Experimental Arthritis Triggers Periodontal Disease in Mice: Involvement of TNF-α and the Oral Microbiota


J Immunol 2011; 187:3821-3830; Prepublished online 2 September 2011;
doi: 10.4049/jimmunol.1101195
http://www.jimmunol.org/content/187/7/3821

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Experimental Arthritis Triggers Periodontal Disease in Mice: Involvement of TNF-α and the Oral Microbiota


Rheumatoid arthritis (RA) and periodontal disease (PD) are prevalent chronic inflammatory disorders that affect bone structures. Individuals with RA are more likely to experience PD; however, how disease in joints could induce PD remains unknown. This study aimed to experimentally mimic clinical parameters of RA-induced PD and to provide mechanistic findings to explain this association. Chronic Ag-induced arthritis (AIA) was triggered by injection of methylated BSA in the knee joint of immunized mice. Anti–TNF-α was used to assess the role of this cytokine. Intra-articular challenge induced infiltration of cells, synovial hyperplasia, bone resorption, proteoglycan loss, and increased expression of cytokines exclusively in challenged joints. Simultaneously, AIA resulted in severe alveolar bone loss, migration of osteoclasts, and release of proinflammatory cytokines in maxillae. Anti–TNF-α therapy prevented the development of both AIA and PD. AIA did not modify bacterial counts in the oral cavity. PD, but not AIA, induced by injection of Ag in immunized mice was decreased by local treatment with antiseptic, which decreased the oral microbiota. AIA was associated with an increase in serum C-reactive protein levels and the expression of the transcription factors RORγ and Foxp3 in cervical lymph nodes. There were higher titers of anti-collagen I IgG, and splenocytes were more responsive to collagen I in AIA mice. In conclusion, AIA-induced PD was dependent on TNF-α and the oral microbiota. Moreover, PD was associated with changes in expression of lymphocyte transcription factors, presence of anti-collagen Abs, and increased reactivity to autoantigens. The Journal of Immunology, 2011, 187: 3821–3830.

The Journal of Immunology

T
he association between autoimmune rheumatic diseases and chronic infectious periodontal disease (PD), the leading cause of tooth loss in humans, has been studied since the 1960s (1); however, it has received increasing attention lately (2–5). Data from several clinical studies suggested a potential relationship between these disorders and indicated that individuals with rheumatoid arthritis (RA) are more likely to experience periodontal problems compared with healthy counterparts (2, 3, 6–13). The major unanswered question is how autoimmune diseases that tend to affect one or few parts of the body (e.g., joints) could interfere with PD.

The clue to understanding this association seems to rely on the pathogenic features shared by RA and PD. The etiologies of RA and PD are distinctly different: one is autoimmune, and the other is infectious. However, they share several common features, including infiltration of inflammatory cells; release of key cytokines, such as TNF-α, in affected tissues; and bone destruction. Thus, in line with these similarities, a “two-hit” model was suggested, in which the commensal oral biofilm (first “hit”) may interact with bone-destructive diseases in another location in the body (second “hit”) to induce PD (14). Other investigators hypothesized that a causal pathway (RA triggers PD and vice versa) and a noncausal pathway (involving genetic, environmental, and behavioral factors) exist (3). Both models are plausible but lack convergent data to support them. The majority of existing studies are low-prevalence case-control trials that vary with respect to design, setting, and methods to ascertain the association (6–10, 15). Mechanistic studies are warranted, and the use of experimental models could be suitable in this way.

In rats, induction of arthritis by injection of adjuvant was associated with spontaneous loss of alveolar bone and increased levels of IL-1β, TNF-α, and metalloproteinases in gingival tissues (16). Trombone et al. (4) used a pristane-induced model of RA in mice to show that a hyperinflammatory genotype was essential to aggravate bacteria-induced PD. Park et al. (5) demonstrated that alveolar bone cells from collagen-induced arthritis mice had increased osteoclastic and decreased bone-forming activity, and these findings were associated with oral bone loss. In this study, we developed a model of chronic Ag-induced arthritis (AIA) in mice to evaluate the effects of arthritis induction in the spontaneous development of PD and investigated the potential contribution of the oral microbiota and TNF-α to PD.

