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Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease

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Introduction: Periodontal disease is a chronic inflammation of the attachment structures of the teeth, triggered by potentially hazardous microorganisms and the consequent immune-inflammatory responses. In humans, the T helper type 17 (Th17) lineage, characterized by interleukin-17 (IL-17) production, develops under transforming growth factor-β (TGF-β), IL-1β, and IL-6 signaling, while its pool is maintained by IL-23. Although this subset of cells has been implicated in various autoimmune, inflammatory, and bone-destructive conditions, the exact role of T lymphocytes in chronic periodontitis is still controversial. Therefore, in this study we investigated the presence of Th17 cells in human periodontal disease.

Methods: Gingival and alveolar bone samples from healthy patients and patients with chronic periodontitis were collected and used for the subsequent assays. The messenger RNA expression for the cytokines IL-17, TGF-β, IL-1β, IL-6, and IL-23 in gingiva or IL-17 and receptor activator for nuclear factor-κB ligand in alveolar bone was evaluated by real-time polymerase chain reaction. The production of IL-17, TGF-β, IL-1β, IL-6, and IL-23 proteins was evaluated by immunohistochemistry and the presence of Th17 cells in the inflamed gingiva was confirmed by immunofluorescence confocal microscopy for CD4 and IL-17 colocalization.

Results: Our data demonstrated elevated levels of IL-17, TGF-β, IL-1β, IL-6, and IL-23 messenger RNA and protein in diseased tissues as well as the presence of Th17 cells in gingiva from patients with periodontitis. Moreover, IL-17 and the bone resorption factor RANKL were abundantly expressed in the alveolar bone of diseased patients, in contrast to low detection in controls.

Conclusion: These results provided strong evidence for the presence of Th17 cells in the sites of chronic inflammation in human periodontal disease.

Key words: immune response; interleukin-17; periodontal disease; receptor activator of nuclear factor-κB ligand; T helper type 17

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Periodontal diseases are chronic inflammatory diseases of the attachment structures of the teeth and are considered one of the most significant causes of tooth loss in adults. Besides being the most prevalent form of bone pathology in humans, periodontal diseases may be a risk factor for life-threatening conditions such as cardiovascular disease and diabetes (15, 16). The bacterial biofilm attached to the tooth surface in close association with periodontal tissues is one the most relevant etiological factors of this disease, although recent findings also point to the participation of herpesviruses like human cytomegalovirus and Epstein–Barr virus in the pathogenesis of periodontal disease (20, 25, 26). In general, patients host a wide diversity of potentially hazardous species of microorganism, which trigger local and systemic inflammatory and immune

responses (2, 9). Adaptive immune responses are vital for the efficient eradication of such infectious agents, although deregulated immune responses might also lead to autoimmune and chronic inflammatory diseases, as occurs in periodontal diseases. The T helper type 1 (Th1)/Th2 paradigm has provided the framework for the understanding of CD4 T-cell biology and the interplay between innate and adaptive immunity. However, recent studies have defined a novel effector CD4 T-cell lineage characterized as an interleukin-17 (IL-17) -producing subset, named Th17, which develops via cytokine signals distinct from, and antagonized by, the products of the Th1 and Th2 lineages (30). This new subpopulation of helper T cells is thought to provide a novel understanding of host defense, immune regulation, and pathogenesis of various diseases.

Interleukin-17, the proinflammatory cytokine produced by the activated Th17 cells (33), has been implicated in numerous autoimmune and inflammatory conditions including rheumatoid arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease (30). Besides IL-17, which is an important regulator of host defense through neutrophil trafficking (34), Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-6, and have probably evolved to enhance host clearance of a range of pathogens distinct from those targeted by Th1 and Th2 responses (30). Although transforming growth factor-β (TGF-β) has long been recognized for its immunoregulatory functions in dampening inflammation, TGF-β and IL-6 signaling together cooperate to drive Th17 commitment, while the cytokine IL-23 amplifies and stabilizes the Th17 phenotype in chronic inflammatory reactions (17). In mice, the differentiation of Th17 cells requires TGF-β and IL-6 whereas for human naive $CD4^+$ T cells, Th17 polarization is induced by IL-1β and enhanced by IL-6 (1). The development of Th17 effectors also shares with some regulatory T cells (Treg) a requirement for TGF-β, establishing an important link between Th17 and Treg development (30). Whereas IL-6 and TGF-β signaling is essential for Th17 development, TGF-β signaling in the absence of IL-6 induces Foxp3-specifying Treg development, and therefore IL-6 appears to divert the development of $Foxp3^+$ regulatory cells towards the Th17 lineage (30).

