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Functional interferences in host inflammatory immune response by airway allergic inflammation restrain experimental periodontitis development in mice

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Abstract

Aims: Periodontal disease (PD) and airway allergic inflammation (AL) present opposing inflammatory immunological features and clinically present an inverse correlation. However, the putative mechanisms underlying such opposite association are unknown.

Material and Methods: Balb/C mice were submitted to the co-induction of experimental PD (induced by *Actinobacillus actinomycetemcomitans* oral inoculation) and AL [induced by sensitization with ovalbumin (OVA) and the subsequent OVA challenges], and evaluated regarding PD and AL severity, immune response [cytokine production at periodontal tissues, and T-helper transcription factors in submandibular lymph nodes (LNs)] and infection parameters.

Results: PD/AL co-induction decreased PD alveolar bone loss and periodontal inflammation while experimental AL parameters were unaltered. An active functional interference was verified, because independent OVA sensitization and challenge not modulate PD outcome. PD+AL group presented decreased tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, -γ, IL-17A, receptor activator of nuclear factor κ-light-chain-enhancer of activated B cells ligand and matrix metalloproteinase (MMP)-13 levels in periodontal tissues, while IL-4 and IL-10 levels were unaltered by AL co-induction. AL co-induction also resulted in upregulated T-bet and related orphan receptor γ and downregulated GATA3 levels expression in submandibular LNs when compared with PD group.

Conclusion: Our results demonstrate that the interaction between experimental periodontitis and allergy involves functional immunological interferences, which restrains experimental periodontitis development by means of a skewed immune response.

Key words: airway allergic inflammation; co-morbidity; cytokine; immune response; inflammatory reaction; periodontal diseases

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Conflict of interest and source of funding Statement

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Periodontal diseases (PD) are the most prevalent human chronic inflammatory disease, where bacterial species hosting periodontal biofilm trigger inflammatory and immune responses involved in tissue damage (Kinane & Attstrom 2005, Sanz & Quirynen 2005). Pro-inflammatory [tumour necrosis factor-α (TNF-α), interleukin (IL)-1β], T-helper type 1 (Th1)--γ (IFN-γ) and Th17- (IL-17A) type cytokines are present at high levels in diseased periodontium and are involved in disease development (Cutler et al. 2000, Graves & Cochran 2003, Honda et al. 2006, Garlet 2010). These cytokines present a catabolic role over the periodontal tissues, mediated by the production of matrix metalloproteinases

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(MMPs) and the osteoclastogenic factor receptor activator of nuclear factor κ-light-chain-enhancer of activated B cells ligand (RANKL) (Graves & Cochran 2003, Taubman et al. 2005, Garlet et al. 2006, 2007, 2008, Garlet 2010). On the other hand, anti-inflammatory, Th2- and Treg-related cytokines such as IL-4 and IL-10 exert the reverse effect, being associated with lower PD severity (Yamazaki et al. 2003, Taubman et al. 2010, 2005, Garlet et al. 2006, McInnes & Schett 2007, Graves 2008, Garlet et al. 2010, Garlet 2010). Interestingly, factors that bias the immunoregulatory balance towards the inflammatory cytokines predominance amplify the severity of PD, which include specific periodontopathogens, hyper inflammatory SNPs and the interaction with systemic conditions (Ferreira et al. 2008, Lamster et al. 2008, de Pablo et al. 2009, Trombone et al. 2009a, Teeuw et al. 2010).

Accordingly, recent studies demonstrate a parallel (and sometimes additive or synergic) development of infectious, inflammatory and autoimmune disorders called co-morbidity, which suggests the existence of similar immunopathogenetic pathways (Somers et al. 2006, Lettre & Rioux 2008, Zhernakova et al. 2009). Clinical studies describe the co-morbidity of PD with diabetes and rheumatoid arthritis (RA), associations further confirmed in and experimental studies (Kasser et al. 1997, Mercado et al. 2001, Lamster et al. 2008, Nilsson & Kopp 2008, Pischon et al. 2008, Trombone et al. 2010). Interestingly, the PD interaction with systemic conditions can be bi-directional, because the control of periodontal infection may reduce the severity of both RA and diabetes (Al-Katma et al. 2007, Lamster et al. 2008, Teeuw et al. 2010, Ortiz et al. 2009). The mechanistic basis of the co-morbidity amplification of PD severity rely on shared common immunopathogenic mechanisms, which involves pro-inflammatory, Th1- and Th17-type cytokines (McInnes & Schett 2007, Graves 2008, Lamster et al. 2008, Teeuw et al. 2010, Trombone et al. 2010).

While similar patterns of immune response may converge to amplify the severity of certain co-morbid conditions, diseases associated with opposing immunological patterns may present an inverse association. In fact, two wide epidemiological studies demonstrate an inverse association between periodontitis and oral pathogens (*Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*) with allergic disease [allergic inflammation (AL)] (Arbes et al. 2006, Friedrich et al. 2008). In immunological terms, it sounds reasonable to conceive that diseases characterized by the production of distinct and mutually inhibitory cytokines present an inverse association. This paradigm fits in the ''hygiene hypothesis'', which postulates that the immune response raised against infections may have protective effects against the allergies development (Strachan 1989, Okada et al. 2010). While cross-sectional data from human studies (Arbes et al. 2006, Friedrich et al. 2008) does not allow a direct cause-effect association, a recent experimental study demonstrate that subcutaneous infection with a periodontal pathogen attenuate airway AL (Card et al. 2010). However, this experimental system does not allow any inference on the possible modulation of PD outcome by allergy (Card et al. 2010), and on how an allergic process could influence the host response at periodontal environment.

In the present study, Balb/C mice was submitted to both ovalbumin (OVA)-induced AL [experimental allergic inflammation (eAL) protocol] and subsequently infected with the periodontopathogen *A. actinomycetemcomitans* [experimental periodontal diseases (ePD) protocol] in order to evaluate the patterns of airway allergy and periodontitis outcome and to investigate the possible mechanisms underlying its interaction.