Materials and Methods

Animals

Experimental groups consisted of 6-wk-old male C57BL/6 mice maintained in the animal facilities of the Department of Microbiology, Instituto de...
Ciências Biológicas, Universidade Federal de Minas Gerais. Mice were housed under standard conditions and had free access to commercial chow and water. All animal experiments were performed according to a protocol approved by the local Institutional Committee for Animal Care and Use (protocol number: 165/2009).

**Chronic AIA**

Chronic AIA was induced, as described earlier (17). C57BL/6 mice were immunized on day −21 with a s.c. injection of 100 μg methylated BSA (mBSA; Sigma-Aldrich, St. Louis, MO) in 50 μl PBS emulsified in 50 μl CFA (Sigma-Aldrich) and supplemented with 4 mg/ml heat-killed Mycobacterium tuberculosis strain H37RA (Difco, Detroit, MI). Further immunization was performed on day −14 with mBSA in IFA (Sigma-Aldrich). In parallel with each Ag-specific immunization, 200 ng Bordeaux pertussis toxin (Calbiochem, La Jolla, CA) was injected i.p. The first challenge with Ag was performed on day 0 by injecting 100 μg mBSA in 20 μl PBS into the left knee joint. Thirty days later, mice were rechallenged by a second intra-articular injection with 100 μg mBSA in 20 μl PBS; knee joints, maxillae, spleen, serum, and inguinal and submandibular lymph nodes (LNs) were analyzed at various time points after this rechallenge (7, 14, 21, 28, 45, and 60 d). Negative controls included mice with systemic mBSA immunization (following the protocols described above) but challenged with PBS injection in knee joints, as well as naive mice. Because no difference was observed between these negative control groups in any of the evaluated parameters, they were grouped as control.

**Experimental PD**

Infectious PD was induced in a group of mice, as previously described (18), to compare this infection-induced PD with the AIA-induced PD. Experimental PD was achieved by oral delivery of 1 × 10^6 CFU culture of the periodontopathogen Aggregatibacter actinomycetemcomitans V4 (anaerobically grown in supplemented heart-mouse blood agar) broth (Sigma-Aldrich) through a micropipette at days 0, 2, and 4. Mice were killed at different time points (7, 14, 30, 45, and 60 d postinfection) for morphometric evaluation of maxillae.

**Anti–TNF-α therapy**

To evaluate the role of TNF-α in AIA-induced PD, mice were subjected to AIA and treated with infliximab (10 mg/kg, i.p. (Remicade; Schering-Plough, Kenilworth, NJ), a chimeric monoclonal anti–TNF-α Ab, 1 d after AIA rechallenge (19, 20). Knee joints, maxillae, and serum of mice were evaluated 14 d later.

**Oral antimicrobial therapy**

To investigate the effects of oral microbital on AIA-induced PD, an additional group received topical delivery of 50 μl chlorhexidine (1-chlorhexidine gluconate 0.12%), an antisepctic agent, plus 2% CMC (4). Chlorhexidine was applied in the mouth of mice every 2 d after AIA rechallenge until day 14, when animals were killed and the maxillae were collected for evaluation of alveolar bone loss, myeloperoxidase (MPO), and quantification of bacterial load by real-time PCR. The control group received 50 μl aqueous CMC with a similar protocol.

**Knee joint evaluation**

At the indicated time points, five mice/group were killed, and the knee cavity was washed with PBS (2 × 5 μl). The total number of leukocytes was counted in a Neubauer chamber. Differential counting was obtained from cytospin preparations (Shandon III; ThermoShandon, Franklin, Genova, Italy) from May–Grunwald–Giemsa. After PBS wash, the periarticular tissue was removed from the joints and used for immunoenzymatic assays.

