Characterization of CD4\(^+\)CD25\(^+\) natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis

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Abstract: Periodontitis is an infectious disease, where putative periodontopathogens trigger chronic inflammatory and immune responses against periodontal structures, in which an unbalanced host response is also determinant to the disease outcome. It is reasonable to assume that patient susceptibility to periodontal tissue destruction could be determined by the balance between the response against periodontopathogens and regulatory mechanisms of these events mediated by suppressive T cells. In the present study, we identified and characterized natural regulatory T cells (Tregs) in the inflammatory infiltrate of human chronic periodontitis (CP) with emphasis on phenotypic analyses that were carried out to address the participation of Tregs in CP. Results showed that patients with CP presented increased frequency of T lymphocytes and CD4\(^+\)CD25\(^+\) T cells in the inflammatory infiltrate of gingival tissues. These cells exhibited the phenotypic markers of Tregs such as forkhead box p3 (Foxp3), CTLA-4, glucocorticoid-inducible TNFR (GITR), CD103, and CD45RO and seemed to be attracted to the inflammation site by the chemokines CCL17 and CCL22, as their expression and its receptor CCR4 were increased in CP patients. Moreover, besides the increased detection of Foxp3 mRNA, diseased tissues presented high expression of the regulatory cytokines IL-10 and TGF-\(\beta\). In addition, the inflammatory infiltrate in CP biopsies was composed of CD25\(^+\)Foxp3\(^+\) and CD25\(^+\)TGF-\(\beta\)\(^+\) cells, thus corroborating the hypothesis of the involvement of Tregs in the pathogenesis of CP. Finally, these results indicate that Tregs are found in the chronic lesions and must be involved in the modulation of local immune response in CP patients. J. Leukoc. Biol. 84: 000–000; 2008.

Key Words: periodontal disease • Tregs • gingival biopsies • immune regulation • inflammation

INTRODUCTION

Periodontal diseases (PD) are chronic inflammatory processes that affect the attachment structures of the teeth and constitute a significant cause of tooth loss in adults. Periodontitis is also one of the most prevalent forms of bone pathology in humans, besides being a modifying factor of the systemic health of patients. This is considered an infectious disease, where putative periodontopathogens trigger chronic inflammatory and immune responses against periodontal structures [1]. Moreover, an unbalanced host response to periodontopathogens is also described to be an important determinant in the disease outcome [2].

The function of T cells in driving periodontitis lesions is complex and has not been fully elucidated. Individual T cells infiltrating gingival lesions can express mRNA for Th1 and Th2 cytokines as well as regulatory cytokines molecules [3, 4]. Furthermore, autoreactive T cells with a Th1 cytokine profile recognizing self-components such as heat shock protein 60 [5] and collagen type I [6] accumulate in gingival lesions of periodontitis patients. These findings imply that T cells may be actively involved in the immunopathology of PD. In this context, it is reasonable to assume that patient susceptibility to periodontal tissue destruction could be determined at least in part by the balance between the response against periodontopathogens—a feasible autoimmunity—and regulatory mechanisms of these events mediated by regulatory T cells (Tregs) [7].

Natural Tregs are key regulators of immune responses, express CD4 and CD25 surface markers, and regulate the activation, proliferation, and effector function of activated conventional T cells in several immunological settings such as immunopathology, autoimmune diseases, transplantation, tumor immunity, and infectious disease [8–13]. Besides CD25, Tregs may also express several other phenotypic molecules, including glucocorticoid-inducible TNFR (GITR), CTLA-4, CD103, CD45RO [11, 13, 14], cell surface TGF-\(\beta\)1 [11, 15], and forkhead box p3 (Foxp3) [16]. The functional mechanism of Tregs action is not clear, but the ligation of CTLA-4 and GITR and involvement of anti-inflammatory cytokines such as IL-10 and TGF-\(\beta\) have been suggested [11, 13, 17]. In addition,
novel mechanisms of the suppressive activity of Tregs were recently described, such as the production of the inhibitory cytokine IL-35 [18] and the presence of granzyme B and perforin, which were demonstrated to be relevant for Treg-mediated suppression of tumor clearance in vivo [19].