Material and Methods

OVA-induced allergy (eAL) and (ePD)

Experimental groups comprised 8-week-old Balb/C mice ($N = 8$ per experimental group), bred and maintained in the animal facilities of the Department of Biochemistry and Immunology of FMRP/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 CFU of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (AA) JP2 (anaerobically grown in supplemented agar medium, TSBV) 2% carboxymethylcellulose PBS diluted culture, placed in the oral cavity of mice with a micropipette with 48 h intervals, as described previously (Garlet et al. 2005). The day of initial AA inoculation was defined as day 0 of the experimental timeline (Fig. 1).

Experimental airway allergy was achieved by OVA-sensitization with two doses of OVA (grade V; Sigma, St. Louis, MO, USA) (performed at days −24 and −10) (Fig. 1). The first dose of OVA (100 μg) was administered subcutaneously in the presence of 1.6 mg alum adjuvant in a 200 μl final volume. The second dose (50 μg) was injected intraperitoneally in 100 μl saline 14 days after the first sensitization. Seven days after the sensitization (day −3), mice were challenged with OVA (100 μg OVA/50 μl saline) intranasally. Seventy-two hours after OVA challenge, mice were inoculated with AA (days 0, 2 and 4) following two OVA challenges (days 12 and 28). Seventy-two hours after the last OVA challenge, samples were collected.

The chronological progression of eAL and ePD protocols was designed to allow the infection of mice presenting an active allergic process (i.e. sensitized and challenged with OVA). Negative controls included sham-infected mice (C-PD group), which received heat-killed bacteria in 2% carboxymethylcellulose solution; mice sensitized with OVA and non-challenged (C-AL group) and mice non-sensitized but challenged with OVA (data not shown). Mice were killed and the samples collected to the different experimental analysis at day 31 (after 55 days post-initial OVA sensitization and 31 days after first microbial inoculation).

Periodontal disease severity was measured by the evaluation of the extent of alveolar bone loss in arbitrary units of area as described previously (Garlet et al. 2005); and by the quantification of leucocytes present in the lesion site performed in a Neubauer chamber as described previously (Garlet et al. 2005).

The allergy severity was determined by determination of cell counts in bronchoalveolar lavage fluid (BALF) and serum antibody detection. BALF was obtained following five consecutive 1 ml injections of RPMI-1640 medium into the lungs. Total volume of 5 ml was centrifuged (400 *g*, 10 min.) and cells were counted in a Neubauer chamber; 40,000 cells were centrifuged onto microscope slides and stained with panoptic (Clorzap – Hemogram Ind.,

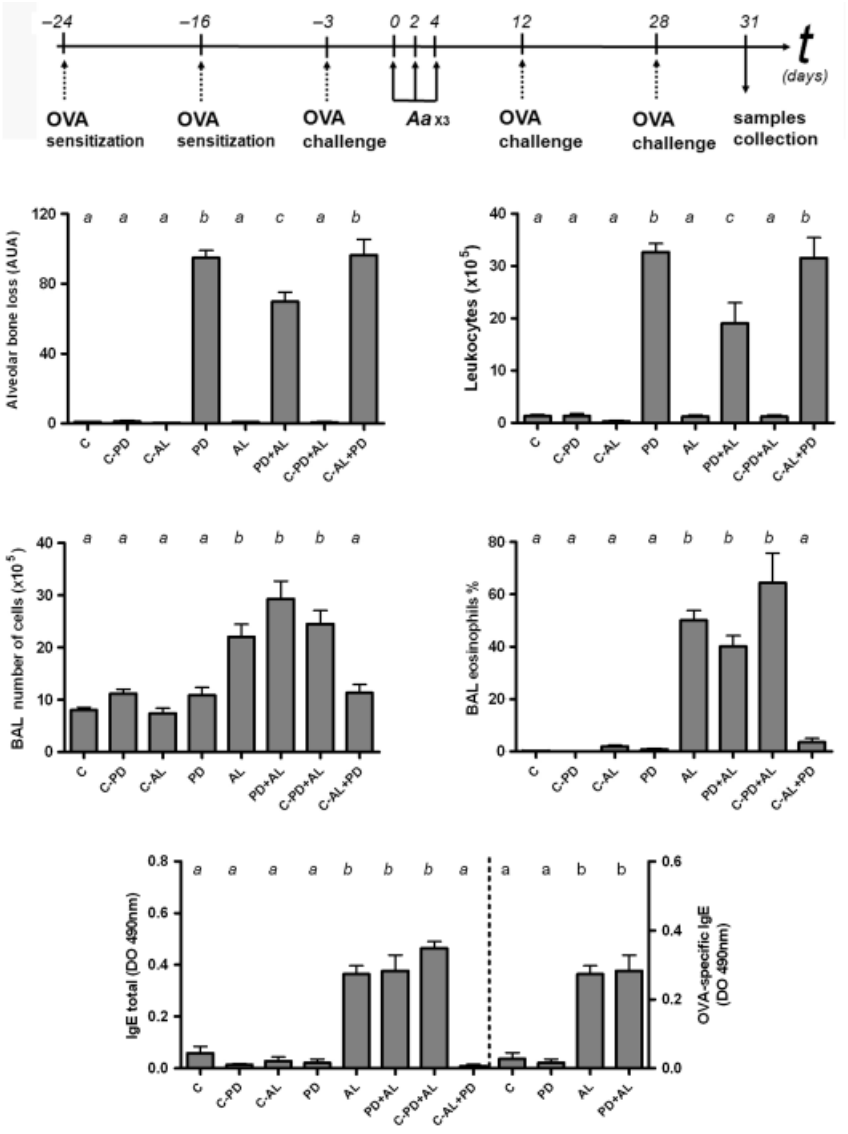

Fig. 1. Interference of OVA-induced airway experimental allergy in *Actinobacillus actinomycetemcomitans*-induced periodontal disease. Balb/C mice infected orally with *A. actinomycetemcomitans* [experimental periodontal disease (PD) induction protocol] and sensitized and challenged with OVA [experimental allergy (AL) protocol], as exemplified in the experimental flowchart; PD, AL as well control (C), sham-infected (C-PD group) and OVA-sensitized but non-challenged (C-AL group) groups 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); and for the severity of experimental allergy (measured as the total BAL counts, % of BAL eosinophils and total and OVA-specific IgE levels). One-way ANOVA followed by Bonferroni's test: groups with different letter designations are statistically different ($p < 0.05$). OVA, ovalbumin; AL, allergic inflammation; BAL, bronchoalveolar lavage.