Knee joints of five mice per group were also collected for histological evaluation. Samples were fixed in 10% buffered formalin (pH 7.4), decalcified for 30 d in 14% EDTA, embedded in paraffin, sectioned, and stained with H&E or toluidine blue (TB). Two sections/knee joint were microscopically examined by a single pathologist (T.A.S.) and scored in a blind manner for different parameters, as follows: severity of synovial hyperplasia, intensity and extension of inflammatory infiltrate, and bone erosion. The grades were summed to obtain an arthritis index (ranging from 0 to 8) (21). TB-stained slides were used to estimate joint proteoglycan loss, as described previously (22). Images of the joint surface of each sample were digitalized and evaluated using Image J software (National Institutes of Health, Bethesda, MD). Cartilage proteoglycan content is reported as the percentage of the TB-stained area in relation to the total evaluated cartilage surface.

**Quantification of neutrophil and macrophage enzymes**

Quantification of MPO, a neutrophil enzyme marker, and N-acetylglucosaminidase (NAG), a macrophage enzyme marker, was performed as described earlier (23). MPO and NAG activities in homogenized knee joint and maxillary tissues (upper molars, alveolar bone, and buccal and palatal gingival tissues) were evaluated by enzymatic reaction, measured by absorbance at 450 nm. The MPO and NAG contents were expressed as relative units calculated from standard curves based on MPO and NAG activities from 5% casein peritoneal-neutrophil and 3% thioglycollate peritoneal-macrophage enzymes, respectively, assayed in parallel.

**Protein extraction and ELISA**

The concentrations of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, tumor growth factor-β, RANKL, and osteoprotegerin (OPG) were measured in knee joint, maxillary tissues, and serum using commercially available kits, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The results were expressed as picograms of cytokines (SEM) normalized for 100 mg tissue.

**Real-time PCR**

The extraction of total RNA from inguinal and submandibular LNs was performed with TRIzol reagent (Invitrogen, Rockville, MD), and the cDNA synthesis was accomplished as described earlier (18). To allow quantification of the bacteria present in the oral biofilm, the extraction of bacterial DNA was performed from maxillary tissues, which were frozen in liquid nitrogen.
nitrogen, mechanically fragmented and homogenized in sterile Milli-Q water, and subsequently submitted to DNA extraction with DNA Purification System (Promega Biosciences, San Luis Obispo, CA). Real-time PCR quantitative mRNA or DNA analyses were performed in a Mini Opticon system (Bio-Rad, Hercules, CA), using SYBR Green PCR Master Mix (Invitrogen), 100 nM specific primers, and 2.5 ng cDNA or 5 ng DNA in each reaction. Primer sequences and reaction properties are shown in Table I. For mRNA analysis, the relative level of gene expression was calculated with reference to β-actin using the cycle threshold method. Bacterial DNA levels were determined using the cycle threshold method and normalized to the mean value of the control group, which was set as 1.

Detection of serum anti-collagen I Abs

The estimation of anti-collagen I total IgG in serum of mice was determined as described earlier, with some modifications (24). Ninety-six-well microplates were incubated overnight at 4˚C with 100 μl/well of 20 μg/ml solution of murine collagen I (kind gift of Dr. G.T. Kitten, Universidade Federal de Minas Gerais) diluted in PBS. The plates were washed with PBS/0.05% Tween, blocked with 5% dry milk, incubated for 1 h at room temperature, and washed. Serial serum dilutions (1:2–1:100) were incubated for 1 h at 37˚C, washed, and incubated for 1 h at 37˚C with 100 μl biotinylated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL). After washing, 100 μl/well streptavidin-HRP was added and incubated at room temperature for 20 min. The wells were washed, and 100 μl/well substrate buffer (o-phenylenediamine dihydrochloride; Sigma-Aldrich) was added and incubated for 20 min at room temperature. The enzymatic reaction was stopped by 1 M H2SO4, and the absorbance was measured at 492 nm. Results are expressed as OD.

