects, which not imply a genetic resistance to the disease development, but reflect the control of the etiologic factors of disease).[16,42–44]

Besides the shared genetic susceptibility and the noncausal hypothesis, a functional interference in the host response against periodontopathogens by RA was verified. Investigating the potential mechanisms underlying the functional interference of RA on PD severity,

our results show that the inflammatory immune response was not only amplified (even higher levels of pro-inflammatory cytokines IL-1β and TNF-α) by RA/PD parallel induction, but also was skewed toward a Th1 and Th17 response (increased IFN-γ and IL-17 levels). Furthermore, the increased ePD severity correlates with the significant increase in the levels of RANKL and MMP-13, and with the increase of RANKL/OPG and MMP-13/TIMPs ratios, characteristically associated with periodontal destruction.[13,19,45] Looking for the reason of the altered adaptive response because of RA/PD interaction, we found that the submandibular LNs (the major draining LNs of oral cavity), which are significantly enlarged by the ePD induction, presented an even higher enlargement because of the pristane injection (data not shown), being an indicative of systemic immune reactivity to the PIA. Furthermore, the pattern of the transcription factors responsible for T-cell polarization expressed in submandibular LN was modulated by the PIA, the levels of Tbet and RORγ being significantly increased in AIRmax mice submitted to both PIA and ePD protocols. Therefore, it is possible that a systemic immune activation because of PIA alters the balance of T-helper differentiation in the submandibular LN environment, and these skewed polarization correlates with the increased levels of Th1- and Th17-type cytokines observed in periodontal tissues. Indeed, PIA model is characterized by a broad autoimmune reactivity to a broad spectrum of autoantigens, associated with a robust influx of inflammatory cells and production of bone resorptive cytokines at the joints, but possible alterations in other tissues were not investigated.[46,47] Other possible mechanism involved in the upregulation of ePD severity by PIA is represented by the increased levels of inflammatory cytokine TNF-α in the serum of AIRmax mice submitted to the PIA protocol (data not shown). The circulating cytokines would prime circulating leukocytes, facilitating its migration to periodontal tissues and also resulting in increased responsiveness to the triggering by bacterial factors such as lipopolysaccharide.[14,48] In accordance with such hypothesis, systemic administration of TNF-α results in increased severity of ligature-induced PD in rats.[49] A mechanistic explanation for this observation is that TNF-α can interfere with monocytes and dendritic maturation and with the subsequent T-helper polarization, as anti-TNF therapy of chronic inflammatory autoimmune disease models results in impaired development of Th1 and Th17 responses.[50–52]

Interestingly, AIRmax mice that received pristane injection, but were not submitted to the oral inoculation of periodontopathogens, also developed a significant alveolar bone loss. These findings support the hypothesis that RA could co-induce and just not exacerbate PD.[14] In the co-induction scenario, the unbalanced immune response because of PIA development triggers PD development in response to the commensal subgingival microflora, which in standard homeostatic conditions would not be harmful to the host.[14] In fact, the control of normal oral flora with local/topic antiseptic bactericidal agent CHX abrogated PIA-induced alveolar bone loss. Interestingly, the CHX treatment abrogated the migration of leukocytes to the periodontal tissues when compared with the mice presenting the normal oral flora (data not shown or new figure). Therefore, it is possible to suggest that the leukocyte migration triggered by the normal oral biofilm, when associated with altered immune responsiveness because of experimental RA, results in alveolar bone loss even in the absence of classic periodontopathogens. Similarly, alloxan-induced diabetes in rats triggers, or co-induces, the onset of alterations that are typical of periodontal diseases even in the absence of aggressive factors such as ligatures or inoculation of classic periodontopathogens.[53] In this context, it is important to consider that experimental arthritis development does not alter the pattern of periodontal infection (the *A. actinomycetemcomitans* and overall bacterial load—data not shown), ruling out the possible increased responsiveness because of an increased microbial challenge. Indeed, as earlier discussed, clinical studies show that RA/PD interaction is only partially dependent on oral hygiene status and the control of oral flora.[4,5,15,16,35] Another well-defined situation of co-induction, described as a two-hit model, is acute respiratory distress syndrome. In acute respiratory distress syndrome, ventilator-induced lung injury does not predictably result in lung tissue destruction, but does promote the accumulation of PMN leukocytes in the lung alveoli (the first hit), which followed by the injection of a relatively low dose of endotoxin (the second hit) induces PMNL degranulation, irreversible lung destruction and death.[54]

Causal hypothesis also consider that PD development because of RA development could be secondary, being mediated by RA-triggered alterations such as systemic osteoporosis and Sjogren's syndrome.[14,16,55,56] Regarding systemic osteoporosis, our results show that a trend toward higher levels of circulating calcium in AIRmax strain throughout experimental RA (data not shown) could be indicative of higher systemic bone resorption activity. However, no significant changes in bone density or architecture were observed (data not shown), the possible occurrence of osteorosis in AIRmax mice being unclear. However, further studies specifically designed to evaluate the development of osteoporosis throughout PIA and its potential influence over PD outcome are required to solve this question. Regarding a possible influence of Sjogren's syndrome-like condition, which could interfere in salivary gland biology and consequently predispose to or intensify PD development,[16,57] there are no changes in submandibular and parotid glands of mice with PIA and/or PD (data not shown).

