Inhibitory Signals Mediated by Programmed Death-1 Are Involved With T-Cell Function in Chronic Periodontitis

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Background: Inhibitory signals mediated via molecules such as programmed death-1 (PD-1) play a critical role in downmodulating immune responses and maintaining peripheral tolerance. We investigated the involvement of cytokines and PD-1 engagement in mediating the T-cell unresponsiveness to bacterial and ubiquitous antigens in periodontal diseases.

Methods: Gingival and peripheral blood samples from healthy individuals and patients with chronic periodontitis were collected and used for the subsequent assays. Leukocytes in the lesion site and blood were evaluated using flow cytometry. The production of interferon-γ, interleukin-10, and transforming growth factor-β proteins was evaluated by enzyme-linked immunosorbent assay (ELISA), and the presence of PD-1+ cells in the inflamed gingiva was confirmed by immunofluorescence confocal microscopy for CD4 and PD-1 colocalization.

Results: T cells from patients with chronic periodontitis proliferated poorly in response to *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) antigen. T-cell unresponsiveness was not associated with imbalanced cytokine production. However, T cells from patients with chronic periodontitis expressed significantly higher levels of PD-1 either upon isolation or after culture with antigens. Moreover, PD-1 blocking did not result in significant T-cell proliferation in cells cultured with phytohemagglutinin or bacterial antigens. The blockade of PD-1 resulted in the increased production of IFN-γ. In addition, CD4+ and CD8+ T cells expressing PD-1 accumulated in lesions with chronic periodontitis.

Conclusion: These data show that PD-1 engagement could be involved in the modulation of IFN-γ production by T cells in patients with chronic periodontitis. *J Periodontol 2009;80:1833-1844.*

KEY WORDS

Cytokines; interferon-gamma; interleukin-10; lymphocyte activation; periodontitis; T-lymphocyte subsets.

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Periodontal disease is a common, complex disease with a variable clinical presentation. The most prevalent form is chronic periodontitis, which can be further characterized by extent and severity (degree of clinical attachment loss). Patients with chronic periodontitis usually show cellular immune hyporesponsiveness to bacterial antigens.[1-3] The suppression of T-cell function by a number of periodontopathogens (*Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* [*Aa*; previously *Actinobacillus actinomycetemcomitans*], and *Fusobacterium nucleatum*) was demonstrated.[1-3]

The mechanisms involved in the modulation of this phenomenon are poorly defined but include low interleukin (IL)-2 production,[4] low IL-2 receptor expression,[5] and an imbalance in cytokine production.[6] Other possible causes include functional T-cell inactivation, inactivation or dysfunction of other immune cell populations, and elimination of T cells as a consequence of activation-induced cell death.[5] T-cell unresponsiveness may also be regulated through costimulatory receptors. Among them, programmed death-1 (PD-1) has been broadly implicated as a critical downregulator of T-cell activation.[7-11] Engagement of PD-1 by PD-1 ligand 1 or 2 (PD-L1 or PD-L2) leads to the inhibition of T-cell proliferation and the production

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of cytokines such as IL-2 and interferon (IFN)-γ.[11] Consistent with the inhibitory role of PD-1, PD-1-deficient mice develop a severe autoimmune disease.[12-15] Concerning periodontal disease, there are no reports addressing the involvement of PD-1 in the suppression of the T-cell response.

Because peripheral blood mononuclear cells (PBMCs) from patients with chronic periodontitis proliferate poorly in response to bacterial antigens,[1-3,16-18] we hypothesized that this inhibition of T-cell activation could involve signals via costimulatory receptors. Therefore, in this study we investigated the involvement of PD-1 engagement in the mechanism that mediates the T-cell hyporesponsiveness observed in patients with chronic periodontitis.

MATERIALS AND METHODS

Patients With Chronic Periodontitis and Healthy Subjects

PBMCs and tissue samples were obtained from 20 patients with chronic periodontitis (12 females and 8 males; age range, 41 to 96 years; mean age = 44.4 ± 12.89 years) as well as 20 age-matched healthy volunteers (14 men and 6 women; age ranged 20–44 years). Healthy subjects (low scores of bleeding on probing <10% of sites and no sites with probing depth >3 mm or presenting attachment loss), and patients with chronic periodontitis (moderate to advanced periodontal disease for at least one tooth per sextant with probing depth >6 mm, attachment loss >3 mm, and radiographic evidence of extensive bone loss) were selected from patients scheduled for treatment at the Bauru School of Dentistry, University of São Paulo, Brazil, using inclusion and exclusion criteria, as previously described.[19,20] Inclusion criteria included partially or fully dentate patients (at least 14 natural teeth, including 10 posterior teeth and excluding third molars), systemically healthy with no evidence of known systemic modifiers of periodontal disease (type 1 and 2 diabetes mellitus, osteoporosis, and medications known to affect periodontal tissues). Exclusion criteria included those patients who would not give informed consent; patients with a significant medical history indicating evidence of known systemic modifiers of periodontal disease as described above; pregnant or lactating females; and patients who had taken systemic antibiotic, anti-inflammatory, hormonal, or other assisted drug therapy in the last 6 months prior to the study, or who had received previous periodontal therapy in the last 2 years. The protocol of this study was approved by the institutional Ethics Committee, and all subjects provided informed written consent.

Media and Reagents

All human cells were grown in Roswell Park Memorial Institute (RPMI) 1640[¶] supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate.[#] Phytohemagglutinin (PHA)[**] and carboxyfluorescein succinimidyl ester (CFSE)[††] were purchased.

Antibodies (Abs) and Flow-Cytometry Analyses

For immunostaining, peridinin chlorophyll protein complex (PerCP) and phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated Abs against CD3 (UCHT1), CD4 (SK3), CD8 (RPA-T8 and HIT8a), CD19 (HIB19), CD28 (CD28.2), PD-1 (MIH4), CD152 (BNI3), and the respective mouse and rat isotype controls were used.[‡‡] The cell acquisition was performed on a flow cytometer using software.[§§] For blocking, unconjugated anti-PD-1[‖‖] and respective isotype controls[¶¶] were used.