Spleen cell cultures

The protocol used for stimulation of cell cultures with collagen was adapted from Berg et al. (25). Whole-spleen cells from individual mice were harvested 14 d after AIA rechallenge and cultured in triplicate in 96-well plates at 10^5 cells/ml in the presence of collagen 1.5 μg/well, Con A 2 μg/well, or culture medium (RPMI 1640, Flow Laboratories, Irvine, U.K.). Culture supernatants were collected at 48 h, and cytokines levels were determined by ELISA, as described above.

C-reactive protein

Quantification of C-reactive protein levels was determined in serum samples using a commercially available agglutination kit, according to the manufacturer’s instructions (Labtest Diagnóstica, São Paulo, Brazil).

Statistical analysis

Data are presented as mean ± SEM, and the statistical significance among control, AIA, and treated groups was analyzed by ANOVA, followed by the Newman–Keuls post hoc analysis. Tests were performed with GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA). Results with p < 0.05 were considered statistically significant.

Results

Chronic AIA

Intra-articular challenge of immunized mice with mBSA induced significant infiltration of mononuclear cells in the synovium and peri-articular tissues, synovial hyperplasia (Fig. 1 B), and lacunae of bone...
resorption (data not shown) from 7 to 28 d after challenge. This inflammatory process was also marked by loss of proteoglycan in joint cartilage (Fig. 1F) and reduction of the nociceptive threshold (data not shown) compared with control mice (Fig. 1E). From days 28 to 60 after challenge, joint inflammation evolved to a more chronic phase, with fibrous tissue (Fig. 1C) and recovery of proteoglycan content (Fig. 1G). These parameters were confirmed by histological score, as demonstrated by the arthritis index (Fig. 1H) and quantification of proteoglycan content in joint cartilage (Fig. 1I). An increase in the number of total leukocytes (Fig. 1K) and mononuclear cells (Fig. 1L) was also observed in the synovial cavity during the course of AIA. In agreement with the quantification of cells in the joint cavity, levels of NAG (Fig. 1M), a marker of macrophage infiltration, and MPO (Fig. 1N), a marker of neutrophil influx, were increased in periarticular tissues in the first weeks after the second Ag challenge. Levels of IL-1β (Fig. 1O) and TNF-α (Fig. 1P) were quantified at day 14 after challenge and found to be elevated in periarticular tissues. In contrast, no signs of inflammation or tissue disturbance were observed in the contralateral unchallenged joint of mice subjected to AIA.

Blockade of TNF-α represents an important therapy for the treatment of inflammatory disorders, including RA. The role of TNF-α in chronic AIA was evaluated by using infliximab, an anti-TNF Ab used in the clinic but also shown to be effective in experimental animal models (19, 20). As seen in Fig. 1, treatment with infliximab had major effects on the parameters evaluated, including arthritis index, proteoglycan loss, infiltration of cells, and cytokine release, indicating that TNF-α has a pivotal role in this model.

AIA-induced PD

Induction of AIA resulted in severe alveolar bone loss just 14 d after articular challenge of immunized mice with Ag (Fig. 2B, 2I). Alveolar bone loss in animals subjected to AIA was similar to that induced by oral infection with the periodontopathogen A. actinomycetemcomitans (Fig. 2C, 2I). Alveolar bone loss began rapidly and seemed to correlate with progression of arthritis, reaching a plateau from days 21 to 60 after rechallenge (Fig. 2I). AIA-induced PD was characterized by increases in the number of osteoclasts (Fig. 2F, 2K) and local levels of MPO (Fig. 2L). Moreover, there was a significant increase in the expression of inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-17,
FIGURE 4. AIA-induced PD is dependent on oral microbiota. AIA mice were treated with vehicle or the antimicrobial agent chlorhexidine (0.12% in 2% CMC, oral application, every 2 d after joint rechallenge) and killed 14 d later. A, 16S bacterial load was quantified by real-time PCR in maxillae. Alveolar bone loss (B) and MPO levels (C) in maxillae were reduced. IFN-γ (Fig. 3 A–E), and RANKL (Fig. 3G) in maxillary tissues at day 14 after AIA induction. In contrast, levels of IL-10 (Fig. 3F) and OPG (Fig. 3H) were reduced.