Curitiba, Brazil). Percentages of eosinophils, lymphocytes, macrophages and neutrophils were determined from each sample. IgE total and OVA-specific antibodies were evaluated by sandwich ELISA using biotin-conjugated anti-mouse Ab according to manufacturer's instructions (BD Biosciences-PharMingen, San Diego, CA, USA).

Protein extraction and cytokine ELISA

Measurements of cytokines and chemokines in periodontal tissues were performed as described previously (Garlet et al. 2008). For protein extraction, palatal periodontal tissue of five mice was homogenized in phosphate-buffered saline pH 7.4, centrifuged at 4000 *g* 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), and the results were expressed as picograms of cytokine (± SD) per milligram of periodontal tissue, for two independent experiments.

Real-time polymerase chain reaction (PCR) reactions

The extraction of total RNA from periodontal tissues (upper molars with whole surrounding buccal and palatal periodontal tissues) and submandibular lymph nodes (LNs) were performed with Trizol reagent (Invitrogen, Rockville, MD, USA) and the cDNA synthesis were accomplished as described previously (Garlet et al. 2008). In order 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 nitrogen, mechanically fragmented and homogenized in sterile Milli-Q water (Invitrogen, Rockville, MD, USA) with Ultra Turrax (IKA, Staufen, 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 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 primers 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 (Ct) method. Bacterial DNA levels were determined using the Ct method and normalized by the tissue weight.

Quantification of antimicrobial mediators

Quantification of antimicrobial mediators was performed as described previously (Garlet et al. 2008). The levels of serum C reactive protein (CRP) were determined in serum samples using a commercially available agglutination kit (Labtest Diagnóstica, São Paulo, Brazil), as described previously (Garlet et al. 2008). Myeloperoxydase (MPO) activity in homogenized periodontal

Table 1. Primer sequences and real-time PCR properties

Target	Sense and anti-sense sequences	T_a (°C)	T_m (°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
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
RORγ	TGACGGCCAACTTACTCTTGG GCCTGGTTTCCTCAAAACGA	53	49	59
CXCR3	ATC TACCTATCAGCCAACTACGA TCAGAGAGCAA ATGTGGATGT	60	79	379
CCR4	CTT GCACCAAGGA AGGTAT AG CATAGACAGA TACCTAGG	58	81	116
CCR8	GGCAGCTTCACAGAAGCTTTG GAATACCACAGCTGGCTTGGA	58	79	74
CCR6	TGCCCACGTCAAGGAGTATTT TGCAAGATTGGAGCACTTGC	59	80	52
iNOS	CGTCATTTCTGTCCGTCTCT TTGCTGGCTGATGGCTGGCG	56	82	390
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495
Aggregatibacter actinomycetemcomitans	ATGCCAACTTGACGTTAAAT AAACCCATCTCTGAGTTCTTCTTC	60	78	557

T_a, annealing temperature; T_m, melting temperature; bp, base pairs of amplicon size; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; RANKL, receptor activator of nuclear factor κ-light-chain-enhancer of activated B cells Ligand; RORγ, related orphan receptor γ; OPG, Osteoprotegerin; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction.

tissue was measured as by enzymatic reaction, measured through the absorbance at 450 nm, and presented as OD; described previously (Garlet et al. 2008). The serum titre of total and *A. actinomycetemcomitans*-specific IgE and IgG was measured by ELISA as described previously (Garlet et al. 2008).

Statistical analysis

Data are presented as means ± SD, and the statistical significance between experimental and control groups was analysed by ANOVA, followed by Bonferroni's post-test, performed with GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Values of $p<0.05$ were considered statistically significant.

Results

A. actinomycetemcomitans infection of allergic mice results in low severity experimental periodontitis

We first evaluated the severity of ePD developed by allergic mice after *A. Actinomycetemcomitans* infection (Fig. 1). The co-induction of ePD and eAL resulted in decreased alveolar bone loss and inflammatory cell migration to periodontal tissues, while no significant alterations were observed in mice individually sensitized (C-AL group) or challenged (data not shown) with OVA. When AL development was investigated, our results demonstrate that AL group (sensitized and challenged with OVA) presented a significant increase in total BAL cell count, BAL eosinophils percentage and total and OVA-specific IgE levels as expected (Kips et al. 2003, Lloyd 2007), but eAL parameters were not modulated by the PD co-induction. These results demonstrate that only mice with active airway allergy develop a less severe PD.

ePD/eAL interaction result in altered cytokine production in periodontal tissues

We next investigated the expression of pro- and anti-inflammatory and prototypical T-helper cytokines, in order to determine the mechanisms responsible for the differential response demonstrated by allergic mice after ePD/eAL co-induction (Fig. 2). ELISA analyses demonstrated that IFN-γ, IL-17, IL-1β and TNF-α levels were significantly lower in PD+AL group when compared with PD. A non-statistically significant trend towards higher IL-4 levels was observed in PD+AL group, while no significant changes were observed in the levels of IL-10 comparing PD+AL with PD groups. OVA-challenged group (data not shown) presented a similar response pattern than OVA-sensitized (C-AL group). No significant levels of cytokines were found in the tissues of control mice not submitted to oral infection protocol.