Although the participation of T cells in the pathogenesis of periodontitis has been widely investigated from different standpoints, the exact role of T cells in the destruction of the periodontium is still controversial. In periodontitis, Th2 cells seemed to be predominant (32), and their common precursor, Th0 cells, can also be found (14), although recent studies suggest that Th1 cells play a crucial role in the bone resorption that occurs in the periodontium (28). Recently, data from our group (7) and from others (21) demonstrated the presence of TGF-β and $CD4^+$ $CD25^+$ regulatory T cells in biopsies from patients with chronic periodontitis (CP), which links this CD4 T-cell subset to the pathogenesis of periodontal diseases. Additionally, some studies reported that IL-17 is produced in periodontal lesions (27) and this cytokine is also capable of inducing the receptor activator of nuclear factor-κB ligand (RANKL), the main stimulatory factor for the differentiation and activation of osteoclasts (19). Therefore, we hypothesized that Th17 cells might be present in chronic lesions and so might be involved in bone resorption in periodontal tissues of patients with advanced CP.

Material and methods

Patients and sample collection

Samples of diseased gingiva were obtained from periodontitis tissues resected during periodontal surgery or extraction of periodontally compromised teeth. Healthy gingiva was obtained from patients who underwent tooth extraction for orthodontic reasons or third molar extraction. Alveolar bone fragments were obtained from adult healthy donors (explants); they were discarded from oral surgical procedures (osteotomy), mainly during third molar extraction. Bone samples from patients with periodontitis were obtained after maxillary or mandibular alveolar ridge regularization during periodontal surgery or extraction of periodontally compromised teeth. Twenty patients with CP (11 male and nine female, mean age 42.5 ± 9.2 years, probing depth 6.22 ± 1.31 mm, attachment loss >3 mm) and 14 controls (six male and eight female, mean age 30.4 ± 14.7 years, probing depth 1.51 ± 0.45 mm), selected as previously described (13), who visited the School of Dentistry of Ribeirão Preto University were evaluated in this study. All periodontitis patients presented moderate to advanced periodontal disease with radiographic evidence of extensive bone loss. Patients with periodontitis received basic periodontal therapy (oral hygiene instruction, scaling, and root planing), and the sites that remained positive for bleeding on probing 4 weeks after the basic periodontal therapy were selected for surgical procedures. We considered that the reevaluation of the diseased sites with an interval of 30 days between the conservative and the surgical treatments was adequate to define a non-responsive site. During this period, we did not observe any improvement but instead there was a worsening in clinical conditions of the sampled sites, whereas the greatest part of the treated sites presented no bleeding on probing and a lower probing depth (11, 13). None of the sampled sites bled on probing in the control subjects. Written informed consent was obtained from all patients and controls, the protocol was reviewed and ethical approval was given by University of Ribeirão Preto review board.