Currently, it is known that Tregs can respond to a variety of self-antigens as well as those expressed by microbes. In experimental models, such as infection by *Leishmania*, HSV, and *Schistosoma mansoni*, Tregs seem to play a role in the maintenance of chronic infections, with persistence of pathogens, consequently enabling the disease reactivation [8]. Indeed, in a mouse model of chronic *Leishmania* infection, the transfer of purified Tregs derived from infected mice into other chronically infected animals was sufficient to trigger disease reactivation [8, 20]. On the contrary, Treg-mediated suppression may be beneficial for the host survival by limiting tissue damage in some infectious diseases [21].

Accordingly, in the present study, we identified the inflammatory infiltrate and characterized the presence of Tregs in human chronic periodontitis (CP) with emphasis on phenotypic analyses that were carried out to address the putative participation of Tregs in CP.

**MATERIALS AND METHODS**

**Study population and clinical examination**

Patients with CP and controls (donors of healthy gingival tissues) were submitted to anamnesis and to clinical, periodontal, and radiographic examination. Inclusion criteria comprised partially or fully dentate patients, systematically healthy with no evidence of known systemic modifiers of PD (type I and 2 diabetes mellitus, osteoporosis, and medications known to influence periodontal diseases). Exclusion criteria comprised patients who did not give informed consent, patients with a significant medical history indicating evidence of known systemic modifiers of PD as described above, pregnant or lactating females, and subjects who had taken systemic antibiotic, anti-inflammatory, hormonal, or other assisted drug therapy in the last 6 months prior to the study. Smokers were not specifically excluded. CP patients had moderate-to-advanced PD (probing depth >3 mm; attachment loss, >3 mm; radiographic evidence of extensive bone loss). All patients were scheduled for periodontal treatment at the Department of Periodontics, University of Ribeirão Preto (UNAERP) Dental School (Brazil), and after the diagnostic phase, they received basic periodontal therapy, which consisted of oral hygiene instruction, scaling, and root planning. Gingival biopsies were obtained at the time of periodontal surgery or the extraction of teeth with severe periodontal involvement. The control group was comprised of subjects presenting clinical, healthy gingival tissues (low scores of bleeding on probing under 10% of the sites; no scaling, and root planning). Gingival biopsies were taken during surgical procedures for wisdom-tooth removal. The clinical features of the groups are summarized in Table 1.

**TABLE 1.** Clinical Features of CP and Control Patients

<table>
<thead>
<tr>
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<th>CP</th>
<th>Control</th>
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<tr>
<td>Distribution</td>
<td>44 (24 m/20 f)</td>
<td>22 (11 m/11 f)</td>
</tr>
<tr>
<td>Smokers/nonsmokers</td>
<td>22/22</td>
<td>3/19</td>
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<tr>
<td>Age (mean±SD)</td>
<td>42.5 ± 9.2</td>
<td>30.4 ± 14.7</td>
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<tr>
<td>Probing depth (mm/sampled site)</td>
<td>6.22 ± 1.31</td>
<td>1.51 ± 0.45</td>
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*Patients with radiographic evidence of extensive bone loss. m, Male; f, female.

The experimental protocol used was approved by the Institutional Review Board of UNAERP. Informed consent was obtained from all subjects before performing the studies.

**Immunohistochemical analysis**

Gingival tissue samples were snap-frozen and stored in liquid nitrogen until analysis. Cryostat frozen sections (5 μm) were applied to poly-L-lysine microscope slides (Sigma-Aldrich, St. Louis, MO, USA), and fixed with cold acetone. An avidin-biotin peroxidase technique was used to reveal CD3, CD4, CD8, CCL17, and CCL22 expression in tissues, and the slides were counterstained with Mayer's hematoxylin.

**Antibodies and flow cytometry analysis**

For immunostaining, PE-, PC-, or FITC-conjugated antibodies against CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M52E), CD19 (HIB 19), CD25 (M-A251), CD11c (B-ly4), CD45RO (UCHL 1), CD152 (BN1.3.1), and the respective mouse and rat isotype controls were used (BD Biosciences Pharmingen, San Jose, CA, USA). PE-conjugated mouse mAb anti-human GITR (110416) was purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD103 (Ber-ACR8) and purified anti-Foxp3 were purchased from eBioscience (San Diego, CA, USA) and Abcam (Cambridge, MA, USA), respectively. The cell acquisition was performed on a FACSort flow cytometer using and CellQuest software (BD Biosciences Pharmingen). Anti-human TGF-β, CD3, CD4, CD8, CD25, CCL17, CCL22 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Foxp3 (Abcam) were used for immunohistochemistry/confocal assays.