Bacterial Strain, Culture Conditions, and Antigens

The virulent *Aa* JP2 strain used in this study was anaerobically grown in supplemented agar medium (tryptic soy-serum-bacitracin-vancomycin), as described previously.[21] For production of bacterial antigens, the cells were disrupted by ultrasonic treatment and centrifuged for 10 minutes at 12,000 × g. Supernatants were collected, and the total amount of protein was determined.

Isolation of Leukocytes

Peripheral blood was harvested with heparin (50 U/ml) from healthy subjects and patients with chronic periodontitis. PBMCs were isolated using density gradient centrifugation,[##] washed, counted, and labeled with specific Abs for phenotypic analysis in the flow cytometer. The number and phenotype of leukocytes isolated from controls and patients with chronic periodontitis were similar. To characterize the leukocytes present in the lesion site, the biopsies of gingival lesions (5 mm) from patients with chronic periodontitis were collected and incubated for 1 hour at 37°C in RPMI 1640 medium containing 50 μg/ml enzyme blend.[***] The tissues were dissociated for 4 minutes in the presence of RPMI 1640 with 10% serum and 0.05% DNase[†††] using a mechanical disaggregation system [‡‡‡] according to the manufacturer's instructions. Tissue homogenates were

¶ Invitrogen Life Technologies, Grand Island, NY.
# Sigma-Aldrich, St. Louis, MO.
** Invitrogen Life Technologies.
†† Molecular Probes, Invitrogen Life Technologies.
‡‡ R&D Systems, Minneapolis, MN.
§§ FACSort flow cytometer using CellQuest software, BD Biosciences, San Jose, CA.
‖‖ R&D Systems.
¶¶ R&D Systems.
Ficoll-Hypaque, Amersham Pharmacia Biotech, Uppsala, Sweden.
*** Liberase CI, Boehringer Ingelheim Chemicals, Indianapolis, IN.
††† Sigma-Aldrich.
‡‡‡ Medimachine, BD Biosciences.

filtered using a 30-μm cell strainer.§§§ The leukocyte viability was evaluated by trypan blue exclusion and used for cell activation or immunolabeling assays.

T-Cell Stimulation

PBMCs isolated as described were counted and suspended at 5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.‖‖‖ The cell suspensions were distributed (1 ml/well) in 24-well tissue-culture plates¶¶¶ and cultured for 96 hours at 37°C in a humidified 5% CO_2 atmosphere in the presence or absence of *Aa* antigen (*Aa*Ag) (0.5 μg/ml) or PHA (1 μg/ml). For PD-1 blocking assays, soluble anti-PD-1 (2 μg/ml) or isotype control (mouse immunoglobulin [Ig] G; 2 μg/ml) was added to cultures on the day of stimulation with antigen. These cultured cells were used to assay the expression of PD-1. Supernatants were collected to evaluate cytokine production.

T-Cell Proliferation Assay

For cell division measured by carboxyfluorescein succinimidyl ester (CFSE) dilution, purified PBMCs were labeled with 2 μM CFSE for 5 minutes in RPMI at 37°C. Labeling was stopped by addition of fetal bovine serum (FBS), and cells were washed and resuspended in complete medium. A total of 1×10^6 CFSE-labeled PBMCs were incubated for 96 hours. At the end of the stimulation, T cells were acquired by flow cytometry. T-cell proliferation was measured by CFSE fluorescence dilution of the electronically gated T-cell population. CFSE profiles were analyzed using the proliferation platform of a software program.### The proliferation index was calculated as the mean values of cell proliferation in the presence of antigen divided by the mean values of cell proliferation in the absence of antigen.

Cytokine Assays

Cell-culture supernatants were harvested after 96 hours of culture in the presence or absence of *Aa*Ag (0.5 μg/ml) or PHA (1 μg/ml), and the cytokines (IFN-γ, transforming growth factor [TGF]-β, and IL-10) that had been secreted were measured by the enzyme-linked immunosorbent assay (ELISA) set.**** Optical densities were measured at 450 nm using a microplate ELISA reader.††††

Immunofluorescence Analyses and Confocal Microscopy

Gingival biopsies were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 12 hours at room temperature. The specimens were trimmed, dehydrated in graded ethanol, and embedded in paraffin. Serial sections (5 μm) were cut and mounted on glass slides precoated with 0.1% poly L-lysine.‡‡‡‡ Slides for double-immunofluorescence staining were blocked with 15% FBS/PBS. After being washed, the slides were incubated with the primary antibody, washed again, and incubated with the appropriate fluorochrome-conjugated (Texas red, PE, or fluorescein D) secondary antibodies as well as 4′6′-diamidino-2-phenylindole. After being washed, the slides were mounted using 90% glycerol/PBS and analyzed using a confocal microscope.§§§§ A program‖‖‖‖ was used for image processing. Secondary antibodies alone were used as negative controls.

Real-Time Polymerase Chain Reactions (PCRs)

The extraction of total RNA from periodontal tissues was performed with reagent¶¶¶¶ following the protocol recommended by the manufacturer, and the complementary DNA was synthesized using 3 μg RNA through a reverse transcription reaction.#### Quantitative real-time PCR was performed in a sequence detection system using the a DNA amplification.***** A specific reagent mix,††††† 100 nM specific primers, and 2.5 ng cDNA were used in each reaction. The primer sequences, the predicted amplicon sizes, and the annealing and melting temperatures for the primers, designed using the software,‡‡‡‡‡ are depicted in Table 1. The standard PCR conditions were 95°C (10 minutes), followed by 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes), and by the standard denaturation curve. For mRNA analysis, the relative level of gene expression was calculated in reference to beta-actin expression in the sample using the cycle-threshold method. Negative controls without cDNA and without reverse transcriptase were also performed.

Statistical Analyses

Data obtained from flow cytometry and the cell proliferation assay were expressed as mean ± SEM. The difference in the intergroup values between the patient and healthy control groups was analyzed using the unpaired Mann-Whitney test.§§§§§ The difference of cytokine-production response under PD-1 blocking conditions was analyzed using the paired Wilcoxon matched test.‖‖‖‖‖ Statistical analyses were performed using software.¶¶¶¶¶ All values were considered significantly different at $P < 0.05$.