Besides the effect of RA over PD, clinical studies also show that PD could increase RA severity.[11,12] Indeed, numerous infectious agents, including periodontal bacteria, and antibodies against them have been implicated as contributory to the etiology of RA.[16,58,59] In addition, oral bacterial DNAs were found in synovial fluid of RA patients, suggesting that oral pathogens could have a perpetuating effect in joint disease.[57] However, no changes in the severity of experimental RA (PIA) were observed at clinical or molecular analysis (data not shown). One may argue that *A. actinomycetemcomitans* is more associated to aggressive PD, whereas RA/PD interaction is primarily described to involve chronic PD. However, *A. actinomycetemcomitans* is also present in a significant number of chronic PD sites and was supposed to be a possible link between PD and RA in earlier studies.[31,60] Recently, the focus has been turned to other periodontopathogens, such as *P. gingivalis*, which are supposed to have a major function in PD/RA

interaction through active peptide citrullination, which could be involved in loss of self-tolerance and RA development.[61] However, even with the substitution of the infectious agent of ePD to *P. gingivalis*, no alterations in the severity of experimental RA were observed, whereas the increase of ePD was similar to that of *A. actinomycetemcomitans*. One possibility to this lack of association is that ePD presents a lower or narrow systemic effect than PIA, possibly insufficient to module RA severity. Furthermore, no evidences of *A. actinomycetemcomitans* or *P. gingivalis* dissemination to arthritic joints were observed (data not shown), in contrast to clinical studies that describe the presence of bacterial DNA in arthritic joints.[57,62] Another possibility is that the distinct kinetics of both chronic models (when PD is induced, PIA is already in development) are difficult for the observation of a putative effect over experimental RA severity.

Taken together, the results presented herein are the first experimental demonstration of the clinical association between RA and PD described in clinical studies. Our results show that the interaction between ePD and PIA involves functional interferences comprising the amplification of innate immunity and an adaptive response skewed to an increased Th1/Th17 responsiveness. In addition, a shared susceptibility genotype prone to hyper-inflammatory responsiveness is involved in RA/PD interaction. However, further studies using different models of both PD and RA, as well longitudinal and genetic investigations in human beings, certainly will contribute to unravel the immunopathological and genetic basis of the complex co-morbidity network between RA and PD. This knowledge may allow us to direct the development of strategies to prevent and treat both RA and PD, aimed to modulate the host immune response to minimize the local and systemic consequences of both conditions.

Materials and methods

PIA and ePD

Experimental groups comprised 8-week-old AIRmin and AIRmax mice (lineages generated, bred, and maintained at 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.

Experimental arthritis was achieved by the two intraperitoneal injections of pristane (0.5 ml; Sigma, St Louis, MO, USA) administered 30 days apart (at days –30 and 0), and signs of arthritis were assessed by ankle joint measurement with a micrometer over a 90-day period. Periodontal infection was achieved by oral delivery of 1×10^9 CFU of a diluted culture of *Aggregatibacter (Actinobacillus) actinomycetemcomitans* JP2 (anaerobically grown in supplemented agar medium, TSBV) or *P. gingivalis* ATCC 49417 (anaerobically grown on supplemented blood agar) in 100 µl of phosphate-buffered saline with 2% carboxymethylcellulose, placed in the oral cavity of mice with a micropipette, as earlier described,[63] at days 0, 2, and 4. Negative controls included noninfected and sham-infected mice, which received heat-killed bacteria in 2% carboxymethylcellulose solution. To eliminate the possible effects of normal mice oral microbiota, an additional group was created by the topical delivery of 50 µl CHX (1-chlorhexidine gluconate 0.12%) solution, plus carboxymethylcellulose 2%, which was topically applied in the mice teeth each 2 days throughout the experimental period. A control group received only the application of 50 µl aqueous carboxymethylcellulose 2% solution with a similar protocol. At day 60 (after 90 days post-initial pristane injection and 60 days after first microbial inoculation) mice were killed and the samples collected to the different experimental analysis. Evaluation of the extent of alveolar bone loss was performed as earlier described[63] in arbitrary units of area.