Because treatment with anti–TNF-α greatly ameliorated AIA, we evaluated whether such treatment would affect development of PD after induction of AIA. Alveolar bone loss (Fig. 2D, 2J), osteoclast recruitment (Fig. 2H, 2K), local MPO levels (Fig. 2L), and production of proinflammatory cytokines (Fig. 3) were significantly ameliorated by blockade of TNF-α. Moreover, the treatment was associated with increased maxillary levels of IL-10 (Fig. 3F) but had no effect on levels of OPG (Fig. 3H).

AIA-induced PD is dependent on oral microbiota

Because PD is primarily triggered by infection, and there was no inoculation in AIA animals that developed PD spontaneously, we hypothesized whether AIA-induced PD would depend on the oral microbiota. To address this possibility, we orally applied the clinically prescribed antimicrobial agent chlorhexidine topically to mice subjected to AIA. Induction of AIA in mice had no effect on bacterial load in the oral cavity (Fig. 4A). Oral application of chlorhexidine greatly reduced the oral microbiota, as assessed by measuring 16S bacterial DNA (Fig. 4A). Significantly, this treatment was associated with prevention of alveolar bone loss (Fig. 4B) and a decrease in MPO levels in maxillae (Fig. 4C). In these animals, despite the improvement in periodontal conditions, knee joint inflammatory signs were similar to those observed in vehicle-treated mice: number of total leukocytes (AIA [14 d]+vehicle: 2985 ± 506 versus AIA[14 d]+chlorhexidine: 2494 ± 297; p < 0.05) and mononuclear cells (AIA[14 d]+vehicle: 2324 ± 515 versus AIA[14 d]+chlorhexidine: 2063 ± 251; p > 0.05) in synovial cavity and histological arthritis index (AIA[14 d]+vehicle: 3.87 ± 0.13 versus AIA[14 d]+chlorhexidine: 3.75 ± 0.25; p > 0.05). We also evaluated bacterial load at the periodontal sites in animals treated with anti–TNF-α therapy and found that it had no effect on the local number of bacteria (AIA[14 d]+vehicle: 1.4 ± 0.2 versus AIA[14 d]+infliximab: 1.5 ± 0.2; p > 0.05).

Evaluation of Th transcription factors in inguinal and submandibular LNs

In view of the results indicating that this model of AIA triggers signs of PD, in a way dependent on the injection of Ag in the knee joint, on TNF-α, and oral bacteria, we then investigated systemic alterations that could be involved in the association between PD and AIA. In inguinal LNs, which drain the joint region, expression of the Th transcription factors tBET, GATA3, RORγ, and Foxp3 mRNA (Table I) increased significantly after Ag challenge (Fig. 5A–D). There was an increase in the expression of all transcription factors that tended to peak at day 14, with the exception of GATA3, which peaked at day 21 after Ag induction. However, when evaluating submandibular LNs, which drain the oral region, a pattern distinct from that in the inguinal region was observed. AIA increased the expression of RORγ and induced a decrease, followed by an increase, in Foxp3 during the course of the disease (Fig. 5G, 5H), whereas tBET and GATA3 remained unaltered in these LNs (Fig. 5E, 5F).