MMPs/tissue inhibitor of metalloproteinase (TIMPs) and RANKL/osteoprotegerin (OPG) expression in periodontal tissues

We next investigated whether the lower periodontitis severity seen in PD+AL group could be due a modulation in MMPs/TIMPs and RANKL/OPG expression (Fig. 3). The qPCR data demonstrated a significantly lower MMP-13 mRNA expression in gingival tissues from PD+AL mice, while TIMP-1 mRNA levels were not modulated by AL co-induction. Similar patterns of expression to that described to MMP-13 were observed to MMP-1, MMP-2 and MMP-9 (data not shown), while TIMP-2 and TIMP-3 mRNA levels were also not modulated by AL co-induction (data not shown) similarly to described to TIMP-1. When the expression of osteoclast regulatory factors was analysed, we found that RANKL expression was decreased in PD+AL, while OPG expression was not modulated by eAL.

eAL significantly alters the pattern of T-helper transcription factors expression in submandibular LNs

Investigating the possible mechanism involved in the skewed immune response due AL/PD interaction, we found that the allergy development resulted in a marked alteration in the expression pattern of the transcription factors responsible for T cell polarization in submandibular LNs (Fig. 4). AL induction resulted in a significant increase of GATA3 levels while T-bet and related orphan receptor γ (RORγ)

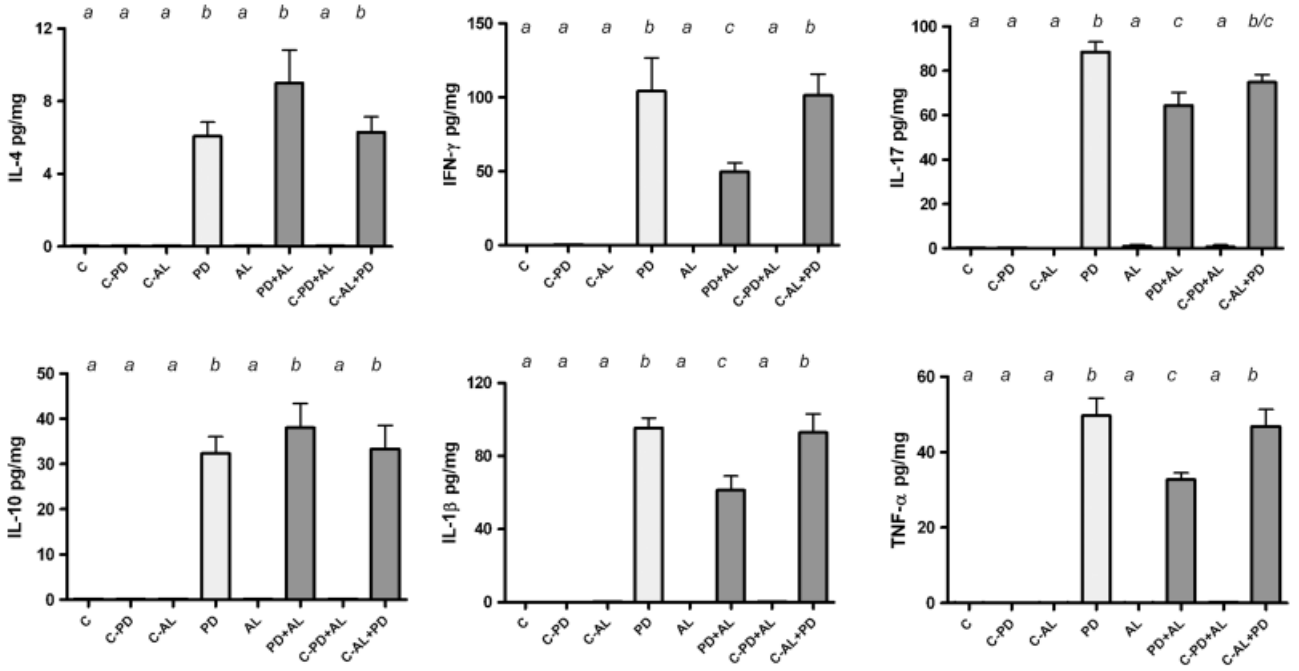

Fig. 2. Cytokine response in periodontal tissues of C57Bl/6 mice after the induction of experimental periodontal disease and allergy. Balb/C mice infected orally with *Actinobacillus actinomycetemcomitans* [experimental periodontal disease (PD) induction protocol] and sensitized and challenged with OVA [experimental allergy (AL) protocol], as exemplified in the experimental flowchart; PD, AL as well control (C), sham-infected (C-PD group) and OVA-sensitized but non-challenged (C-AL group) groups were evaluated regarding the levels of IL-4, IFN-γ, IL-17, IL-10, IL-1β and TNF-α protein in periodontal tissues by enzyme-linked immunosorbent assay. The results are presented as picograms of cytokine per milligram of tissue, mean $\pm$ SD. One-way ANOVA followed by Bonferroni's test: groups with different letter designations are statistically different ($p<0.05$). AL, allergic inflammation; OVA, ovalbumin; AL, allergic inflammation; IL, interleukin; TNF-α, tumour necrosis factor-α; IFN, interferon-γ.

expression was significantly reduced in AL+PD group when compared with PD group. The levels of the regulatory T cells transcription factor FOXp3 were not modulated by AL co-induction. In the view of the potential alterations in the pattern of T-helper cell polarization in submandibular LNs environment, we also investigated the patterns of T-helper-related chemokine receptors. The results depict a similar scenario to that described to the transcription factors, where AL co-induction resulted in a significant increase of Th2-related CCR4 and CCR8 receptors expression, while the expression of CXCR3 (Th1-related) and CCR6 (Th17) related were significantly when compared with PD group.

