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ORIGINAL ARTICLE

Programmed Death 1 (PD-1) and PD-1 Ligand (PD-L1) Expression in Chronic Apical Periodontitis

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ABSTRACT

Objective: This study aimed to examine programmed death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) expression on leukocytes from chronic apical periodontitis, and to determine the levels of cytokines in the apical periodontitis lesions.

Methods: Leukocytes from healthy gingival tissue (n=16) and chronic apical periodontitis (n=10) were evaluated using flow cytometry. The PD-1 and PDL-1 expressions were evaluated using flow cytometry. The cytokine levels were evaluated by enzyme-linked immunosorbent assay. Data were analyzed using one-way ANOVA. The statistical significance level was set at $P<0.05$.

Results: Results showed that the apical periodontitis lesions are more infiltrated by PD-1$^+$ and PDL1$^+$ lymphocytes than the control samples. In addition, the PDL-1 expression was detected on macrophages in the apical periodontitis lesions, and was significantly higher compared to leukocytes from healthy gingival tissue. The IFN-γ, TGF-β, IL-10, and TNF-α levels were significantly higher in the apical periodontitis lesions compared to control samples.

Conclusion: The PD-1, PD-L1, and CTLA-4 molecules are evident in apical periodontitis, and can be an important immune checkpoint in chronic periapical periodontitis.

Keywords: Chronic periapical periodontitis, cytokines, lymphocytes, PD-1, PDL-1

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HIGHLIGHTS

- Chronic inflammatory immune response leads to exhaustion or depletion of effector T cells.
- Recent studies have suggested that engagement of PD-1 with their ligands, PD-L1 and PD-L2, impairs effector T-cell function resulting in an exhaustion phenotype.
- The correlation between co-inhibitory receptors and T-cell exhaustion in chronic apical periodontitis has not been described.
- PD-1 and PD-L1 are expressed at higher levels by CD4+ T cells during chronic apical periodontitis.
- PD-1 signals may have an inhibitory effect on T-cell activation during chronic apical periodontitis.

INTRODUCTION

Root canal infection occurs because of tissue necrosis caused by the inflammatory response of the pulp to invading bacteria from caries or dental trauma (1). Infection is controlled by adaptive immune response, and unbalanced immune response is described as an important determinant in the disease outcome (2). Chronic infections in general are difficult to overcome because long inflammatory immune response leads to exhaustion or depletion of effector T cells (3). Failure to eliminate the pathogen results in loss of effector functions by T cells, such as reduced proliferative potential and cytokine secretion [i.e. interleukin (IL) 2, tumor-necrosis factor (TNF), and interferon gamma (IFN-γ)] (4). T-cell exhaustion has been described in many experimental models of disease and in humans with chronic infections or cancer (4). The most well defined feature of exhausted T cells is their expression of multiple co-inhibitory receptors, which in turn highly correlates with their degree of unresponsiveness (5). Co-inhibitory molecules associated with exhausted T cells include programmed death protein 1 (PD-1), TIM3, CTLA-4, BTLA, CD160, LAG3, and 2B4 (5).

PD-1 is a negative regulator of T-cell activation (6), and is a characteristic marker of exhausted T cells during chronic infections (4, 7). Blockade of PD-1 has been suggested as an approach to enhance T-cell responses and control viral infections (8, 9). PD-1 is expressed on $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells, B cells, and activated monocytes. It negatively regulates T-cell receptor signals (10). PD-1 interacts with the B7H1 (programmed death ligand 1, PD-L1) and B7-DC (programmed death ligand 2, PD-L2) (3, 5, 10). PD-L1 is expressed by APCs, and its expression is upregulated by IFN-γ, IL-12, GM-CSF, and IL-4 (3, 5, 10). Upon engagement by its ligand (PD-L1 or PDL-2), the PD-1 signals lead to the inhibition of T-cell proliferation and downregulation of cytokine production (3, 5, 10). The PD-1 pathways play an important role in T-cell exhaustion during HIV infection (9). Blocking the PD-1 signaling during animal models improves survival (11). The PD-1 signaling has a critical role in limiting the effectiveness of antigen-specific T cells during other persisting infections and cancer (12). However, the correlation between PD-1 signals and T-cell exhaustion in lesions from patients with chronic apical periodontitis has not been described.