§§§ BD Biosciences.
‖‖‖ Invitrogen Life Technologies.
¶¶¶ Costar, Corning, Lowell, MA.
CellQuest software, BD Biosciences.
**** BD Biosciences.
†††† Spectra Max 250, Molecular Devices, Sunnyvale, CA.
‡‡‡‡ Sigma-Aldrich.
§§§§ Leica TCS-SPE, Leica Microsystems, Mannheim, Germany.
‖‖‖‖ Adobe Photoshop (CS8), Adobe Systems, San Jose, CA.
¶¶¶¶ Trizol, Invitrogen Life Technologies, Rockville, MD.
Superscript III, Invitrogen Life Technologies.
***** ABI Prism 7000 Sequence Detection System using the SybrGreen System, Applied Biosystems, Warrington, U.K.
††††† SybrGreen PCR MasterMix, Applied Biosystems.
‡‡‡‡‡ PrimerExpress, Applied Biosystems.
§§§§§ INSTAT Software, GraphPad, La Jolla, CA.
‖‖‖‖‖ INSTAT Software, GraphPad.
¶¶¶¶¶ INSTAT Software, GraphPad.

Table 1.

Primer Sequences and Reaction Properties

Target	Forward (F) and Reverse (R) Sequence	bp
TGF-β	F: TACCTGAACCCGTGTTGCTCT R: ATCGCCAGGAATTGTTGCTG	330
IFN-γ	F: ATGAAATATACAAGTTATATCATG R: TGTTTCGAGGTCGAAGAGCATCCC	501
IL-10	F: AGATCTCCGAGATGCCTTCA R: CCGTGGAGCAGGTGAAGAAT	307
β-actin	F: ATGTTTGAGACCTTCAACA R: CACGTCAGACTTCATGATGG	495

bp = base pairs of amplicon size.

RESULTS

PD-1 Expression by PBMCs From Patients With Periodontal Disease

Previous studies[1,17,22] determined that T-cell proliferative responses from patients with chronic periodontal disease are depressed in response to bacterial antigens and PHA. Our results indicate that PBMCs from patients with chronic periodontitis were unable to proliferate in response to *Aa*Ag (Fig. 1B). However, patients with chronic periodontitis exhibited a higher T-cell proliferative response to PHA similar to healthy subjects (Figs. 1A and 1B). To investigate the possible participation of costimulatory molecules in T-cell hyporesponsiveness, we evaluated the expression of PD-1 in PBMCs obtained from healthy subjects and patients with chronic periodontitis. In freshly isolated CD4+ T cells from patients with chronic periodontitis, the expression of PD-1 was significantly higher ($P<0.01$) than in PBMCs from healthy individuals (6.2% ± 5.7% and 2.4% ± 2.03%, respectively; see Figs. 1D and 1E). In addition, the expression of PD-1 in CD4+ T cells from patients with chronic periodontitis was higher than in PBMCs from healthy subjects when the cells were cultured with *Aa*Ag ($P<0.005$, Figs. 1F and 1H). The percentage of CD8+PD-1+ T cells was higher in leukocytes from patients with chronic periodontitis (1.27% ± 1.02%) than in PBMCs from healthy individuals (0% ± 0.53%) (Fig. 1C). As shown in Figure 1, *Aa*Ag and PHA induced low levels of PD-1 in CD8+ T cells from healthy subjects and patients with chronic periodontitis (Fig. 1G).

Cytokine Production by PBMCs From Patients With Chronic Periodontitis

To evaluate whether the decreased T-cell proliferation from patients with chronic periodontitis, in vitro, could be due to a disturbance in the balance of cytokine production, we assayed IFN-γ, IL-10, and TGF-β production in supernatants from PBMCs cultured with *Aa*Ag or PHA. Although it was not statistically significant, PHA induced minor levels of IFN-γ production in PBMCs from patients with chronic periodontitis compared to the cells from healthy subjects (Fig. 2A). Differently, *Aa*Ags induced similar levels of IFN-γ production by PBMCs from patients with chronic periodontitis and healthy subjects (Fig. 2A). Although a statistically significant difference was not found, the level of IL-10 produced by cells cultured with PHA was lower in supernatants of PBMCs from patients with chronic periodontitis than in supernatants from healthy subjects (Fig. 2B). However, PHA and *Aa*Ags did not induce a significant TGF-β production by PBMCs from patients with chronic periodontitis and healthy subjects (Fig. 2C).

Effect of PD-1 Interaction on T-Cell Activation

Because increased PD-1 expression in PBMCs from patients with chronic periodontitis was found, we investigated whether this molecule could be involved in the mechanism that mediates the T-cell unresponsiveness. We cultured leukocytes with PHA or *Aa*Ag in the presence or absence of anti-PD-1 monoclonal antibody, which blocks PD-1 engagement, and cell proliferation was quantified. Blockade of PD-1 engagement slightly increased the T-cell proliferative response to either *Aa*Ag or PHA in cultures from healthy subjects (Figs. 3A and 3C). However, the blockade of PD-1 engagement did not significantly restore the proliferation of T cells from patients with chronic periodontitis when the cells were cultured with *Aa*Ag (Figs. 3B and 3C).

To gain insight into the functional inactivation of T cells by PD-1, T-cell production of IFN-γ in the cultures previously described was measured. The results show that IFN-γ production was significantly increased when the cells from patients with chronic periodontitis ($P<0.001$) and healthy subjects ($P<0.001$) were cultured with anti-PD-1 monoclonal antibody and *Aa*Ags (Figs. 3D through 3F). The blockade of PD-1–PD-L interaction increased the IL-10 production in cultures of PBMCs from healthy individuals (Figs. 3G through 3I). Overall, these data showed that, although PD-1 activation did not affect the proliferative response in patients with chronic periodontitis, the production of IFN-γ was increased after the inhibition of signals mediated by this molecule.