**Isolation of leukocytes**

To characterize the inflammatory infiltrate present in the lesion site, the biopsies of gingival lesions from CP patients were collected and incubated 1 h at 37°C in RPMI-1640 medium, containing 50 μg/ml liberase CI enzyme (Roche Diagnostics GmbH, Mannheim, Germany). Next, the tissue was dissociated for 4 min in the presence of RPMI 1640 with 10% FCS and 0.05% DNase (Sigma-Aldrich) using a Medimachine (BD Biosciences Pharmingen), according to the manufacturer’s instructions. The tissue homogenates were filtered using a 30-μm cell-strainer (Falcon, BD Biosciences Pharmingen). The leukocyte viability was evaluated by trypan blue exclusion, and cells were used for immunolabeling assays.

**Real-time PCR**

Total RNA was extracted from whole fragments of gingival biopsies using the Promega RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions. cDNA was synthesized using 1 μg RNA through a RT reaction (Mooney murine leukemia virus RT, Promega). Real-time PCR quantitative mRNA analyses were performed on the ABI Prism 7000 sequence detection system using the SYBR Green fluorescence quantification system (Applied Biosystems, Warrington, UK). The standard PCR conditions were 95°C for 10 min, 40 cycles for 1 min at 94°C, 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of human primers were designed in PrimerExpress software (Applied Biosystems) using nucleotide sequences present in the GenBank database and were as follows: β-actin, sense: TGGTACAACTGATTGAGCAG, antisense: ATGAAAGCATGCTAACATCTCA; IL-10, sense: AGATCT-CCGAGAATCCCTTTATG, antisense: CCGTGGAGACT- GGAAGATT; TGF-β, sense: ATVCGCGTGGTGGTGAATC, antisense: TGACT- TATCCCTGCTGTCACAG; Foxp3, sense: CCCCATTACAGGACTCTCT, antisense: CTTCCTCCTTCCTCAGCACA; CCL1, sense: CAGTGGAGACCTCATGCCTATT, antisense: AAGGCCAGAGGAAATGTTG; CCL17, sense: AGGATCTAACGGAGGAACCT, antisense: ATCTCCCTCACTGCTGTCCTC; CCL22, sense: TGCCGT-GATTAGCTGCTGTTAG, antisense: AAGGCCAGCTTACAGAGT; CCR4, sense: GGATATAGCAGATACCACCCTC, antisense: CTAG- CTTGCTGCTGTCACA; CCR5, sense: AGATCTCTGATCCCTTGGT; CCR5, sense: AGATGCACTGCTTAGT, antisense: AAATCGTCTCTCAGACCA; CCR6, sense: TCGAGTCCCTTGGT; CCR6, sense: AGATGCACTGCTTAG, antisense: AAATCGTCTCTCAGACCA.

The SYBR Green PCR master mix (Applied Biosystems), 0.1–0.2 μM specific primers and 2.5 μg cDNA, was used in each reaction. Threshold for positivity of real-time PCR was determined based on negative controls. Calculations to determine the relative level of gene expression were made according to the instructions from the user’s bulletin (PN 4306359) of Applied Biosystems by reference to the β-actin in each sample, using the cycle threshold
Confocal microscopy

Slides for double-immunofluorescence staining (CD25/TGF-β and CD25/Foxp3) were postfixed with acetone and blocked with 15% FCS/PBS. After being washed, slides were incubated with the primary antibody, washed again, incubated with the secondary antibody, followed by another washing step, and finally, incubated with streptavidin-Alexa Fluor 488 or 546 (Molecular Probes, Eugene, OR, USA) and 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes). After being washed, the slides were mounted using Fluormount (EM Sciences, Hatfield, PA, USA) and analyzed using a confocal microscope (TCS SP5, Leica Microsystems, Bannockburn, IL, USA). Adobe Photoshop (Version 4.0) was used for image processing. Secondary antibodies alone were used as negative controls.

Statistical analysis

Statistical analysis was performed using unpaired one-tailed t-test (InStat, GraphPad Software, San Diego, CA, USA), and values were considered significantly different at \( P < 0.05 \).