Considering apical periodontitis as a long-lasting inflammatory disease, it seems reasonable to hypothesize the mechanisms involved with occurrence of exhausted T lymphocytes at such lesions. The purpose of this study was to analyze the potential participation of PD-1 in the development of human chronic apical periodontitis. Our hypothesis was that bacterial persistence and chronic pulp infection is facilitated by exhaustion of T cells that express the inhibitory receptor PD-1.

MATERIALS AND METHODS

Patients with apical periodontitis and healthy volunteers

This study was approved by the Institutional Review Boards at the University of São Paulo (School of Dentistry of Bauru). Written informed consent regarding the use of specimens was given by all volunteers. All studies were performed in accordance with the relevant guidelines and regulations. Sixteen patients with chronic apical periodontitis (six men and ten women; age range 41-69 years, mean age=58.42±2.25 years) participated in the study. Radiographic examination was performed to evaluate the presence of periapical pathology (13). Samples were collected from patients referred to the Endodontics Clinic of School of Dentistry of Bauru (FOB/USP) and Postgraduate Endodontics Clinic of CPO Uningá Bauru/Brazil for root canal therapy. Medically compromised patients (i.e. those using systemic antibiotics, anti-inflammatory drugs, and hormone therapy) and patients with preexisting conditions such as periodontal disease, and pregnant or lactating women were excluded from the study. At the time of surgery, one part of the tissue sample was sent to histopathological analysis, and the other part was sent to isolation of leukocytes and supernatant collection. The samples from 16 lesions were diagnosed as cysts (n=4) and granulomas (n=12). Samples of healthy periodontal tissues were used as control samples and taken from patients undergoing procedures unrelated to periodontal disease, such as the extraction of premolars for orthodontic reasons (n=10, five men and five women; age range 27-60 years).

Isolation of leukocytes

To characterize the leukocytes present in the lesion site, the samples of periodontal tissue collected from patients were incubated with 50 µg/mL collagenase (Boehringer Ingelheim Chemicals, São Paulo, Brazil) at 37°C for 60 min. One cycle of cellular dissociation were performed for 4 min using a Medimachine (BD Biosciences, CA, USA). The tissue homogenates were briefly centrifuged, and the cell suspension was passed through a 30 µm cell strainer using the plunger from a 2 ml syringe (BD Bioscience). All cell counts were determined using a Neubauer chamber. Dead cells were excluded on the basis of trypan blue staining (14).

Flow cytometry analysis

Single cell suspensions isolated from human periodontal tissue were used for flow cytometry analysis. Cells were stained with surface antibodies (eBiosciences, San Diego, CA, USA) as previously described (14). Fixed cell suspensions were collected using FACSCalibur flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ). Data were analyzed using CellQuest software (BD Biosciences).

ELISA

IFN-γ, TGF-β, and IL-10 were determined in whole periodontal tissue homogenates using a standardized sandwich ELISA technique, according to the manufacturer's instructions (BD Pharmingen Corp., San Diego, CA).

Statistical analysis

All results were expressed as mean±SEM. One-way ANOVA was used for statistical analysis of all experiments to determine the difference between the apical periodontitis lesions and healthy samples (controls). Data analysis was performed using the GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA). All values were considered significantly different at $P<0.05$.

RESULTS

Phenotypic characterization of leukocytes in the gingival tissue from patients with chronic apical periodontitis

Flow cytometry examination showed higher leukocytes numbers isolated from the apical periodontitis lesions when compared with healthy control gingival tissue (Fig. 1a). Analysis of apical periodontitis tissue confirmed that most leukocyte cells (75.3%) were $CD3^+$ T cells. Among $CD3^+$ cells, the proportion of $CD4^+$ T cells was higher in the apical periodontitis lesions compared to control samples (Fig. 1b). Results showed similar frequencies of $CD8^+$ T cells, B cells ($CD19^+$), and macrophages ($CD14^+$) in the lesions of apical periodontitis and control samples (Fig. 1b). Frequencies of $CD4^+$ T cells expressing CD45RO, CD25, and CTLA-4 were significantly higher in the apical periodontitis lesions than in cells from the control samples (Fig. 2a). In addition, only $CD8^+$ CTLA-4 frequencies were significantly higher in the chronic apical periodontitis lesions compared to cells from control samples (Fig. 2b). Interestingly, the CD28 expression was lower in $CD8^+$ T cells from chronic apical periodontitis. Our data indicate that apical periodontitis leads to higher amounts of infiltrating leukocytes in tissue, especially