RNA extraction and real-time polymerase chain reaction

Samples obtained were processed for RNA extraction using the RNA extraction Kit (Promega, Madison, WI) and complementary DNA was synthesized using 1 μg RNA in a reverse transcription reaction, as previously described (13). Real-time polymerase chain reaction (PCR) quantitative messenger RNA (mRNA) analyses were performed in an ABI-Prism7000 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK), and the calculations for determining the relative level of gene expression by reference to β-actin were performed by the cycle threshold method, as previously described (13). The standard PCR conditions were 95°C for 10 min, 40 cycles of 94°C for 1 min, 56°C as the temperature of annealing for 1 min, and 72°C for 2 min, followed by the standard denaturation curve. The sequences of the primers were designed based on nucleotide sequences in the GenBank database and were as follows: β-actin, sense TGACAA AACCTAACTTGCGC, antisense ATAA AGCCATGCCAATCTCA; IL-17A, sense CAATGACCTGGAAATACCCAA, antisense TGAAGGCATGTGAAATCGAGA; TGF-β, sense ATTGAGGGCTTTCGCCT TAG, antisense TGTGTTATCCCTGCTG TCACA; IL-1β, sense GGAACCCCAGA GCGAAATACA, antisense CCTGAAGA ATGCCTCCTCACA; IL-6, sense AAATT CGGTACATCCTCGAC, antisense CAG GAACTGGATCAGGACTT; IL-23p19, sense GCTTCAAAATCCTTCGCAGC,

antisense TCTGAGTGCCATCCTTGAG CT; RANKL, sense CAGAAGATGGCA CTCACTGCA, antisense CACCATCGCT TTCTCTGCTCT. Results were depicted as the mean mRNA expression from triplicate measurements normalized by internal control β-actin, analyzed using a Mann–Whitney test, performed with the GRAPHPAD PRISM 4.0 software (GraphPad Software Inc., San Diego, CA). Values of $P < 0.05$ were considered statistically significant. Gene expression of the Th17-related cytokines IL-17A, TGF-β, IL-1β, IL-6, and IL-23p19 as well as of RANKL was evaluated in periodontitis samples and compared to that in healthy controls.

Immunohistochemical analysis

For immunohistochemistry, gingival tissue samples were snap-frozen in liquid nitrogen, 5-μm cryostat frozen sections were applied to poly-L-lysine microscope slides (Sigma-Aldrich, Saint Louis, MO), and fixed with cold acetone. An avidin–biotin peroxidase technique was used to reveal IL-17A (antihuman IL-17A antibody; R&D Systems Minneapolis, MN), TGF-β (antihuman TGF-β antibody; Santa Cruz Biotechnology, Santa Cruz, CA), IL-1β (antihuman IL-1β antibody; Santa Cruz Biotechnology, Santa Cruz, CA), IL-6 (antihuman IL-6 antibody; R&D Systems) and IL-23 (antihuman IL-23p19 antibody; R&D Systems) expression in CP and healthy tissues, and the slides were counterstained with Mayer's hematoxylin, as described previously (6). Because of the small size of the fragments obtained, not all the biopsies collected for gene expression analysis could also be used for immunohistochemistry. For this reason, we used five different tissues for the immunohistochemistry assays. Therefore, the data shown are not always a result of serial sections from the same patient.

Immunofluorescence/confocal analysis

Slides for double immunofluorescence staining (antihuman CD4, BD Biosciences Pharmingen, San Jose, CA, and antihuman IL-17A, R&D Systems) were postfixed with acetone and blocked with 0.1% phosphate-buffered saline–Tween containing bovine serum albumin (4 mg/ml). After washing, the slides were incubated with the primary antibody, washed again, incubated with the secondary antibody streptavidin-alexafluor 488 or streptavidin-Cy3 (Molecular Probes, Eugene, OR) and 4′, 6′-diamidino-2-phenylindole (DAPI, Molecular Probes). After a further wash, the slides were mounted using Fluormount (EM Sciences, Hatfield, PA) and analyzed using a confocal microscope (TCS SP5; Leica Microsystems, Bannockburn, IL). ADOBE PHOTOSHOP (version 4.0) was used for image processing. Secondary antibodies were used alone as negative controls.