RESULTS

Characterization of cellular infiltrate in CP

First, to characterize the lymphocyte infiltrate in CP, frozen tissues were stained specifically for surface markers. Results showed increased accumulation of inflammatory cells in CP biopsies when compared with controls, and the infiltrate was composed mainly by CD3+ T lymphocytes, in special CD4+ and CD8+ cells, as demonstrated in Figure 1A. To have a more accurate quantification and to evaluate if the inflammatory infiltrate of CP gingival tissues could be related to the disease pathogenesis, gingival tissues were collected and processed for flow cytometry, and cells were stained with specific antibodies for characterization of the leukocyte populations in CP.

As demonstrated in Figure 1, B and C, tissues from both groups of patients presented leukocyte infiltrate composed of T lymphocytes (CD3+, CD3-CD4+, CD3+CD8+), B cells (CD19+), macrophages (CD14+), and dendritic cells (CD11c+), although controls presented noticeable, diminished infiltrate in comparison with diseased tissues. Among the different leukocytes quantified, we observed that the frequency of CD3+ T lymphocytes in CP was significantly higher than in control (\( P < 0.05 \)), and we could not observe any relevant difference in the proportion of B cells, macrophages, or DC between both groups (Fig. 1C).

Detection of CD4+CD25+ T cells in CP gingiva

In accordance with the great extent of CD3+ T lymphocytes and the presence of CD4+ cells in periodontitis patients, we next asked if CD4+CD25+ T lymphocytes could be present in the inflammatory infiltrate of CP gingival tissues. We found that periodontitis patients presented an increased accumulation of CD4+CD25+ leukocytes in gingival tissues as compared with healthy controls (Fig. 2A; \( P < 0.05 \)). Additionally, the presence of CD25+ cells in CP tissues was confirmed by immunofluorescence analysis of this population, as observed in Figure 2B. However, as regulatory and activated T lymphocytes may express the α-chain of IL-2R (CD25), we then phenotyped the CD4+CD25+ T cells with specific markers of Treg populations. Therefore, we evaluated the expression of the molecules Foxp3, CTLA-4, GITR, CD45RO, and CD103 in these leukocytes and in the CD4+ T cells not expressing CD25. Notably, although some cells from the CD4+CD25+ population presented Treg markers in CP patients, a higher frequency of CD4+CD25+ lymphocytes expressed the specific molecules

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Fig. 1. Phenotypic characterization of leukocyte infiltrate in gingival biopsies of CP patients and healthy controls. (A) Immunohistochemical staining shows positive cells expressing CD3, CD4, and CD8 markers in the inflammatory infiltrate of CP and control patients. (B) Leukocytes obtained from gingival biopsies were analyzed via their forward- and side-scatter (FSC and SSC) properties and gated on lymphocyte (R1) or monocyte (R2) regions. (C) Frequency of CD3+, CD3-CD4+, CD3-CD8+, CD19-, CD14-, CD103, CD14-5, and CD11c- cells as determined by flow cytometry. Data are the mean ± SEM results for 22 patients, and CP and eight healthy control subjects were tested individually. *, \( P < 0.05 \), compared with the healthy control subjects. Original scale bars = 20 μm.
that characterize natural Tregs, as demonstrated in Figure 2C ($P < 0.05$). These results showed that the majority of CD4$^{+}$CD25$^{+}$ cells in CP patients presented a Treg phenotype, suggesting their involvement in the pathogenesis of the disease.

**Migration of CD4$^{+}$CD25$^{+}$ T cells to the gingival lesions in CP**

As we detected the presence of CD4$^{+}$CD25$^{+}$ T cells in diseased tissues, it was important to know the mechanisms that could guide these cells to the periodontal lesions. As Tregs usually migrate in response to CCL1, CCL17, and CCL22 and may express chemokine receptors such as CCR4 and CCR8 [22], we investigated the expression of these molecules in CP. We found that the levels of CCL17, CCL22, and CCR4 were increased significantly in CP biopsies when compared with controls (Fig. 3, A–E; $P < 0.05$). Moreover, CCL17 protein was mainly associated with epithelia, endothelial cells, fibroblasts, and small leukocytes such as lymphocytes, and CCL22 was apparently produced by larger cells such as histiocytes in the inflammatory infiltrate of CP patients. We could not observe relevant production of these chemokines in control biopsies (Fig. 4). These results indicate that CD4$^{+}$CD25$^{+}$ Tregs may migrate to CP gingival, especially in response to chemokines such as CCL17 and CCL22 produced by the leukocytes infiltrating periodontal lesions.