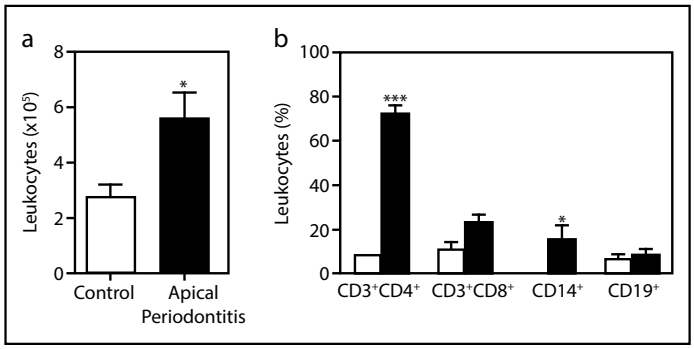

Figure 1. Apical periodontitis is associated with increased immune cell infiltration in lesions. The frequencies of total leukocytes, macrophages, and B and T cells were determined using immunostaining and FACS analysis. (a) The absolute number of leukocytes isolated of the control tissue (n=10, open bar) and apical periodontal lesions (n=10, closed bar). (b) Bars show the frequencies of T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$), B lymphocytes ($CD19^+$), or macrophages ($CD14^+$) isolated of apical lesions. Data are mean±SEM. *P<0.05, ***P<0.001

$CD4^+$ T cells, compared to healthy tissues. Isolated lymphocytes from apical periodontitis displayed activated phenotype in the collected samples.

PD-1 expression in leukocytes from patients with apical periodontitis

To investigate the possible participation of costimulatory molecules in T-cell exhaustion, we evaluated the expression of PD-1 in leukocytes isolated from healthy and chronic apical periodontitis tissues. In freshly isolated $CD4^+$ T cells, the expression of PD-1 was significantly higher (P<0.01) than in leukocytes from healthy gingival tissue (Fig. 3c). The percentage of $CD8^+PD\text{-}1^+$ T cells was higher in apical lesions than in healthy tissues (Fig. 3c). PD-L1 is a major co-inhibitory molecule identified on stromal cells of lymphoid and non-lymphoid organs (3). When we analyzed the PDL-1 expression, the results show higher frequencies of T cells and macrophages expressed PDL-1 in apical periodontitis samples (Fig. 3d). Moreover, macrophages showed higher PDL-1 expression than other immune cells analyzed.

Cytokines were increased in chronic apical periodontitis

Chronic infections support the induction of terminally differentiated T cells that shows reduced capacity to produce cytokine (15). The identification of cytokines profiles in chronic periapical periodontitis could provide clues that specific mediators are involved in the development of this chronic infec-