Phenotypic Characterization of Leukocytes in Gingival Tissue From Patients With Periodontal Diseases

The leukocytes derived from gingival tissue obtained from patients with chronic periodontal disease and healthy individuals (n = 20 for both groups) were characterized (Fig. 4A and 4B). The results showed that

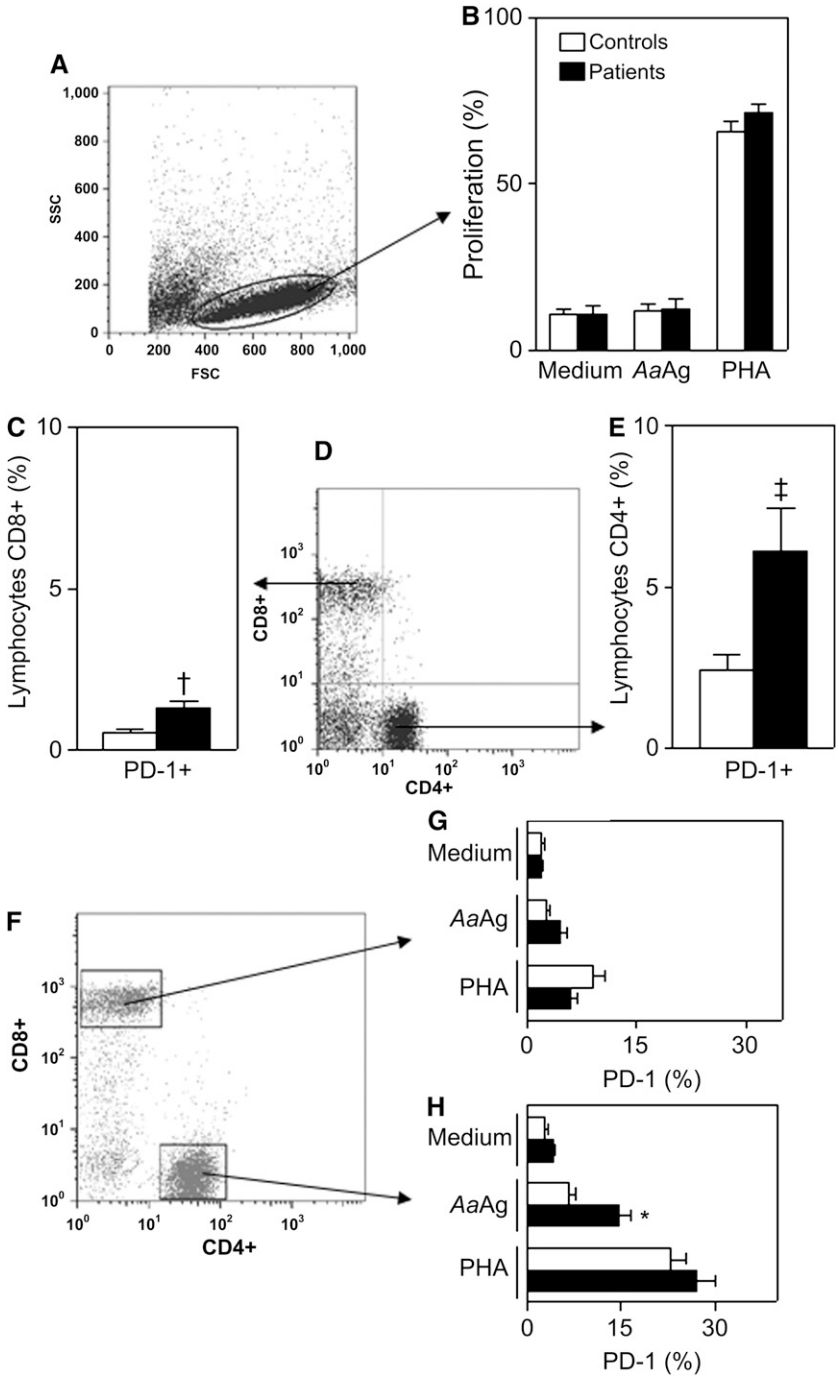

Figure 1.

PD-1 expression by PBMCs from patients with chronic periodontitis (patients) and healthy subjects (controls). ***A)*** *PBMCs from controls (n = 20) and patients (n = 20) in these analyses were gated on lymphocytes via their forward (FSC) and side scatter (SSC) properties.* ***B)*** *Proliferation was determined after 96 hours of culture by CFSE fluorescence measurement. Results are expressed as the mean ± SEM of the percentage of cell proliferation.* ***C through F)*** *PD-1 expression on CD4+ and CD8+ T cells from freshly isolated PBMCs. The representative flow-cytometry analysis of CD4+PD-1+ and CD8+PD-1+ is shown in D and F.* ***G*** *and* ***H)*** *PD-1 expression on CD4+ and CD8+ T cells after 96 hours of culture in the presence or absence of AaAg (0.5 μg/ml) and PHA (1 μg/ml). Bar graphs (mean ± SEM) indicate the percentage of PD-1 positive cells. *P <0.001, †P <0.01, and ‡P <0.05 compared to controls (unpaired Mann-Whitney test). Open rectangle = controls; black rectangle = patients (B, C, E, G, and H).*

the total numbers of leukocytes isolated were higher in patients with chronic periodontitis (3.35 ± 1.57 × 10^6 cells/biopsy) than in healthy subjects (0.50 ± 0.07 × 10^6 cells/biopsy) (Fig. 4B). CD3+ cells represented the main population in all biopsies (54.81% ± 4.43% of cells in chronic periodontitis and 38.44% ± 4.80% of cells in healthy tissue) (Fig. 4B). The proportion of CD4+ T cells was higher in chronic periodontitis