The production of antimicrobial mediators and the control of experimental *A. actinomycetemcomitans* infection were unaltered by allergic process

In the view of the potential impact of the skewed immune response in the production of antimicrobial mediators and in the control of periodontal infection, we next investigated the control of *A. actinomycetemcomitans* infection in the presence or absence of allergic process (Fig. 5). Our results demonstrate that eAL co-induction not modulated the levels of AA-specific IgE and IgG or the levels of the antimicrobial mediators MPO and inducible nitric oxide synthase (iNOS) (mRNA).

Also, our data demonstrate that the bacterial load levels were not affected by AL co-induction, and that the serum CRP levels were slightly decreased in AL+PD group when compared with PD.

Discussion

Clinical studies demonstrate the inverse association between periodontal disease and AL (Arbes et al. 2006, Friedrich et al. 2008), pathologies characterized by opposing immunological pathways (Finkelman et al. 2010, Garlet 2010). However, these cross-sectional studies do not allow a direct cause-effect association or suggest the possible mechanisms involved in such association. In an experimental setting design to solve some of these questions, the results presented here demonstrate that allergic mice develop a less severe PD after the oral infection with *A. actinomycetemcomitans*, associated with functional interferences in host inflammatory immune response.

Co-morbidity models demonstrate that the associated diseases are linked by functional interferences in the host response due the concurrent pathological processes (Kasser et al. 1997, Mercado et al. 2001, Rosenstein et al. 2004, Bartold et al. 2005, Golub et al. 2006, Nilsson & Kopp 2008, Pischon et al. 2008, de Pablo et al. 2009). In fact, in the PD/diabetes and PD/RA co-morbidity scenarios described previously increased ratios of pro/anti-inflammatory cytokines are associated with increased PD severity (Graves et al. 2007, Preshaw et al. 2007, Trombone et al. 2010). Interestingly, our data demonstrate an opposing scenario, characterized by a lower production of TNF-α, IL-1β, IFN-γ and IL-17A, and a trend towards higher IL-4 production in allergic mice response to AA infection.

TNF-α and IL-1β are classically regarded as destructive cytokines in

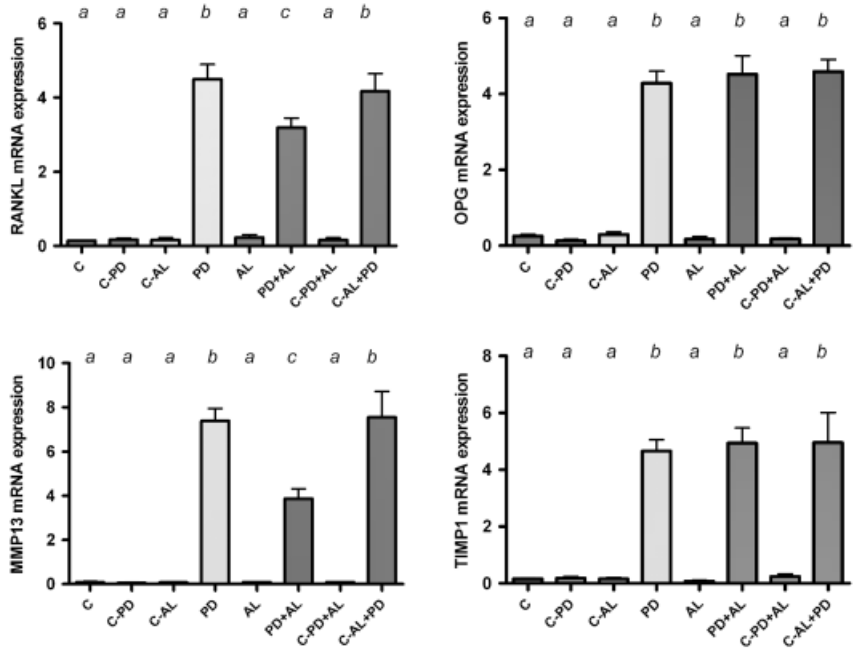

Fig. 3. MMP-13, TIMP-1, RANKL and OPG expression in periodontal tissues of C57Bl/6 mice after the induction of experimental periodontal disease and allergy**.** Balb/C mice infected orally with *Actinobacillus actinomycetemcomitans* [experimental periodontal disease (PD) induction protocol] and sensitized and challenged with OVA [experimental allergy (AL) protocol], as exemplified in the experimental flowchart; PD, AL as well control (C), sham-infected (C-PD group) and OVA-sensitized but non-challenged (C-AL group) groups 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 ± SD. One-way ANOVA followed by Bonferroni's test: groups with different letter designations are statistically different ($p<0.05$). MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor κ-light-chain-enhancer of activated B cells Ligand; OPG, Osteoprotegerin; TIMP, tissue inhibitor of metalloproteinase; AL, allergic inflammation; Ct, cycle threshold; OVA, ovalbumin; PCR, polymerase chain reaction.

PD pathogenesis (Graves 2008). TNF-α acts in cell migration process at multiple levels, inducing the production and upregulation inflammatory cytokines (including IL-1β) and chemokines, being therefore a central mediator of leucocyte influx into periodontal tissues (Garlet et al. 2007, Graves 2008, Trombone et al. 2009a). In addition, TNF-α is involved in the progression of PD as a potent inducer of MMPs and RANKL expression (Graves & Cochran 2003, Garlet et al. 2004, Graves 2008). Interestingly, while redundant at a first sight, TNF-α and IL-1β present interesting additive and complementary roles in inflammatory bone loss process (Wei et al. 2005, Zwerina et al. 2007). In fact, while its individual absence attenuates bone resorption the simultaneous inhibition leads to almost the complete remission of osteoclastic activity (Graves & Cochran 2003, Zwerina et al. 2004, Sartori et al. 2009). Accordingly, our results demonstrate that AL co-induction was associated with a parallel decrease of TNF-α, IL-1β, MMP-13 and RANKL levels in periodontal tissues, what could explain the reduction in the disease severity presented by allergic mice.