**Expression of suppressive cytokines and Foxp3 in CP**

The transcription factor Foxp3 is essential for natural Treg development and function, and the suppressive effects of regulatory lymphocytes may be mediated by cytokines such as TGF-β and IL-10 involved in the induction of tolerance and regulation of immune response [10, 16, 23]. Then, to determine the expression pattern of regulatory molecules in CP, we conducted real-time PCR in gingival samples from diseased and healthy tissues to assay the expression of IL-10, TGF-β, and Foxp3 mRNA. The results demonstrated that CP patients presented increased expression of messages for all of the molecules analyzed when compared with controls (Fig. 5, A–C, $P < 0.05$). This elevated expression of IL-10, TGF-β, and Foxp3 was not exclusive of CP, as patients affected by other forms of periodontal inflammation such as gingivitis and aggressive periodontitis also showed high levels of these regulatory-related molecules (data not shown). These data agree with the previous results described above and suggest that besides the increased presence of CD4$^{+}$CD25$^{+}$ cells, the inflammatory response in PD tissues is underlined by Treg-related molecules that could be mediating the latency and/or the multifactorial inflammation in these patients.

**Confocal analysis of Tregs function in situ**

Finally, to verify if the CD25-expressing leukocytes could indeed be characterized as regulatory cells, we performed confocal microscopy in gingival biopsies of CP patients and analyzed the coexpression of regulatory-like molecules in these cells. As visualized in Figure 6A, CP tissues were shown to have CD25$^{+}$ cells coexpressing the protein Foxp3, thus confirming the presence of natural Tregs in these inflamed, gingival biopsies. Moreover, it is striking to note that the CD25$^{+}$ cells found in CP tissues were an important source of the suppressive cytokine TGF-β found in this biopsies, as CD25$^{+}$ cells also coexpressed this molecule in the gingival inflamma-
tory infiltrate (Fig. 6B). These results demonstrated that periodontitis-inflamed tissues not only presented CD4+CD25+ Tregs, but also, these cells were producing TGF-β, which could be capable of immunosuppressing the immune response in this pathology.

**DISCUSSION**

Although periodontopathogenic bacteria are the primary etiological agents in periodontitis, the ultimate determinant of disease progression and clinical outcome is the host’s immune response, which involves the generation of cytokines, the activation of osteoclasts, and the recruitment of inflammatory cells [24]. Accordingly, in the present work, we phenotyped the leukocytes presented in periodontitis lesions, demonstrating elevated numbers of T and B cells, macrophages, and DC in these tissues. Although there were no statistical differences in the proportion of CD3+CD4+ or CD3+CD8+ cells between diseased and healthy groups, we observed a tendency toward an increase in the percentage of these leukocytes in CP patients as that seen for CD3+ lymphocytes. In addition, regarding the number of cells, the periodontitis biopsies showed increased inflammatory infiltrate that consequently resulted in a great number of all leukocytes phenotyped, including CD3+, CD3+CD4+, or CD3+CD8+ cells in these patients when compared with controls. Moreover, it is of note that another leukocyte population may express the surface molecule CD3 as NKT or γδ T cells, which could also explain the differences observed in the percentage of CD3+ but not CD3+CD4+ or CD3+CD8+ cells. These results agree with others [25], and the exacerbated presence of T lymphocytes suggested a potential role for these cells in the mechanisms of tissue destruction associated with PD.

CD4+CD25+ Tregs play an important role in the regulation of immune responses and immunologic tolerance. These cells are found in the thymus and peripheral blood of humans and mice [13, 26, 27], and a growing body of evidence has shown that Tregs play a critical role in the immune response induced by transplanted organs, tumors, and parasite antigens [13]. In addition, the number of immune cells and their effector functions are under the strict control of Tregs [26]. In periodontitis, the effector immune response has to be regulated to control the bacteria growth and dissemination and to prevent tissue damage. Thus, as natural Tregs preferentially accumulate at sites of infection, limiting the effector immune responses and promoting pathogen survival [8, 20], in this study, we explored the
presence and characteristics of CD4⁺CD25⁺ T cells in the site of lesion in patients with CP.