Results

Gingival tissues from patients with CP presented elevated levels of mRNA expression for the Th17 cytokine IL-17 (Fig. 1A, $P < 0.05$ vs. controls), which corroborated previous data that also reported the presence of this inflammatory cytokine in periodontal lesions (27). In addition, messages for the Th17-inducing cytokines TGF-β, IL-1β, and IL-6 were increased in CP compared with controls (Fig. 1B–D; $P < 0.05$), suggesting that even in the presence of TGF-β, a cytokine with regulatory/antiinflammatory properties, the concomitant high levels of IL-1β and IL-6 in gingiva from CP might provide the suitable milieu for the development of a Th17 subpopulation in this chronic inflammation site. Moreover, IL-23p19, the cytokine involved in the maintenance of the Th17 pool, was also highly expressed in patients with CP, when compared to the low detection in controls (Fig. 1E, $P < 0.05$). Indeed, we were not able to observe IL-17 protein production in the control samples, in contrast to abundant detection in patients with CP, as demonstrated by immunohistochemistry (Fig. 2A and F, respectively). Similarly, the production of TGF-β, IL-1β, IL-6, and

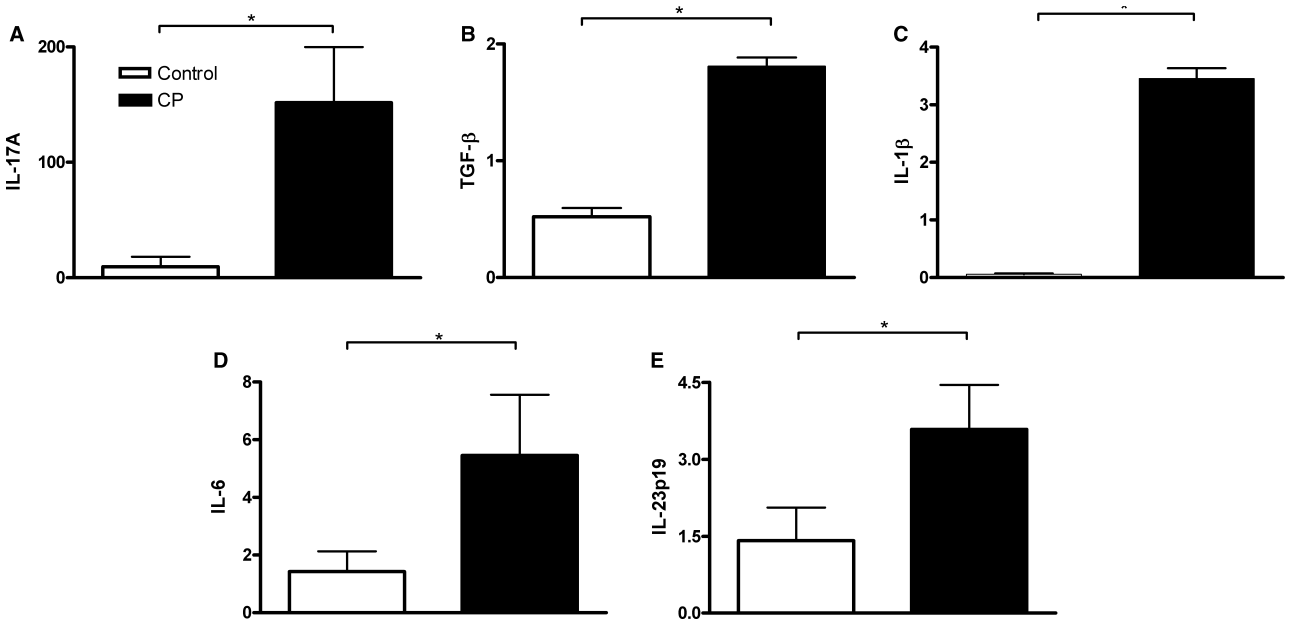

Fig. 1. Quantitative messenger RNA expression of (A) interleukin-17A (IL-17A), (B) transforming growth factor-β (TGF-β), (C) IL-1β, (D) IL-6, and (E) IL-23p19 in patients with chronic periodontitis (CP), and in a healthy control group. Total RNA was extracted, and the levels of cytokine messages were measured quantitatively using a real-time polymerase chain reaction SYBR-green system. The data are presented as the intensity of expression of the individual messenger RNAs, with normalization to β-actin, using the cycle threshold method. The results shown are from one experiment representative of three. Controls were significantly different (*$P < 0.05$, Mann–Whitney test) from patients with CP in all analyses.