Table I. Primer sequences and reaction properties

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense and Antisense Sequences (5’–3’)</th>
<th>At (˚C)</th>
<th>Mt (˚C)</th>
<th>Bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>tBET</td>
<td>CCC CTG TCC AGT CAG TAA CTT CTT CTC TOT TGT GCT GGC T</td>
<td>60</td>
<td>78</td>
<td>115</td>
</tr>
<tr>
<td>GATA3</td>
<td>AGG AGT CTC CAA GTG TGC GAA TGG TGA GAA TGC AGA CAC CAC CT</td>
<td>60</td>
<td>80</td>
<td>124</td>
</tr>
<tr>
<td>Foxp3</td>
<td>CAGTCACTGACAATGTCGGCCATT</td>
<td>62</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>RORγ</td>
<td>TGTGGACAAAGGAACGTGCAGTGCACG</td>
<td>53</td>
<td>49</td>
<td>59</td>
</tr>
<tr>
<td>β-actin</td>
<td>ACTGTTGAGACCTTCTCATCACA</td>
<td>56</td>
<td>75</td>
<td>495</td>
</tr>
<tr>
<td>16S</td>
<td>CGTGCGATGGCGATGGGTGTTA</td>
<td>60</td>
<td>72</td>
<td>69</td>
</tr>
</tbody>
</table>

At, annealing temperature; bp, bp of amplicon size; Mt, melting temperature.
with anti–TNF-α reversed the pattern of polarization of Th transcription factors 14 d after AIA challenge, both in the inguinal (TBET, GATA3, RORγ, and Foxp3) and submandibular LNs (RORγ and Foxp3).

**Systemic reactivity to collagen I**

We also investigated whether any factor soluble in serum could account for PD in animals subjected to AIA. No significant changes in levels of cytokines, including TNF-α, IL-1β, IL-6, IL-17, and IL-10, were observed in serum of mice subjected to AIA compared with controls at 14 d after joint challenge (data not shown). Nevertheless, there was a slight increase in levels of TNF-α (control: nondetectable versus AIA [14 d]: 21.7 ± 8.9 pg/100 mg tissue; p < 0.05) and IL-10 (control: 15.8 ± 3.2 versus AIA [14 d]: 71.8 ± 21.1 pg/100 mg tissue; p > 0.05) in spleen of AIA mice. Serum C-reactive protein levels were increased in AIA animals (control: 3.1 ± 0.4 versus AIA [14 d]: 6.0 ± 0.6 mg/l; p < 0.05), indicating systemic reactivity, but they decreased...
after anti–TNF-α treatment (2.8 ± 0.4 mg/l; \( p < 0.05 \) versus AIA). In addition to these mediators, AIA induced production of anti-collagen I Abs that could be detected in serum and were partially reduced by anti–TNF-α treatment (Fig. 6).

Collagen I is prevalent in periodontium; when it was used to stimulate whole-spleen cell cultures from control and AIA mice, a distinct pattern of response was seen. Supernatants collected from AIA cell cultures presented significantly higher levels of TNF-α and IL-17 than did controls, whereas TGF-β levels were not altered (Table II) after collagen I stimulation. Both cell cultures responded similarly without stimulation and with a nonspecific stimulus, such as Con A (Table II). These findings suggested that arthritis may induce systemic reactivity to specific Ags, and this may contribute to the triggering of oral damage in the presence of oral microbiota.

**Discussion**

The association between RA and PD has long been studied, but conflicting data of several clinical trials hinder a better understanding of this relationship. Indeed, there is a lack of experimental evidence to support the hypotheses raised upon the existing epidemiological findings (3, 13, 14). The major results of this study can be summarized as follows: an experimental model of chronic AIA was shown to be useful in mimicking several features of inflammatory RA and the PD triggered by joint lesion. In this model, AIA-induced PD was associated with low-grade systemic inflammation measured by C-reactive protein, but not with serum parameters, such as cell infiltration and cartilage destruction, the anti–TNF-α receptor p55 results in an impairment in protective immunity to A. actinomycetemcomitans infection in mice, leading to an increase in bacterial load (18), which could explain the gingival inflammation in those patients. Nevertheless, in the current study, the bacterial load of infliximab-treated mice was not altered, and patients taking infliximab were already shown to present a dental plaque index similar to their healthy counterparts (28). The latter

In the current study, AIA spontaneously induced inflammatory PD, without any manipulation of the oral environment (i.e., there was no need for the injection of periodontopathogens). These findings strongly support current hypotheses to explain RA and PD association (3, 14). AIA triggers the release of anti-collagen I IgG. Serum samples of control, vehicle-treated AIA, and infliximab-treated AIA mice were collected 14 d after joint rechallenge and assayed for total anti-collagen I IgG. Ab titers were enhanced by AIA and partially reversed by infliximab. \( *p < 0.05 \), versus control (group C); \( \#p < 0.05 \), versus vehicle-treated AIA (14 d) group.