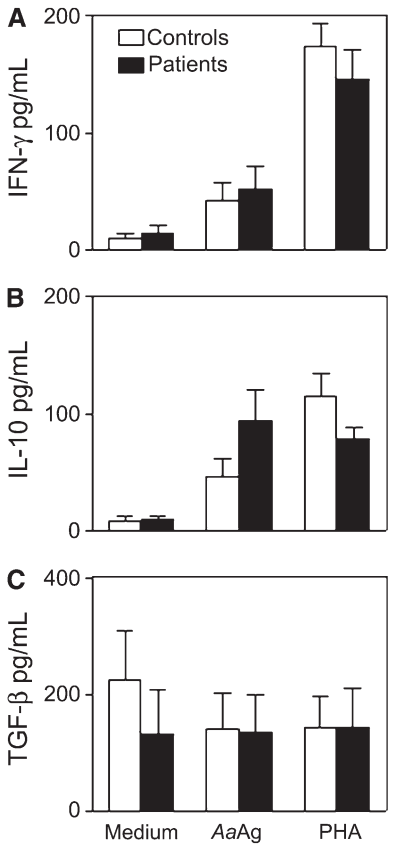

Figure 2.
Cytokine production by PBMCs from patients with chronic periodontitis and healthy subjects. ***A through C)*** *Freshly isolated PBMCs (1×10^6 cells/well) were cultured in the presence or absence of AaAg (0.5 μg/ml) and PHA (1 μg/ml). IFN-γ* ***(A)****, IL-10* ***(B)****, and TGF-β* ***(C)*** *production was analyzed in supernatants after 96 hours of culture. Results are expressed as the mean ± SEM for patients with chronic periodontitis and healthy subjects tested individually.*

lesions (22.07% ± 2.26%) than in control biopsies (8.82% ± 1.50%) (Fig. 4B). In addition, CD8+ T cells (range, 11.53% to 17.38%) were present (Fig. 4B), and a lower proportion of CD19+ cells was also detected in all the biopsies (Fig. 4B). When gating was performed on CD4+ T cells (Fig. 4D), they expressed PD-1 in different levels (35.14% ± 5.58% in chronic periodontitis biopsies and 5.66% ± 1.32% in control biopsies) (Fig. 4E). Similar levels of PD-1 expression were observed for CD8+ T cells but in a lower proportion when compared to CD4+ T cells (Fig. 4C). The presence of CD4+PD-1+ and CD8+PD-1+ T cells was also confirmed by immunofluorescence (Figs. 4F and 4G, respectively).

Expression of Cytokines in Gingival Tissue From Patients With Chronic Periodontitis

The relative intensity of mRNA expression for IL-10, TGF-β, and IFN-γ in the gingival tissue of patients with chronic periodontitis was examined. The mRNA for IFN-γ and TGF-β was more intensely expressed in gingival tissue from healthy subjects than in patients with chronic periodontitis ($P < 0.05$) (Fig. 5A). Although without a statistically significant difference, the level of IL-10 expression was lower in gingival tissue from patients with chronic periodontitis than in healthy subjects. In addition, TGF-β was detected in the biopsies by immunofluorescence staining (Fig. 5B), which revealed that macrophages are an important source of this cytokine in chronic periodontitis lesions (data not shown). Immunostaining of lesions revealed that CD4+ T cells were an important source of IFN-γ (Fig. 5C). IFN-γ was not detected in the control biopsies by immunofluorescence staining (data not shown).

DISCUSSION

These data show, for the first time to our knowledge, that engagement of the downregulatory molecule PD-1 was clearly involved in modulating T-cell function in patients with periodontal disease. The results of this study confirmed results from previous studies[1,2] showing that PBMCs from patients with chronic periodontal disease did not proliferate in response to bacterial antigen. Cell-mediated immunity is crucial for host defense in periodontal disease,[23-27] so the mechanism underlying the T-cell unresponsiveness seen in patients with chronic periodontitis was investigated. We did not find a significant imbalance in the levels of IFN-γ, IL-10, and TGF-β produced by PBMCs after antigen and mitogen stimulation, which could explain the T-cell unresponsiveness.

Recently, emerging evidence showed that the costimulatory molecule PD-1 is a crucial modulator of T-cell activation because it provides an additional stimulus for both IL-2 production and IL-2 receptor expression.[28] Considering that cells from patients with periodontal disease showed decreased IL-2 production,[29] we hypothesized that PD-1 expression could be upregulated in T cells from patients with chronic periodontitis, as it has been demonstrated to occur in T cells from patients infected with other pathogens, such as human immunodeficiency virus (HIV).[30-32] In fact, in the present study, PD-1 expression was significantly increased in cells from patients with chronic periodontitis but not from healthy subjects. Moreover, stimulation with *Aa*Ag or PHA additionally increased PD-1 expression in leukocytes from patients with chronic periodontitis, a finding that suggests that specific stimulation prompted these cells to become unresponsive. Interestingly, increased expression of PD-1 was also observed in cells from patients with hepatitis[33] and from patients with HIV infection.[30-32] In HIV-infected patients, the proliferation of T cells was inversely correlated with PD-1 expression, which suggests that PD-1 may be