However, while pro-inflammatory cytokines have been systematically associated with PD progression, the role of T-helper-related cytokines remains controversial (Gaffen & Hajishengallis 2008, Garlet 2010). The Th1-type cytokine IFN-γ is present at high levels in periodontal lesions and associated with higher PD severity (Garlet et al. 2003, Honda et al. 2006, Dutzan et al. 2009). While described as an anti-osteoclastogenic factor *in vitro* (Gowen & Mundy 1986, Gao et al. 2007, Garlet 2010), the prominent IFN-γ pro-inflammatory activity *in vivo* overcomes the direct anti-osteoclastogenic effect (Gao et al. 2007). Accordingly, IFN-γ stimulates osteoclast formation and bone loss *in vivo* via RANKL+cells chemoattraction and T cell activation (Baker et al. 1999, Teng et al. 2005, Gao et al. 2007, Garlet et al. 2008, Repeke et al. 2010). Th17 cells are also present in chronic PD lesions, and are regarded as mediators of PD progression via IL-17-mediated RANKL production (Kotake et al. 1999, Takahashi et al. 2005, Vernal et al. 2005, Sato et al. 2006, Cardoso et al. 2009, Ohyama et al. 2009, Yago et al. 2009). However, experimental studies in rodents demonstrate the IL-17 deficient mice may present increased or decreased bone lesions in response to periodontal pathogens challenge (Yu et al. 2007, Oseko et al. 2009). Interestingly, both IFN-γ and IL-17 can exacerbate inflammatory periodontal disease by activating adjacent cells to produce IL-1β and TNF-α, generating therefore a positive loop for inflammatory reaction amplification and lesion exacerbation (Beklen et al. 2007, Gao et al. 2007, Garlet et al. 2008). Therefore, it is possible to suggest that IFN-γ and IL-17 can converge into a pro-inflammatory and osteoclastogenic pathway, a hypothesis that must be confirmed by future studies.

Despite of the individual role of the potential destructive cytokines, its detrimental role can be antagonized by the Th2-prototypical cytokine IL-4, which suppress the polarization of Th1 cells and Th17 cells and its signature cytokines production (Agnello et al. 2003, Jarnicki & Fallon 2003, Appay et al. 2008, Bluestone et al. 2009, Newcomb et al. 2009). In fact, a trend towards a higher IL-4 production in periodontal tissues of allergic mice parallel with the downregulation of IFN-γ and IL-17. In addition, IL-4 is also able to interfere in the MMPs/TIMPs and RANKL/OPG ratios (Ihn et al. 2002, Giannopoulou et al. 2003, Saidenberg-Kermanac'h et al. 2004), reinforcing its potential protective role. However, some studies suggest a destructive role for IL-4, which would be mediated by its capacity to stimulate B cells, regarded as a RANKL source in periodontal environment (Gemmell et al. 2002, Gemmell & Seymour 2004, Kawai et al. 2006, Han et al. 2009). However, while B cells seem to contribute to alveolar bone loss, they are not essential to this process (Yamaguchi et al. 2008), being the exact role of IL-4 in PD pathogenesis still unclear.

In the view of the significant changes in the cytokine milieu in periodontal tissues, we next investigated the possi-

ble impact of AL co-induction in the submandibular LNs, the major draining LNs of oral cavity. Our results demonstrate that the transcription factor GATA3 (Th2-related) expression levels were significantly increased while T-bet (Th1-related) and RORγ (Th17-related) were decreased in allergic mice. Similarly, the expression pattern of chemokine receptors characteristically expressed by distinct T-helper subsets was also significantly modulated in allergic mice. Interestingly, the co-induction of RA, which increases the severity of ePD, modulated the pattern of transcription factors expression in submandibular LN throughout ePD in an opposed way than described here (Trombone et al. 2010). Accordingly, previous studies demonstrate that diabetes, RA and inflammatory intestinal disease co-morbidity result in the increase the prevalence and/or severity of PD (de Pablo et al. 2009, Smolik et al. 2009, Oz et al. 2010). Interestingly, the occurrence of co-morbidity is associated with convergent similar immunopathogenetic pathways, while conditions presenting opposing patterns of immune response may present an antagonistic effect (Somers et al. 2006, Lettre & Rioux 2008, Osada et al. 2009, Zhernakova et al. 2009). Indeed, diabetes, RA and inflammatory intestinal disease are described as conditions where the production of pro-inflammatory, Th1- and Th17-type cytokines is prominent, while airway allergy is usually characterized by a Th2 biased response (Kasser et al. 1997, Mercado et al. 2001, Nilsson & Kopp 2008, Pischon et al. 2008). However, direct comparisons with PD must be careful in the view of the complex and still unclear role of T-helper subsets in PD pathogenesis discussed previously (Gaffen & Hajishengallis 2008, Garlet 2010). It is also important to consider that Th17 cells were also recently implicated in AL pathogenesis (Oda et al. 2005, Finkelman et al. 2010). However, several reports point out for the role of IL-17 in the protection against allergic disorders (Nakae et al. 2002, Murdoch & Lloyd 2010). It has been shown that IL-17 is mainly expressed in the airways and lung lymphoid associated tissue in allergic mice (Oda et al. 2005, He et al. 2007, Suzuki et al. 2007, Lajoie et al. 2010), but there are no studies comparing the development of systemic and local Th17 response during the development of allergic responses.