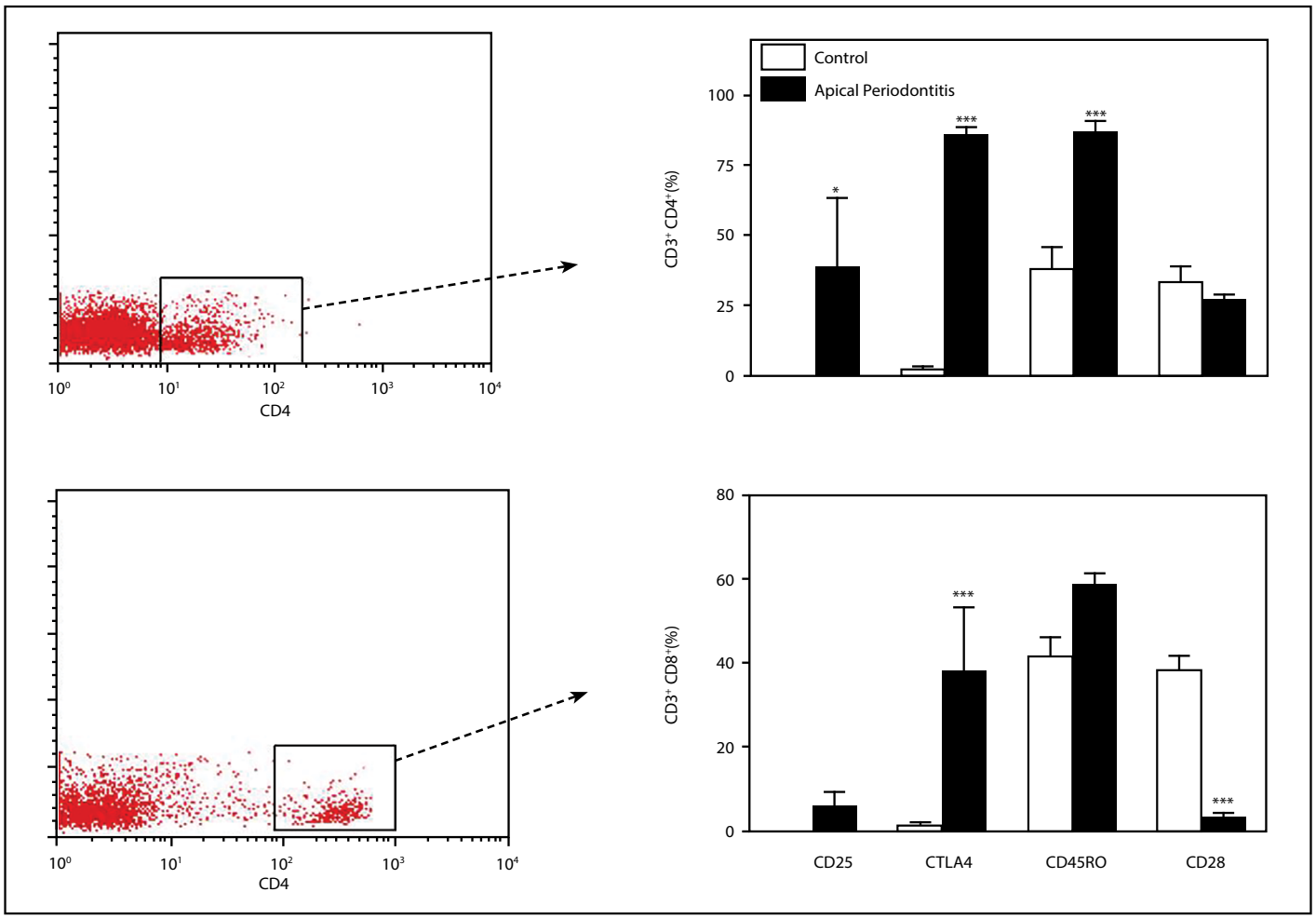

Figure 2. Phenotypic characterization of lymphocytes in the gingival tissue from patients with chronic apical periodontitis. (a) Bar graphs show expression of CD25, CTLA-4, CD45RO, and CD28 in $CD4^+$ T lymphocytes, and histograms show gated $CD4^+$ T cells isolated of apical lesions. (b) Bar graphs show expression of CD25, CTLA-4, CD45RO, and CD28 in $CD8^+$ T lymphocytes, and histograms show gated $CD8^+$ T cells isolated of apical lesions. Data are mean±SEM. *P<0.05, **P<0.01; ***P<0.001