One aspect that must be emphasized is that the frequency of CD4⁺CD25⁺ cells in gingival biopsies was strongly augmented in CP when compared with controls. More strikingly, these cells exhibited phenotypic characteristics of Tregs, as confirmed by the expression of specific Treg markers such as CTLA-4, GITR, CD103, CD45RO, and Foxp3, preferentially in the CD25⁺ population obtained from CP patients. Although these markers are not uniquely expressed by natural Tregs, their level of expression and constitutive nature have been useful in the characterization of CD4⁺ T cells with regulatory properties. For instance, GITR was already identified as a constitutively expressed marker for Tregs, and CTLA-4 has been shown to trigger the induction of the enzyme indoleamine 2,3-dioxygenase when interacting with its ligands on DC, thus leading to the production of metabolites, which have potent, immunosuppressive effects in the local environment of the DC [11]. Additionally, in the peripheral blood of healthy adults, the majority of Tregs, identified as CD4⁺CD25⁺Foxp3⁺ T cells, was shown to have the memory CD45RA⁻CD45RO⁺ phenotype [28], and the expression of CD45RO was also detected in Tregs present in skin lesions from patients with chronic mycosis [10]. Aside from these markers, CD4⁺CD25⁺ T cells may also express CD103, an integrin in the peripheral blood of healthy adults, the majority of Tregs, identified as CD4⁺CD25⁺Foxp3⁺ T cells, was shown to have the memory CD45RA⁻CD45RO⁺ phenotype [28], and the expression of CD45RO was also detected in Tregs present in skin lesions from patients with chronic mycosis [10]. Aside from these markers, CD4⁺CD25⁺ T cells may also express CD103, an integrin involved in Treg migration to lesion sites, especially in protozoa and fungal infections [10, 29]. In the present study, the reduced inflammatory infiltrate and special CD4⁺CD25⁺ cells in control biopsies did not permit the evaluation of Treg markers in healthy individuals, although the data shown in this work indicated that cells with characteristics of natural Tregs accumulate within the gingiva of periodontitis patients and may play an important role in the maintenance of the chronicity in this disease.

Besides the expression of integrins such as CD103, it is possible that periodontopathogens may have evolved strategies to establish conditions favoring Treg recruitment to the sites of infection through triggering the secretion of chemokines that attract these cells to the lesions. In fact, the migration of CD4⁺CD25⁺ T cells to periodontitis gingiva seemed to be dependent on CCL17 and CCL22 expression, mainly by specific cells of the local inflammatory infiltrate. These results are in agreement with previous data from our group that showed the production of these chemokines in the lesions of leishmaniasis [9] and paracoccidioidomycosis patients [10] and correlated it to the presence of Tregs in these tissues. Although we could not correlate the frequency of CD4⁺CD25⁺ cells with chemokine expression, we could observe, in these immunohistochemical studies, that the patients with higher inflammatory infiltrate were the same who showed increased chemokine (or cytokine) staining, thus demonstrating a correlation between the parameters evaluated. Therefore, we believe that the production of both chemokines during the course of the inflammatory response could dictate the extent, severity, and duration of the inflammatory process by modulating recruitment of Tregs expressing CCR4 or CCR8 to the gingival tissues [22]. Moreover, CCR4 and/or CCR8 may guide Tregs to sites of antigen presentation in inflamed areas to attenuate T cell activation,
therefore maintaining the latent and chronic inflammatory response in CP. In contrast to our findings, another work showed diminished Foxp3+CD25+ cells in PD [30]. In the present study, we argue against this possibility, as the levels of chemokine and chemokine receptors related to Treg migration were increased in the tissues evaluated here.

We previously demonstrated that periodontitis patients exhibit higher expression of the bone resorption factor receptor activator of NF-κB ligand (RANKL) when compared with healthy controls and that the expression of its inhibitor osteoprotegerin was higher in CP and was associated with lower IFN-γ and higher IL-10 expression, compared with aggressive periodontitis [31]. On the other hand, others postulated that diminished Foxp3+CD25+ cells in PD are associated with pathogenic RANKL expression by activated lymphocytes and a negative correlation between concentrations of soluble RANKL and IL-10 in diseased gingiva [30]. Therefore, it is possible that not only the presence of regulatory cells but also the balance between cytokines and bone resorption factors determine the chronic, stable, or progressive nature of the lesions and regulate the severity of PD.