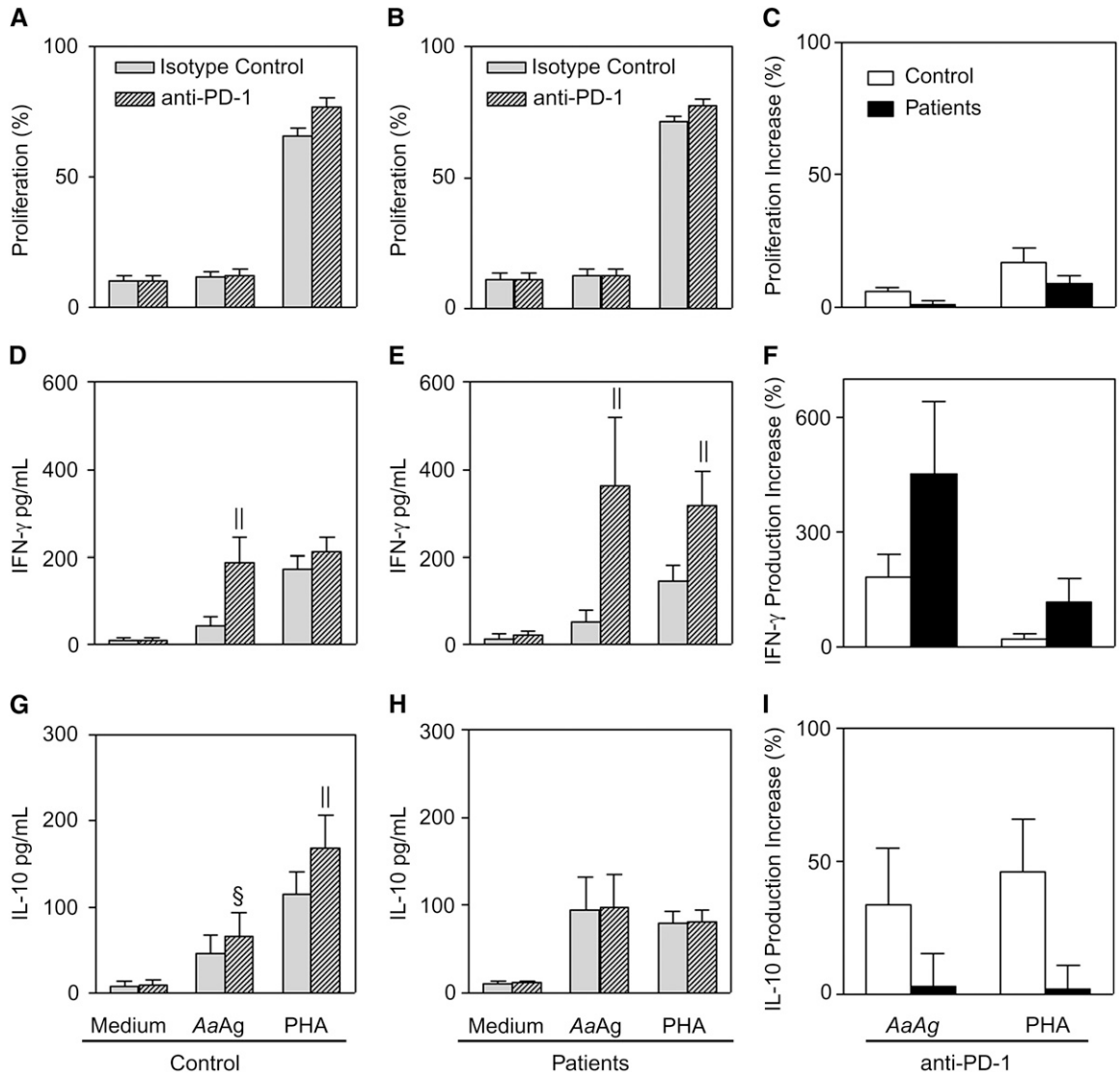

Figure 3.

*Effect of PD-1 interaction on T-cell activation. CFSE-labeled PBMCs **(A** and **B)** or freshly isolated PBMCs **(C through F)** (1×10^6 cells/well) were cultured in the presence or absence of AaAg (0.5 μg/ml) and PHA (1 μg/ml) for 96 hours. For PD-1 blocking assays, soluble anti-PD-1 (2 μg/ml) or isotype control (mouse IgG [2 μg/ml]) was added to cultures in the day of stimulation with antigen. Proliferation **(A through C)** was determined by CFSE fluorescence measurement, and IFN-γ **(D through F)** and IL-10 **(G through I)** production was analyzed in supernatants after 96 hours of culture. **(C, F, and I)** Bar graphs (mean ± SEM) indicate the proliferation and production of cytokine from patients with chronic periodontitis and healthy subjects calculated based on the PHA or AaAg-mediated T-cell activation cultured without anti-PD-1. Significant at $^{\S}P < 0.01$ and $^{//}P < 0.001$ (paired Wilcoxon matched test).*

involved in the elimination and anergy of T cells in HIV-1 infection.[30-32] Also, increased PD-1 expression was correlated with decreased proliferative activity and IFN-γ production in T cells from patients with chronic hepatitis B.[33] In contrast with what was observed in HIV-infected patients, the present study showed that the addition of anti-PD-1 neutralizing monoclonal antibody to the cultures of PBMCs from patients with chronic periodontitis did not significantly restore the T-cell proliferative response (Fig. 3A).

The blockade of the PD-1–PDL-1 interaction was unable to significantly restore the proliferative response of T cells from patients with chronic periodontitis. Although this result is not fully understood, one possible explanation is that PD-1 expression remains increased in these cells (Fig. 1) and controls T-cell exhaustion. The coexpression of other inhibitory receptors by *Aa*-specific T cells, for example cytotoxic T-lymphocyte antigen-4 (CTLA-4), represents another possible explanation for the impaired T-cell proliferation in these cultures. This conclusion is reinforced by recent results that showed T cells from patients with chronic periodontitis expressed higher levels of CTLA-4, and the blockade of