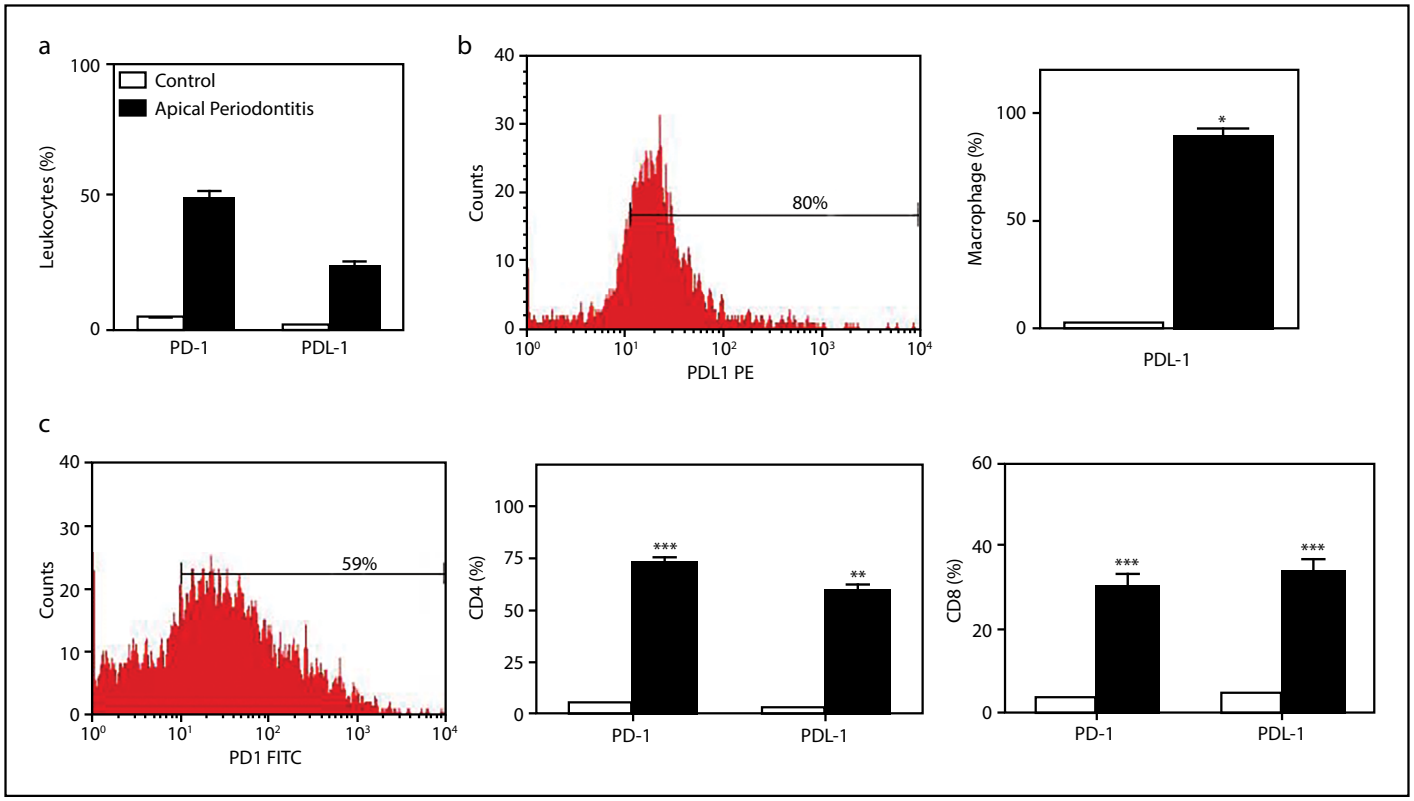

Figure3.Apicalperiodontitisisassociatedwithincreased PD-1expressioninCD4⁺lymphocytes.(a)Bargraphshowsexpressionof PD-1andPD-L1 intotalleukocytesisolatedfromlesions.(b)Bargraphshowsexpressionof PD-L1inmacrophages,andhistogramshows PD-L1expressioningated macrophages isolated from chronic apical periodontitis. Data are mean±SEM. P<0.05, **P<0.01; ***P<0.001