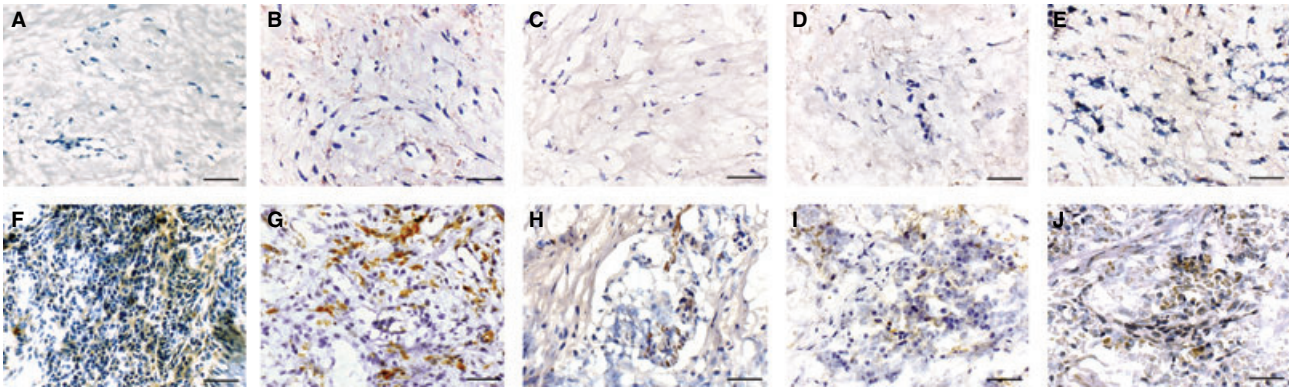

Fig. 2. Immunohistochemical detection of T helper type 17 (Th17) -related cytokines in patients with chronic periodontitis (CP). Tissue samples from controls (A–E) and patients with CP (F–J) were snap-frozen in liquid nitrogen and 5-μm cryostat sections were used for the detection of (A,F) interleukin-17A (IL-17A), (B,G) transforming growth factor-β (TGF-β), (C,H) IL-1β, (D,I) IL-6, and (E,J) IL-23 proteins with the utilization of specific antibodies and an avidin–biotin peroxidase technique. Slides were counterstained with Mayer's hematoxylin. Results are representative of five independent experiments. Scale bar = 50 μm.

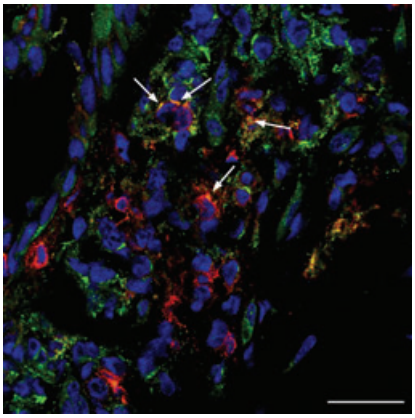

Fig. 3. Immunofluorescence/confocal localization of T helper type 17 cells in chronic periodontitis tissues. Frozen biopsy samples were fixed, incubated with anti-interleukin-17A (IL-17A) and anti-CD4 antibodies, followed by secondary antibodies–fluorochromes and then stained with 4′,6′-diamidino-2-phenylindole (DAPI - blue). IL-17-positive and CD4-positive cells appear stained green and red, respectively. Colocalization of CD4/IL-17 is shown in yellow (arrows). Tissues were analyzed by confocal microscopy and staining shows one experiment representative of three. Scale bar = 25 μm.

IL-23 protein was also reduced in healthy tissues (Fig. 2B–E) in comparison to the elevated production in periodontitis (Fig. 2G–I), which supports the idea that Th17 cells can be present in the diseased gingival environment. In fact, we detected $CD4^+$ leukocytes producing IL-17 in gingival biopsies from patients with CP, as visualized by confocal microscopy (Fig. 3), confirming the presence of Th17 cells in the scenario of periodontal disease.

Chronic periodontitis is characterized as a chronic inflammation of the attachment structures of the teeth mainly based on RANKL-mediated bone destruction.