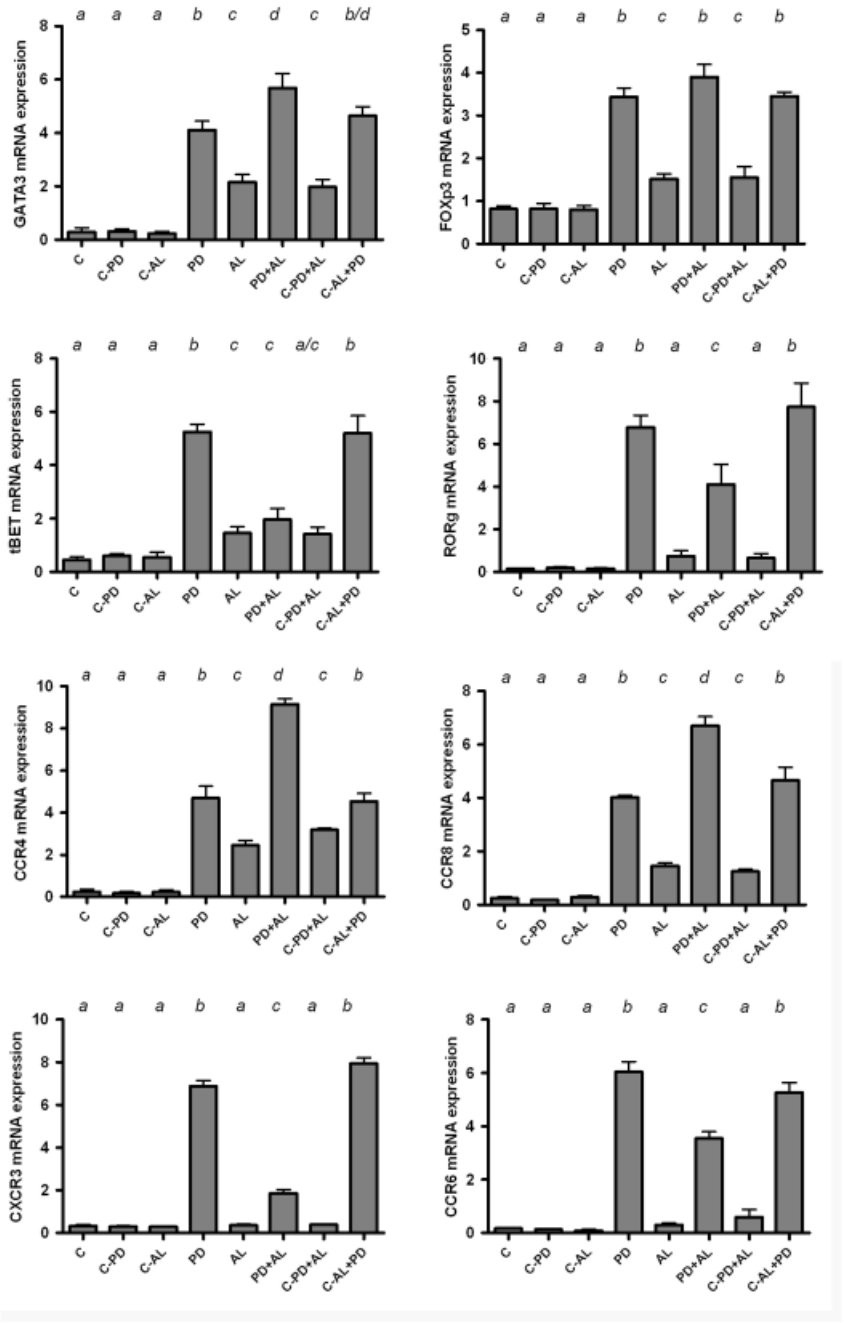

Fig. 4. Expression of T-helper subsets-related transcription factors and chemokine receptors in the submandibular lymph nodes of C57Bl/6 mice after the induction of experimental periodontal disease and allergy. Balb/C mice infected orally with *Actinobacillus actinomycetemcomitans* [experimental periodontal disease(PD) induction protocol] and sensitized and challenged with OVA [experimental allergy (AL) protocol], as exemplified in the experimental flowchart; PD, AL as well control (C), sham-infected (C-PD group) and OVA-sensitized but non-challenged (C-AL group) groups were evaluated for the levels of GATA3, FOXp3, T-bet and RORγ, and for the T-helper-related chemokine receptors CCR4, CCR8, CXCR3 and CCR6, 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 ± SD. One-way ANOVA followed by Bonferroni's test: groups with different letter designations are statistically different ($p < 0.05$).AL, allergic inflammation; PCR, polymerase chain reaction; OVA, ovalbumin; Ct, cycle threshold; RORγ; related orphan receptor γ.