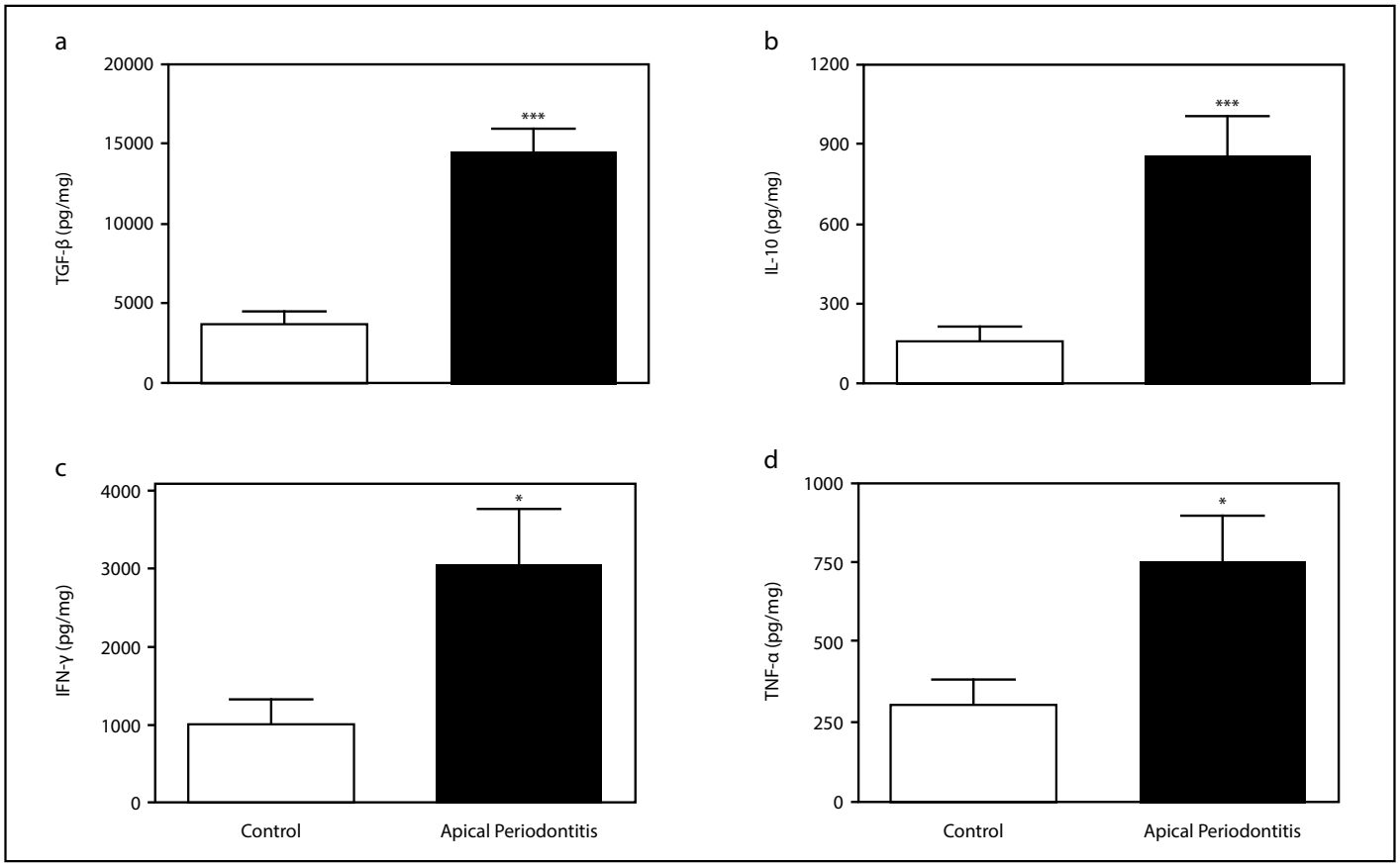

Figure4.Cytokinesinchronicapicalperiodontitis.TheIFN-γ,TNF-α,IL-10,andTGF-βlevelsweremeasuredinsupernatantsofgingivalsamples using the ELISA assay. Data are mean±SEM. P<0.05, **P<0.01; ***P<0.001

tion. Thus, we chose to determine the levels of cytokines in the apical periodontitis lesions. IL-10, IFN-γ, TNF-α, and TGF-β were detectable in the chronic apical periodontitis lesions, and the amount of TGF-β was significantly higher compared to the control (Fig. 4).

DISCUSSION

Apical periodontitis is an immune response to the bacterial content of infected root canals (1, 16, 17). These inflammatory lesions are infiltrates of lymphocytes, monocytes, macrophages, plasma cells, and mast cells. Among the mononuclear cells found in these tissues, lymphocytes are known as the prevailing cell type (16), especially T cells (17). It has been suggested that most T lymphocytes found in apical lesions are resting (18), which could be a result of the chronicity of persistent infection as apical periodontitis. The protective effector function of T cells may be compromised by inhibitory receptors such as CTLA-4 and PD-L1 (3, 5). Here, this study demonstrated that PD-1 and PD-L1 are expressed at higher levels by $CD4^+$ T cells during chronic apical periodontitis.

Alterations within the T-cell repertoire are present during the apical periodontitis progression (19). The absolute $CD4^+$ and $CD8^+$ T cell numbers are increased during disease progression (20), although the relatively higher increase in the $CD4^+$ subset may be observed (17). In accordance with these findings, we reported here the preferential accumulation of T $CD4^+$ in the apical periodontitis lesions. CD4 T cells play critical roles in mediating adaptive immunity to a variety of pathogens (21). However, higher numbers of $CD4^+$ T cells can result in exacerbated inflammation during chronic infections, suggesting that enhanced $CD4^+$ T cell responses should be sought carefully (22). Because the presence of T cells has been associated with the apical periodontitis lesions progression and chronicity (23), our data suggest the major infiltration of these cells in the apical periodontitis lesions may be related to the exacerbated inflammation and chronicity.