![FIGURE 6. AIA triggers the release of anti-collagen I IgG. Serum samples of control, vehicle-treated AIA, and infliximab-treated AIA mice were collected 14 d after joint rechallenge and assayed for total anti-collagen I IgG. Ab titers were enhanced by AIA and partially reversed by infliximab. \( *p < 0.05 \), versus control (group C); \( \#p < 0.05 \), versus vehicle-treated AIA (14 d) group.](Image 89x625 to 241x732)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Spleen Cell Culture Sample</th>
<th>Collagen I (15 μg/well) (Mean ± SEM; pg/ml)</th>
<th>No Stimulus (RPMI 1640) (Mean ± SEM; pg/ml)</th>
<th>Con A (2 μg/well) (Mean ± SEM; pg/ml)</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Control</td>
<td>1.3 ± 1.2</td>
<td>15.4 ± 5.4</td>
<td>210.8 ± 76.9</td>
</tr>
<tr>
<td></td>
<td>AIA</td>
<td>108.7 ± 17.6*</td>
<td>26.7 ± 12.9</td>
<td>368.6 ± 120.2</td>
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<td></td>
<td></td>
<td>0.0003</td>
<td>0.589</td>
<td>0.616</td>
</tr>
<tr>
<td>IL-17</td>
<td>Control</td>
<td>55.5 ± 24.7*</td>
<td>107.6 ± 79.6*</td>
<td>342.8 ± 71.5</td>
</tr>
<tr>
<td></td>
<td>AIA</td>
<td>285.4 ± 55.9*</td>
<td>43.3 ± 38.7</td>
<td>336.1 ± 182.7</td>
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<td></td>
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<td>0.005</td>
<td>0.488</td>
<td>0.973</td>
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<tr>
<td>TGF-β</td>
<td>Control</td>
<td>296.1 ± 6.3*</td>
<td>295.6 ± 8.5</td>
<td>293.7 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>AIA</td>
<td>314.4 ± 15.7</td>
<td>283.3 ± 6.5</td>
<td>288.2 ± 17.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.311</td>
<td>0.280</td>
<td>0.796</td>
</tr>
</tbody>
</table>

\( *p < 0.05 \), control versus AIA; unpaired Student's t test

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contrasting results may be accounted for by potential clinical variables, such as differences in the periodontal microbial environment and the responsiveness of the patient to the medication. TNF-α blockade has been a major breakthrough in the therapy of RA during the past years, but this should not detract from the fact that in more than half of the patients in clinical trials the complete remission of RA is rare (30). Therefore, it seems that only an efficacious treatment of RA may prevent the development of other associated diseases, such as PD.

The control of oral microbiota to avoid the development of AIA-induced PD also became evident in the current study. Although the quantitative oral bacterial load was not affected by AIA, its control by the antimicrobial agent chlorhexidine, widely prescribed in dental offices, abrogated alveolar bone loss without altering signs of AIA. Accordingly, several studies showed that RA patients have oral hygiene status similar to healthy individuals, although the periodontal condition is worse (2, 9, 28). The present results showed the clinical importance of treating both RA and PD.