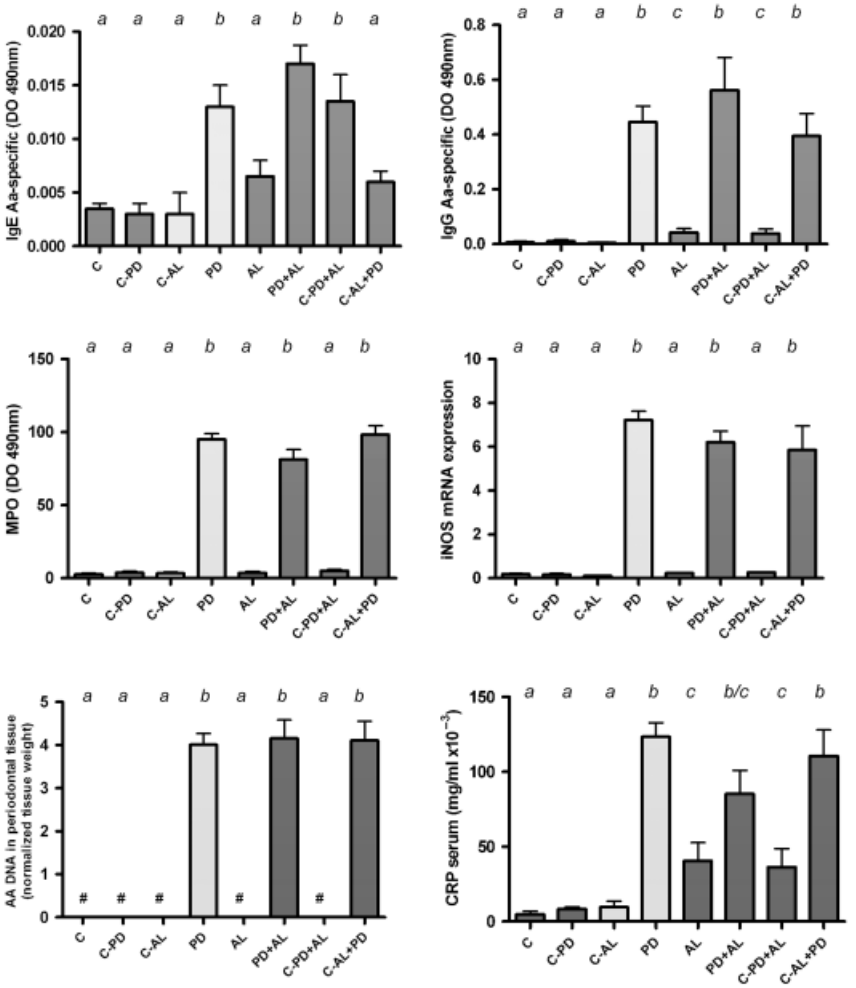

Fig. 5. Antimicrobial mediators production and *Actinobacillus actinomycetemcomitans* infection parameters after the induction of experimental periodontal disease and allergy. Balb/C mice infected orally with *A. actinomycetemcomitans* [experimental periodontal disease (PD) induction protocol] and sensitized and challenged with OVA [experimental allergy (AL) protocol], as exemplified in the experimental flowchart; PD, AL as well control (C), sham-infected (C-PD group) and OVA-sensitized but non-challenged (C-AL group) groups were evaluated for AA-specific IgE and IgG levels; 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; *A. actinomycetemcomitans* load in periodontal tissues, quantified by real-time PCR using the SybrGreen system and normalized by tissue weight (# non-detected); serum levels of C reactive protein, presented as mg/ml $\times$ 10^{-3}. One-way ANOVA followed by Bonferroni's test: groups with different letter designations are statistically different ($p < 0.05$). AL, allergic inflammation; MPO, myeloperoxydase; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction; OVA, ovalbumin; Ct, cycle threshold; AA, (*Actinobacillus*) *actinomycetemcomitans*.

Additionally, in our experimental model, we observe local production of IL-17A, but not systemic secretion of IL-17 (data not shown), suggesting that this cytokine activities in airway AL are restricted to the response site and consequently would not interfere in the host response at distinct sites such as the periodontal tissues. Nevertheless, considering the complex cytokine network operating in PD pathogenesis and the controversies regarding the role of Th1, Th2 and Th17 type cytokines described previously, further studies are required to precisely determine the mechanistic basis by which experimental airway AL attenuates PD development.

In the view of the potential impact of the cytokine production modulation described previously in the control of periodontal infection (Garlet et al. 2007, 2008), we next investigated the infection parameters in the presence or absence of allergic process. However, the AA load in oral cavity [as well the total bacterial load (16S DNA), data not shown] was not modulated by the AL co-induction, as well the levels of the antimicrobial mediators MPO and iNOS and the levels of AA-specific antibody. While previous studies demonstrated that the absence of TNF-α and IFN-γ (found to be decreased in allergic mice) resulted in impaired control of experimental periodontal infection, variations in host responsiveness of higher magnitude than those described here do not impair the periodontal infection control (Garlet et al. 2007, 2008, Trombone et al. 2009b).

It is also important to mention that the parameters of airway AL severity were not altered by periodontal infection. One hypothesis to explain this finding is that ePD presents a lower or narrow systemic effect than AL, possibly insufficient to module allergic process outcome. Accordingly, a previous study demonstrates that the influence of ePD is limited to submandibular LNs, confirming its narrow systemic effect (Trombone et al. 2010). An additional hypothesis refers to the kinetics of experimental diseases induction. While the subcutaneous *P. gingivalis* infection before allergen sensitization reduced airway inflammatory cell and cytokine content, the after sensitization infection (similar to our study design) not affect airway inflammatory endpoints (Card et al. 2010). Consequently, the first condition established seems to determine a hierarchical dominance in the polarization of immune response that can affect the subsequent host response. This hypothesis is agreement with the hygiene theory originally formulated in 1989, which suggested that infection in early infancy presented protective effects against the development of allergies (Strachan 1989, Card et al. 2010, Okada et al. 2010). Therefore, a potential protective effect of PD against AL may depend on the relative chronology of infection and allergic process onset.

The results presented herein demonstrate the control of ePD development by active airway allergic process, being the first experimental confirmation of the inverse clinical association between PD and AL described in clinical studies. The AL/PD interaction involves functional immunological interferences comprising alterations in the patterns of T cell transcription factors expression

in LN environment and modulation of local host response in periodontium. However, the AL outcome was not modulated by the latter induction of PD, suggesting that the kinetics of disease onset may account in the outcome of diseases interaction. Further studies using different models of both PD and AL and distinct kinetics of disease induction, as well longitudinal investigations in humans, certainly will contribute to unravel the mechanistic basis of the interaction network between PD and AL.

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Clinical Relevance

Scientific rationale for the study: Periodontal disease and allergy (AL) present opposing inflammatory immunological features and have been found to clinically present an inverse correlation. However, the putative mechanisms underlying such opposite association are unknown.

Principle findings: An active interference in the inflammatory immune response by airway AL significantly decreased ePD severity, being associated with alterations in the local production of tissue destructive cytokines and changes in the patterns of T cell subsets transcription factors expression in submandibular LNs.

Clinical implication: The results presented here suggest the mechanistic basis for the previously described inverse clinical association between PD and AL.