T-cell exhaustion is a state of dysfunction that commonly occurs during chronic infections due to the persistence of inflammation (6). Based on the current view, the exhausted phenotype of T cells in human apical lesions was investigated. Almost half of the leukocytes found on apical lesions expressed PD-1, whereas PD-L1 was detected on one-fifth of cells. However, not only T lymphocytes but also macrophages express PD-L1. In line with our results, in patients with chronic periodontitis, T cells expressed significantly higher levels of PD-1 either upon isolation or after culture with antigens (24). Recent studies revealed that the engagement of PD-1 with their ligands, PD-L1 and PD-L2, impairs effector T-cell function such as proliferation, cytotoxic activity, and cytokine production, resulting in an exhaustion phenotype that has been observed in different chronic infections, including cancer and HIV infection (9, 25, 26). This study demonstrated that PD-1 and PD-L1 are expressed at higher levels by $CD4^+$ T cells during chronic apical periodontitis. In the context of inhibitory molecules, a range of additional molecules such as CD25 and CTLA-4 are also upregulated on T cells in apical periodontitis, and may contribute to the phenomenon. When CTLA4 is upregulated, CD28 expression is subsequently downregulated by endocytosis (5). In fact, our results showed that higher frequencies of $CD8^+$ T cells do not express CD28. Similar phenotypes can be observed in T cells upon long-term exposure to tumor antigen (5). However, the mechanisms involved with the upregulation of inhibitory receptors by effector T cells during chronic apical infections have yet to be investigated. Cytokines play a key role in the pathogenesis periapical lesions. Cytokines from T helper 1 profile (IFN-γ and TNF-α) have been associated with lesion progression, whereas T helper 2 cytokines (IL-4, IL-10, and TGF-β) are described to attenuate the tissue damage (27). In this study, lesion supernatant showed increased levels of either anti-inflammatory TGF-β and IL-10 and modest amounts of proinflammatory IFN- γ and TNF-α. Although we did not observe a direct effect of the PD-1–PD-L1 pathway in this study, it is notable that IFN-γ, as a proinflammatory cytokine, could be induced even in the presence of higher PD-L1 expression by lymphocytes in chronic apical periodontitis. IFN-γ can also upregulate molecules that impair T-cell responses by interfering with metabolic pathways (28). Studies have suggested the existence of a correlation between the PD-1–PD-L1 pathway and the production of IL-10 (29). CTLA-4 binding may lead to TGF-β production (30). These data might explain the high levels of TGF-β in apical granulomas and radicular cysts (31). The presence of exhausted $CTLA4^+$ lymphocytes might be due to the upregulation of TGF-β apical periodontitis. PD1/PDL1 signals induced high levels of IL-10 production that in turn inhibited the function of $CD4^+$ T cells (32). The IL-10-mediated inhibition of $CD4^+$ T cell effector function was shown in chronic LCMV infection in mice (29). It should also be considered that in this context, not only lymphocytes but other leucocytes such as plasma cells and macrophages as well as resident cells such as endothelial cells and fibroblasts would be involved in cytokine production, which in turn proliferate because of chronic inflammatory processes (33, 34). However, because the antigenic source remains in contact with living tissues during apical periodontitis, it is reasonable to suggest that proinflammatory cytokine production will remain high because of the continuous exposure to bacterial products along the existence of the apical disease.

Limitations of these data include the small sample size of 16 patients. Furthermore, while data regarding costimulatory molecules expression were obtained from the apical periodontitis lesions, and this allowed us to compare correlation with T cells unresponsiveness, we could not observe a direct effect of the PD-1–PD-L1 pathway. Even with the above-mentioned limitation, our results demonstrate that PD-1 and CTLA-4, which are associated with T-cell exhaustion, are expressed in higher levels in chronic periapical periodontitis. This confirms our hypothesis that exhausted T lymphocytes are present in the apical periodontitis lesions.

CONCLUSION

This study showed that PD-1, PD-L1, and CTLA-4 molecules are detected in the apical periodontitis lesions and can be an important immune checkpoint in chronic periapical periodontitis. The contribution of costimulatory molecules to the immunopathogenesis of chronic apical periodontitis needs to be be investigated further.

Disclosures

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethics Committee Approval: CAAE number: #02084712.7.0000.5417

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