In addition to TNF-α and oral microbiota, AIA-induced PD was associated with the presence of serum anti-collagen I Abs. Collagen I is one of the major components of periodontal tissues (31) and, it was shown, mainly for RA, that Abs binding to self-Ags can initiate an inflammatory reaction in tissues, possibly through the release of tissue-degrading enzymes (32) and cytokines (33) by the infiltrating cells (34). In this study, the production of proinflammatory cytokines, such as TNF-α and IL-17, was enhanced by cells of AIA mice when stimulated by collagen I. Thus, it seems reasonable to hypothesize that the increased concentrations of proinflammatory cytokines and osteoclast activators in a quantitatively nonaltered bacterial environment, such as the maxillae of AIA mice, were triggered by anti-collagen I Abs. Abs can also directly cause the destruction of their target tissue through impairment of tissue formation (35), inhibition of collagen fibrillogenesis (36), and disruption of collagen fibrils in the extracellular matrix (37). Once more, there is evidence of increased matrix metalloproteinases in maxillae of AIA mice (4, 16); however, it remains to be determined whether it is triggered by autoantibodies.

Along with this inflammatory milieu in maxillae, submandibular LNs of AIA mice seemed to present an altered pattern of polarization of Th cells. The expression of RORγ transcription factor mRNA was increased in AIA submandibular LNs, distinctly from tBET and GATA3. RORγ is selectively expressed in in vitro-differentiated Th17 cells and in IL-17+T cells present in the lamina propria of naive mice (38). These findings are corroborated by unpublished observations of our research group, indicating that there are greater numbers of CD4+CD25+IL17+ cells in the submandibular LNs and spleen of AIA mice compared with controls. In view of the present results, we cannot ascertain whether this probable polarization was the cause or the consequence of oral inflammation, but it seems to be important in the context of the association between AIA and PD. The increase in RORγ expression occurred concomitantly with the decrease in Fossp5. Indeed, Th17 and regulatory T cell developmental programs of T cells are antagonistically interconnected, and regulatory T cells are involved in the control of PD by release of IL-10 (39). Accordingly, in the current study, maxillae of AIA mice presented decreased levels of IL-10 and higher levels of IL-17. In RA patients, the expression of TNF-α, IL-1β, and IL-17 was predictive of bone tissue destruction (40), and RANKL expression on the surface of Th17 cells induced osteoclastogenesis (41), directly promoting bone erosion (42). Furthermore, osteoblasts respond vigorously to IL-17 by upregulating several proinflammatory cytokines, chemokines, and proteases, and TNF-α, IL-1β, and IL-6 induced by IL-17 might even feedback on the generation and expansion of additional Th17 cells. This pattern of polarization was different from the observed by Trombone et al. (4) in an experimental model of pristane-induced RA. In that study, tBET, GATA3, and RORγ were upregulated, suggesting activation of the Th1, Th2, and Th17 pathways. The systemic challenge discussed in this article may account for this distinction. Nevertheless, despite this discrepancy, the Th17 component also seemed to be important in those experimental conditions (4) and, irrespective of such controversies, there are data suggesting that both Th1 and Th17 cells may be involved in the process of inflammatory bone loss (43). Another important finding was the prevention of this transcription factor polarization by the anti–TNF-α therapy. In fact, TNF-α was already shown to alter the maturation of monocytic and dendritic cells and, subsequently, the pattern of Th responses. Anti–TNF-α therapies resulted in impaired Th17 and Th1 responses in models of autoimmune disorders (44, 45). This effect might have contributed to the periodontal amelioration in AIA mice.

Altogether, the present findings clearly support the two-hit model (14). It is possible to suggest that, in the presence of a “suitable” environment, such as the mouth with its commensal microbiota (first “hit”), the systemic immunomodulated Th17 cells triggered by RA (i.e., autoantibodies, Th polarization, and release of acute-phase proteins) results in greater cell migration and alveolar bone loss, even in the absence of classic periodontopathogens or greater amounts of dental plaque. This hypothesis is further strengthened by the finding that the nonchallenged joints of AIA mice did not present any signs of inflammation.

In conclusion, this study provided new experimental evidence to support the two-hit model. AIA exaggerated the reactivity to Ag of the local oral microbiota and caused spontaneous periodontal bone loss in a TNF-α–dependent manner. Thus, PD triggered by arthritis may be the manifestation of systemic inflammatory bone disease.

Disclosures
The authors have no financial conflicts of interest.

References


37. Arthritis Res. Ther. 8: R22.