Inflammatory cells analysis

The isolation and characterization of leukocytes present in the periodontal lesion site were performed as earlier described.[18] 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 for total inflammatory cell count. Results represent the number of cells (± s.d.) in the periodontal tissues of each mouse, normalized by the tissue weight, for two independent experiments.

Protein extraction and cytokine ELISA

Measurements of cytokines and chemokines in periodontal tissues were performed as described earlier.[18] For protein extraction, palatal periodontal tissue of five mice was homogenized in phosphate-buffered saline pH 7.4, centrifuged at 1000 r.p.m. at 4 °C and the supernatants were stored at −70 °C. The concentrations of cytokines in periodontal extracts were determined by ELISA (R&D Systems, Minneapolis, MN, USA) as follows: IL-1β (sensitivity $>3\,pg\,ml^{-1}$), TNF-α ($>3.4\,pg\,ml^{-1}$), IFN-γ ($>2\,pg\,ml^{-1}$), IL-17 ($>5\,pg\,ml^{-1}$), and IL-10 ($>4\,pg\,ml^{-1}$). The results were expressed as picograms of cytokine (± s.d.) per milligram of periodontal tissue for two independent experiments.

Real-time PCR reactions

The extraction of total RNA from periodontal tissues (upper molars with whole surrounding buccal and palatal periodontal tissues) was performed with Trizol reagent (Invitrogen, Rockville, MD, USA) and the cDNA synthesis were accomplished as described earlier.[18] To allow the 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

Table 1 Primer sequences and reaction properties

Target	*Sense and anti-sense sequences*	*At (°C)*	*Mt (°C)*	*bp*
MMP-13	AGAGATGCGTGGAGAGTCGAA AAGGTTTGGAATCTGCCCAGG	65	85	162
TIMP-1	ACTGCAGGATGGACTCTTGCA TTTCAGAGCCTTGGAGGAGCT	30	82	206
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
OPG	GGAACCCCAGAGCGAAATACA CCTGAAGAATGCCTCCTCACA	57	77	225
INOS	CGTCATTTCTGTCCGTCTCT TTGCTGGCTGATGGCTGGCG	56	82	390
T-bet	CCC CTG TCC AGT CAG TAA CTT CTT CTC TGT TTG GCT GGC T	60	78	115
GATA3	AGG AGT CTC CAA GTG TGC GAA TTG GAA TGC AGA CAC CAC CT	60	80	124
FOXp3	CAGTCACTGCAAATGTCCGGT TGTCGGACACAAAGGAACTGC	62	75	75
RORg	TGACGGCCAACTTACTCTTGG GCCTGGTTTCCTCAAAACGA			
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495
16S	CGCTAGTAATCGTGGATCAGAATG TGTGACGGGCGGTGTGTA	60	72	69
Porphyromonas gingivalis	AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	60	87	404
Aggregatibacter actinomycetemcomitans	ATGCCAACTTGACGTTAAAT AAACCCATCTCTGAGTTCTTCTTC	60	78	557

Abbreviations: At, annealing temperature; bp, base pairs of amplicon size; Mt, melting temperature.

nitrogen, mechanically fragmented, and homogenized in sterile Milli-Q water with Ultra Turrax (IKA, Germany), and subsequently submitted to DNA extraction with DNA Purification System (Promega Biosciences Inc., San Luis Obispo, CA, USA). Real-time PCR quantitative mRNA or DNA analyses were performed in a Mini-Opticon system (Bio-Rad, Hercules, CA, USA), using SybrGreenMasterMix (Invitrogen), 100 nM specific primers (Table 1), and 2.5 ng of cDNA or 5 ng of DNA in each reaction. The primers sequences and reaction properties are depicted in Table 1. For mRNA analysis, the relative level of gene expression was calculated with reference to β-actin using the cycle threshold (Ct) method. Bacterial DNA levels were determined using the Ct method and normalized by the tissue weight.

Quantification of anti-microbial mediators
Quantification of anti-microbial mediators was performed as described.[17] The levels of serum C reactive protein were determined in serum samples using a commercially available agglutination kit (Labtest Diagnóstica, São Paulo, Brazil). Myeloperoxydase activity in homogenized periodontal tissue was measured by enzymatic reaction, measured through the absorbance at 450 nm, and presented as OD. The serum titer of IgG specific to *A. actinomycetemcomitans* was measured by ELISA.

Statistical analysis
Data are presented as means ± s.d., and the statistical significance between the infected and control mice of both strains was analyzed by ANOVA, followed by Bonferroni's post-test, performed with GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA, USA). Values of $P<0.05$ were considered as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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