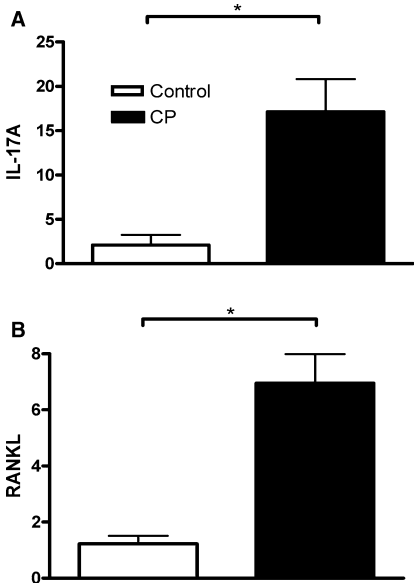

Fig. 4. Quantitative messenger RNA expression of (A) interleukin-17A (IL-17A) and (B) receptor activator of nuclear factor-κB ligand (RANKL) in alveolar bone of patients with chronic periodontitis (CP) and in a healthy control group. Total RNA was extracted and the levels of messages were measured quantitatively using a real-time polymerase chain reaction SYBR-green system. The data are presented as the intensity of expression of the individual messenger RNAs, with normalization to β-actin, using the cycle threshold method. The results shown are from one experiment representative of three. Controls were significantly different (*$P < 0.05$, Mann–Whitney test) from patients with CP in all analyses.

Moreover, because IL-17 induces the expression of RANKL (19) and this cytokine was highly expressed in gingiva from CP, we decided to investigate if these molecules were directly involved in the development of bone-resorption lesions. As demonstrated in Fig. 4, the expression of IL-17 was strongly augmented in diseased bone as well as the osteosclast differentiation factor RANKL, in comparison to controls. These results indicate that besides the presence of Th17 cells in the gingiva, the elevated expression of IL-17 and RANKL in alveolar bone may be in close association to the bone resorption observed in patients with CP, so contributing to disease progression.

Discussion

It is likely that different T-cell subsets predominate at different phases of periodontal disease. Some studies have reported Th1 cells associated with stable lesions and a Th2 response with disease progression whereas others have reported predominant Th1 or reduced Th2 responses in diseased tissues (14). Previous studies from our group demonstrated a time-dependent mixed pattern of polarized immune response in experimental periodontal disease (9, 12) as well as concomitant expression of interferon-γ, tumor necrosis factor-α (TNF-α), IL-10, and IL-4 in diseased human tissues (13). Therefore, although the first steps in deciphering the pathological mechanisms of periodontal disease have been achieved, conflicting and unresolved paradigms still remain obscure in the pathogenesis of periodontal diseases. Consequently, further studies are now required to elucidate the participation of the novel helper T-cell population – Th17 – in the regulation of the inflammatory response and bone resorption in CP. As a result, in the present study, we described the presence of Th17-related cytokines as well as of Th17 cells in diseased gingiva and of bone resorption

factors in samples of diseased alveolar bone in human periodontitis.

The development of this disease may not be simply a consequence of the Th1/Th2 paradigm as previously described, but these profiles of immune response may also be profoundly influenced by other factors like the proinflammatory actions of IL-17, which may exert a major role in the polarization of the immune response in periodontal disease. In this study, the proportion of IL-17-positive cells seemed to be increased in more inflamed tissues. However, there is an overall elevated expression of IL-17 on patients with CP, despite the intensity of cellular infiltration, when compared to controls. These findings are representative of several patients and agree with the results in which the elevated production of Th17-related cytokines is in accordance with the gene expression assays.