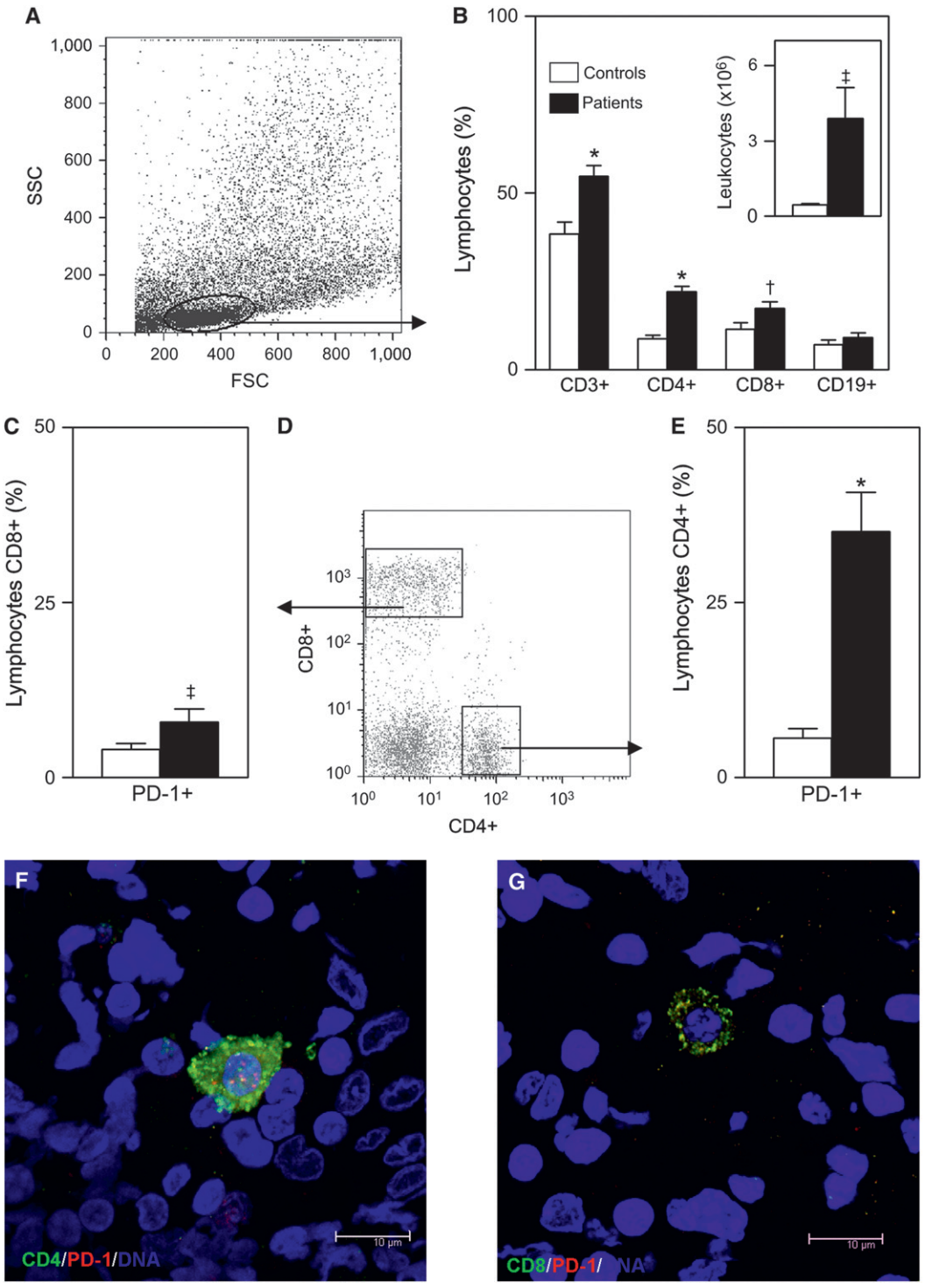

Figure 4.

Phenotypic characterization of leukocytes in gingival tissue from patients with periodontal disease and healthy subjects. The leukocytes derived from gingival tissue obtained from patients with chronic periodontal disease and healthy individuals (n = 20 for both groups) were characterized by flow cytometry. ***A)*** *Gated lymphocytes.* ***B)*** *Total cell numbers were counted, and the frequency of lesion-derived leukocytes that expressed CD3+, CD4+, CD8+ and CD19+ cells was analyzed by flow cytometry.* ***C through E)*** *PD-1 expression on CD4+ and CD8+ T cells from gingival tissue-derived lymphocytes from patients with chronic periodontitis and healthy subjects.* ***F*** *and* ***G)*** *Show the staining for PD1 (red) and either CD4 or CD8 (green) observed in 1 representative experiment. Gingival samples were fixed, incubated with anti-PD1 and either anti -CD4 or CD8, stained with the appropriate fluorochrome-conjugated secondary antibodies as well as 4′,6′-diamidino-2-phenylindole (blue), and analyzed by confocal microscopy. Results are expressed as the mean ± SEM for patients with chronic periodontitis and healthy subjects. *P <0.001, †P <0.01, and ‡P <0.05 compared to controls (unpaired Mann-Whitney test). Open rectangle = controls; black rectangle = patients (B, C, and E).*