Indeed, upon stimulation with bacterial products, inflammatory cells synthesize and secrete a variety of cytokines that modulate the local immune response [25]. Our results showed elevated mRNA expression for IL-10 and TGF-β cytokines in gingival CP tissues, as well as Foxp3. TGF-β is capable of inducing IL-10-secreting CD4+ Tregs from CD4+CD25−CD45RBlow precursors through de novo Foxp3 production, consequently maintaining natural Treg peripheral homeostasis [32]. TGF-β is also strongly associated with the suppression of proinflammatory responses and the faster growth of parasites [8, 33]. On the other hand, IL-10 is correlated with the suppression of the antigen-induced proliferative T cell response [34] and has the ability to inhibit antigen presentation [35]. This cytokine is also involved in Leishmania persistence and reactivation of disease in humans [36, 37]. Moreover, IL-10 and TGF-β have been implicated in the suppressor mechanisms mediated by CD4+CD25+ T cells in autoimmune and infectious diseases [9, 10, 13, 26]. Our data suggest that the CD4+CD25+ T cells found in CP biopsies may be exerting their suppressive functions through secretion of TGF-β and IL-10. In fact, we observed a positive correlation between TGF-β and Foxp3 as well as IL-10 and Foxp3 mRNA levels in the patients with PD (data not shown). In addition, we identified, by immunostaining, leukocytes coexpressing CD25 and Foxp3 as well as CD25 and TGF-β, thus confirming the presence and the production of suppressive cytokines by regulatory cells in CP.

To date, previous studies demonstrated that Tregs are present in PD, and individual T cell clones isolated from a few periodontitis lesions showed mRNA expression for Treg markers [4, 7, 38]. However, although comparing the gene expression in this T cell clone with gingival biopsies, the method used to establish the clones (anti-CD3 antibody and IL-2) may have affected the gene expression profile, and these clones did not suppress the proliferation of CD4+CD25− T cells. Moreover, these studies were performed with clones isolated from a small number of patients, and there were no comparisons with a control, healthy group, which were demonstrated here to present CD25+ T cells.

Recently, it has been shown that Tregs can control a large number of infections by modulating the intensity of the effector immune response [8], which also prevents the induction of immune-mediated lesions. Previous studies from our group demonstrated that CD4+CD25+ T cells were present in lesions of patients with cutaneous leishmaniasis as well as paracoccidiomycosis and exhibited phenotypic and functional characteristics of natural Tregs [9, 10]. One aspect that we could not confirm is whether the Tregs found in periodontitis lesions were directly involved in the development of bone resorption and/or in periodontopathogens persistence. In experimental models, such as infection by Leishmania, HSV, and S. mansoni, Tregs seem to play a role in the maintenance of chronic infections, with persistence of pathogens, consequently enabling the disease reactivation [8]. Another possibility is that in CP-established lesions, Tregs may be recruited in an attempt to hamper exacerbated tissue destruction through extensive inflammatory response or even putative autoimmune mechanisms via a negative-feedback system. Alternatively, these cells may impede protective immunity to pathogens [39]. In this study, we detected a more pronounced inflammatory infiltrate in periodontitis lesions than in controls; however, we could not specifically correlate the higher or lower Treg frequency with the periodontal destruction and clinical course of the disease, as the periodontitis patients evaluated in this study comprised a homogeneous group with a similar clinical condition. However, we believe that in some patients, the levels of Tregs may be related to the development of the disease before the onset of extensive bone loss, and these differences might be clearly observed in cases, in which very different clinical conditions are compared, such as chronic and aggressive PD. Collectively, these data indicate that Tregs found in chronic lesions must be involved in the modulation of a local immune response. However, whether Tregs are associated with pathology for increasing tissue damage or maintaining the chronicity of the disease remains to be determined.

Thus, we demonstrate that natural Tregs defined by their phenotype are present in gingival lesions of patients with the chronic form of periodontitis, thus corroborating with the findings in other inflammatory lesions of human diseases [40]. Future studies are, however, required to understand the relationship among Tregs, effector T cell activation, and the mechanisms underlying the maintenance of periodontopathogens or disease reactivation. We believe that this work is a necessary step toward the design of future therapeutic protocols needed to treat chronic inflammatory diseases and particularly, CP. In this context, it is reasonable to assume that patient susceptibility to periodontal tissue destruction could be determined at least in part by the balance between effector response mediated by activated T cells and regulatory mechanisms mediated by Tregs. Further studies are needed to unravel the relationship between Tregs and different clinical presentations of PD. Finally, understanding this relationship may bring about new therapeutic and prophylactic strategies to avoid the development of CP in susceptible individuals.
REFERENCES