The amount of Th17 cells found in the diseased gingiva was not too high compared with that of all other inflammatory cells in the tissues. However, as Th17 is a key regulatory population involved in the pathogenesis of many diseases, it is possible that a small but functional population of Th17 leukocytes may be enough to induce the breakdown of tissue homeostasis, perpetuation of the inflammatory process, large bone resorpting lesions, and tooth loss. We also found IL-17 single-positive cells in the confocal analysis, suggesting that other cell types may be important sources of this cytokine, like natural killer T (23), $CD8^+$ (29) or $\gamma\delta^+$ cells (5). Moreover, staining can also be seen in the extracellular matrix and may represent the production and secretion of IL-17 by the local inflammatory infiltrate. Indeed, the production of IL-17 was already demonstrated in periodontal lesions (3, 22) and is thought to exacerbate inflammatory periodontal disease by inducing the production of metalloproteases (3) or by activating gingival fibroblasts to produce inflammatory mediators, generating therefore a positive loop for inflammatory reaction amplification (27).

T cells are involved in bone destruction via IL-17 production in rheumatoid arthritis (19), and because IL-17 shares properties with IL-1 and TNF-α, it may directly upregulate osteoclast-mediated bone resorption (18). IL-17 was associated with cells producing RANK and RANKL, besides being capable of inducing RANKL, the main stimulatory factor for the differentiation and activation of osteoclasts (19). In addition, whereas IL-17 is mainly produced by IL-1β-, IL-6-, and TGF-β-activated Th17 cells, RANKL is expressed by osteoblasts, T cells, stromal cells, peripheral blood monocytes, bone marrow cells, and spleen cells (4). In the present study, we could not determine the number of Th17 cells in bone. However, as many cytokines other than IL-17 are capable of inducing RANKL, such as IL-1β and TNF-α, we suggest that, in bone, IL-17 may act in synergy with these cytokines to amplify inflammation. Moreover, because this cytokine is an important pathogenic element in bone-destructive diseases, like in rheumatoid arthritis (24), we propose that it could also be a key element in the pathogenesis of bone destruction in CP. Accordingly, besides demonstrating $CD4^+$ cells producing IL-17 *in situ*, in inflamed gingiva, our results demonstrated, for the first time, elevated mRNA levels for this cytokine as well as RANKL directly on the bone resorption lesions in CP, although the cellular source for this cytokine remained unknown.

Given the growing association of IL-23 and/or IL-17 with host protection in a number of gram-negative bacterial infection models, it is likely that Th17 cells evolved to cope with a range of bacterial pathogens (8) as well as potentially contributing to the clearance of periodontopathogens in human CP. In a murine model of periodontitis, the influence of IL-17 on neutrophil regulation balanced its potential role in bone destruction, revealing the importance of IL-17-induced neutrophil mobilization in the control of the bacterial infection (34). In fact, inflammatory mediators seem to play a dual role in the disease, contributing to the control of periodontal infection while triggering tissue destructive pathways (10).

Interestingly, our results also pointed to a putative dual role of TGF-β in the diseased periodontal environment. TGF-β had been classically considered as an anti-inflammatory cytokine and had been linked to the development of $Foxp3^+$ regulatory T cells, but the newly identified links between TGF-β and Th17 development promise the solution of many conundrums that have precluded a complete understanding of autoimmune pathogenesis (30). Indeed, if this is the case for pathogenic Th17 cells in periodontal disease, it will be imperative to establish the interplay among TGF-β, Treg cells, and Th17 cells in attenuating or promoting inflammation, before attempting novel therapies for CP. In fact, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells can function as inducers of Th17 cells and can differentiate into Th17 cells in the absence of exogenous TGF-β (31). Therefore, in the present work we speculate that Th17 cells in periodontitis may develop under the influence of TGF-β-producing regulatory cells in sites where abundant IL-1β and IL-6 production is detected. Complementary studies in murine models are already being performed by our group to elucidate this and other questions regarding the participation and function of Th17 cells in CP, apart from indicating that they are present in this chronic inflammation.

Finally, our data demonstrated, for the first time, the presence of Th17 cells in the chronic inflammation in human periodontal disease, as well as IL-17 expression in the alveolar bone of patients with CP. Therefore, knowledge concerning the role of CD4 T cell subsets and the interplay among cytokines, inflammatory mediators, and periodontopathogenic microorganisms in the outcome of periodontal diseases may provide the basis for future interventions aimed at limiting the inflammatory process in periodontal tissues and novel therapies to circumvent disease progression.

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