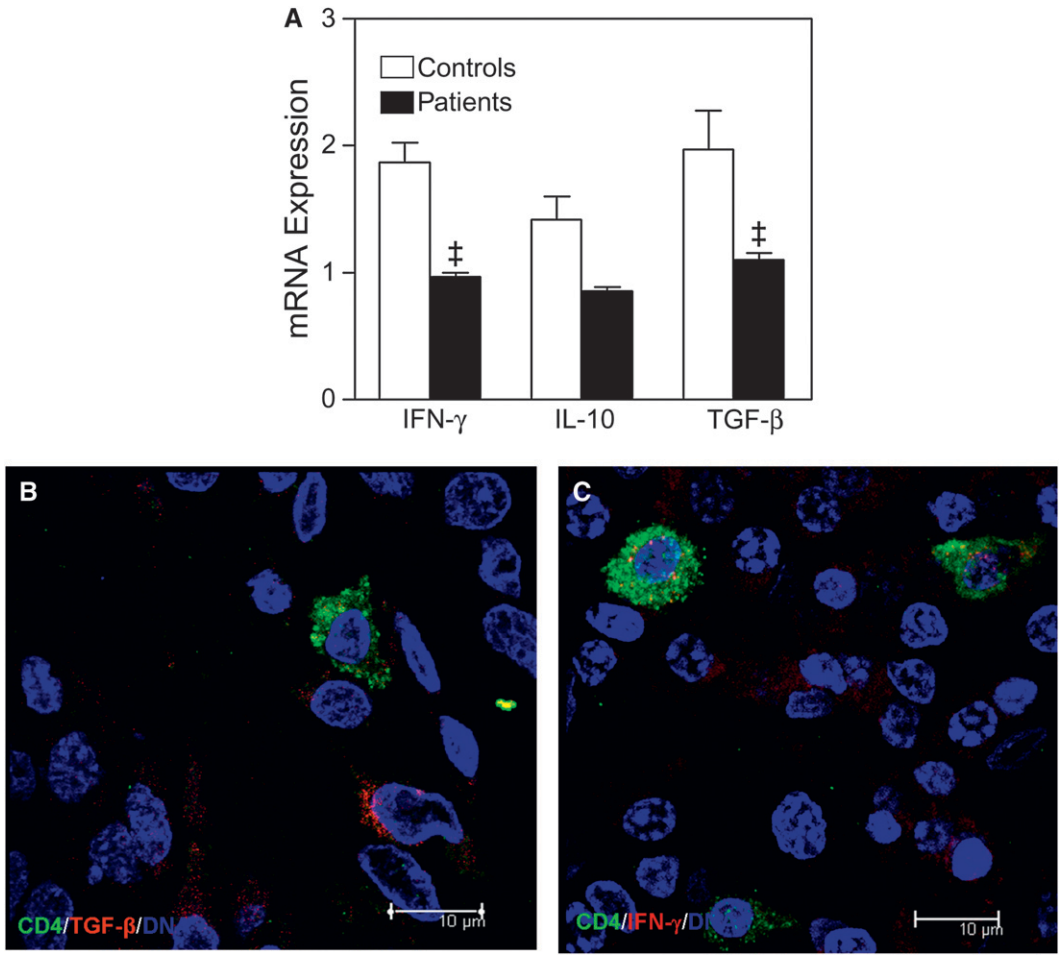

Figure 5.

Cytokine expression in gingival tissue from patients with periodontal disease and healthy subjects. ***A)*** *The levels of IFN-γ, IL-10, and TGF-β mRNA expression in lesions from patients with chronic periodontitis and healthy subjects were measured quantitatively by a real-time PCR system. Results are presented as the target mRNA level normalized to β-actin.* ***B*** *and* ***C)*** *Show the staining for TGF-β or IFN-γ (red) and CD4+ (green) observed in one representative experiment. Gingival samples were fixed, incubated with anti-CD4 and anti-TGF-β or IFN-γ, stained with the appropriate fluorochrome-conjugated secondary antibodies and 4′,6′-diamidino-2-phenylindole (blue), and analyzed by confocal microscopy.* ‡*P <0.05 compared to controls (unpaired Mann-Whitney test).*

CTLA-4 resulted in an increase in the T-cell proliferation.[34] These observations suggest the existence of layers of regulation in place to limit the responsiveness of *Aa*-specific T cells during chronic infection. Nevertheless, the blockade of PD-1 did not result in any improvement of bacterial antigen-driven proliferative T-cell response (Fig. 1). This could be due to the type of bacterial antigens used in the cultures. Similar results were obtained when purified antigen was used, which is considered to be the main antigenic compound of *Aa*.[1] Therefore, the antigen-specific T cells did not proliferate after the PD-1 blockade, which suggests the involvement of other negative regulatory pathways in CD4+ T-cell unresponsiveness in periodontal disease. To better explain, further studies with other inhibitory molecules are needed.

Another important aspect of our study is the finding that the blockade of PD-1 led to an increased IFN-γ production by PBMCs from patients with chronic periodontitis (Fig. 3B). This indicates that T-helper 1 cells do not produce IFN-γ after interaction with specific bacterial antigens because of PD-1 expression on the cell surface. This result is in accordance with the findings of the low mRNA expression of IFN-γ in the gingival lesions of patients with chronic periodontitis (Fig. 5A). It also suggests that PD-1 impairs the proliferation of T cells while allowing them to accumulate in the G_0/G_1 phase of the cell cycle and IFN-γ production, a suggestion in accordance with previously published results about CTLA-4.[35,36] These results concerning T-cell proliferation and IFN-γ production indicate that functional restoration by the PD-1 blockade is incomplete, and defects in CD4+ T cells remain after inhibition of PD-1 signals, which suggests the involvement of other negative regulatory pathways in CD4+ T-cell function during chronic periodontal disease.

Another article[37] showed that PD-1 engagement lead to IL-10 production. IL-10, another cytokine that is able to inhibit T-cell proliferation[38] and macrophage activation,[39] was persistently detected in supernatants of cells and in serum samples of patients with chronic periodontitis, as reported elsewhere.[40,41] Inhibition of PD-1 engagement resulted in no influence over IL-10 production by T cells from patients with chronic periodontitis (Fig. 3C). These results suggest that IL-10 production and PD-1 engagement are independent phenomena in the human immune response against *Aa*. The possibility that they are independent is incompatible with recent results that demonstrate that PD-1 and IL-10 represent similar mechanisms for the regulation of the T-cell response in patients with Sjögren's Syndrome.[42] A recent report[37] suggested an integral role for PD-1 in receiving signals from dendritic cells that control T-cell tolerance versus activation via IL-10 production. Further studies are needed to determine how the signal of PD-Ls to dendritic cells influence either immunity or tolerance in patients with chronic periodontitis (see Keir et al.[43] for a review).

PD-1 expression correlates with disease state and the persistence of functionally exhausted T cells during chronic infections.[30-32] Therefore, in the present study, the presence of CD4+PD-1+ and CD8+PD-1+ T cells in the gingival tissue of patients with periodontal disease was investigated. CD4+ and CD8+ T cells expressing PD-1 were present in sites that developed chronic periodontitis. These results suggest that PD-1+ T cells were effectively accumulated in the sites of periodontopathogen infection. The major frequency of CD4+ and CD8+ T cells expressing PD-1 in lesions from patients with chronic periodontitis could be involved in limiting the T-cell response during this infection. The results regarding cytokine production corroborate this hypothesis. These results also show that CD4+ T cells in chronic lesions expressed IFN-γ. Interestingly, it is assumed that periodontal disease alternates stable stages with bursts of disease progression. Therefore, the inhibitory effect of PD-1 associated with low levels of IFN-γ could be responsible for the non-progressive stages of the disease. Accordingly, high IFN-γ levels are associated with progressive lesions or higher severity forms of periodontitis.[20,44] Additionally, studies[45-48] in rodents demonstrated that IFN-γ was involved in the development of inflammatory reaction and alveolar bone resorption in experimental periodontal disease.

CONCLUSIONS

The data presented in this study indicate that stimuli driven by PD-1 engagement could be involved in the modulation of the immune response during periodontal disease. Therefore, studies to understand the relationship among T-cell activation, proliferation, and apoptosis may lead to significant improvements in the treatment of patients with bacterial infections. PD-1 engagement may be involved in the modulation of T-cell function in patients with chronic periodontitis.

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