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Factors involved in the T helper type 1 and type 2 cell commitment and osteoclast regulation in inflammatory apical diseases

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Introduction: Periapical chronic lesion formation involves activation of the immune response and alveolar bone resorption around the tooth apex. However, the overall roles of T helper type 1 (Th1), Th2, and T-regulatory cell (Treg) responses and osteoclast regulatory factors in periapical cysts and granulomas have not been fully determined. This study aimed to investigate whether different forms of apical periodontitis, namely cysts and granulomas, show different balances of Th1, Th2 regulators, Treg markers, and factors involved in osteoclast chemotaxis and activation.
Methods: Gene expression of these factors was assessed using quantitative real-time polymerase chain reaction, in samples obtained from healthy gingiva (n = 8), periapical granulomas (n = 20), and cysts (n = 10).
Results: Periapical cysts exhibited a greater expression of GATA-3, while a greater expression of T-bet, Foxp3, and interleukin-10 (IL-10) was seen in granulomas. The expression of interferon-γ, IL-4, and transforming growth factor-β was similar in both lesions. Regarding osteoclastic factors, while the expression of SDF-1α/CXCL12 and CCR1 was higher in cysts, the expression of RANKL was significantly higher in granulomas. Both lesions exhibited similar expression of CXCR4, CKβ8/CCL23, and osteoprotegerin, which were significantly higher than in control.
Conclusion: Our results showed a predominance of osteoclast activity in granulomas that was correlated with the Th1 response. The concomitant expression of Treg cell markers suggests a possible suppression of the Th1 response in granulomas. On the other hand, in cysts the Th2 activity is augmented. The mechanisms of periradicular lesion development are still not fully understood but the imbalance of immune and osteoclastic cell activity in cysts and granulomas seems to be critically regulated by Treg cells.

Key words: apical periodontitis; bone resorption; Th1; Th2; Treg cells

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Apical periodontitis is a periradicular tissue disorder caused by etiological agents of endodontic origin, which can result in chronic lesion formation with concomitant resorption of hard tissues and destruction of periradicular periodontal ligament (27, 39, 47). Receptor activator of nuclear factor-κB ligand (RANKL)-mediated osteoclastogenesis plays a pivotal role in bone resorption around the tooth apex. RANKL is required for differentiation and activation of osteoclasts, whereas the decoy receptor osteo-

protegerin (OPG) inhibits it (5, 19, 35, 51). The upregulated expression of RANKL in periapical lesions is correlated with lesion expansion (15, 44). Moreover, an imbalance in RANK–RANKL–OPG levels has been observed in several other pathologies, such as osteoporosis, osteopetrosis, rheumatoid arthritis, periodontal diseases, and altered tooth eruption (5, 17, 35, 41, 44). Factors other than the RANKL system, such as the chemokines stromal cell-derived factor (SDF)-1α/CXCL12 and β-chemokine CKβ8/CCL23 (46) which are chemotactic for osteoclast precursor cells and their respective receptors CXCR4 and CCR1, are also involved in the bone loss process by guiding osteoclast precursors cells from the bone marrow to sites of resorption, where they undergo fusion and differentiation (4, 11, 46, 50, 52).

The interaction between the immune system and the skeletal system has been intensively studied in many diseases. The presence of abnormal and prolonged activation of the immune system in some diseases such as rheumatoid arthritis and periodontitis has been described, suggesting the distinct activation of the immune system in bone destruction. In general, immune responses to bacteria are considered to be a host-protective mechanism against pathogenic bacteria. In inflamed periapical sites, as in other inflammatory diseases, two patterns of response can be generated, the T helper type 1 (Th1) and Th2 responses, characterized by the production of interleukin-2 (IL-2), IL-12, and interferon-γ (IFN-γ), and by IL-4, IL-5, IL-6, IL-10, and IL-13, respectively (12). Evaluating the chemokine and chemokine receptors expression, a concomitance of the Th1 and Th2 patterns has been shown in cysts and granulomas (36, 37). On the other hand, experimental models suggest a hierarchy of Th2 cytokines in the immunomodulation of apical periodontitis, because the absence of Th1-type cytokines (IFN-γ and IL-12), does not interfere with lesion development (32), whereas deficiency of the Th2 cytokines IL-6 (3) and IL-10 (33) increases the extension of apical lesions. Other studies, suggest that the Th2 response seems to be dominant in human periapical regenerating lesions, while in apical granulation tissues, the Th1 response is predominant (7, 13).

Accordingly, it has been shown that the development of a Th1/Th2 balance can be regulated by transcription factors (40). T-bet, expressed by T cells, natural killer cells, B cells, monocytes/macrophages, and dendritic cells (21, 40) appears to induce Th1 development. Conversely, GATA-3 is a transcription factor that is highly expressed in T cells and that promotes Th2 while inhibiting Th1 differentiation (20, 26, 54).While Th1/Th2 responses are induced by cytokines, both types of effector responses are regulated by a heterogeneous family of cells, which are known as regulatory T (Treg) cells. They form a subset of 5–10% of $CD4^+$ T cells and were originally characterized on the basis of CD25 expression (31). More recently, it was shown that expression of the forkhead transcription factor (Foxp3) is both required and sufficient for the regulatory phenotype, so it appears to be the master regulator of the Treg cell lineage (9, 29). Both Th1 and Th2 responses can be suppressed by Treg cells through contact-dependent mechanisms and/or the production of IL-10 and transforming growth factor-β (TGF-β) (28).

Controversy surrounds the balance of factors involved in T-cell regulation and activation of bone resorption in periapical cysts and granulomas. The present study was undertaken to examine whether there is a different balance of Th1 (T-bet and IFN-γ) regulators, Th2 (GATA-3, IL-10 and IL-4) regulators, Treg marker and product (Foxp3 and TGF-β), and factors involved in osteoclast chemotaxis and activation (SDF-1α/CXCL12, CXCR4, CCR1, CKβ8/CCL23, RANKL, and OPG) in human inflammatory chronic endodontic infections.

Materials and methods

Human subjects

Healthy gingival tissues ($n = 8$) and periapical tissues ($n = 30$) were obtained as previously described (37). The Committee of Ethics in Research of the School of Dentistry of Ribeirão Preto, University of São Paulo, approved the study (Protocol number 2003.1.1120.58.6) and informed consent was obtained from all patients. All the subjects of this study were adult patients with radiographic evidence of periapical alveolar bone loss and indication for tooth removal who had been referred to the Faculties of Dentistry of the University of São Paulo and of the University of Ribeirão Preto. The mean age of patients was 45 years with a range from 32 to 60 years. The patients had not taken any medication for 2 months prior to the surgery and were apparently free of systemic diseases. Only two cases were endodontically treated. All cases were free of symptoms. The specimens from 30 lesions were diagnosed as cysts ($n = 10$) and granulomas ($n = 20$) according to the presence of fully developed cavities lined by stratified squamous epithelium with variable thickness and a fibrous capsule in cysts, whereas periapical granulomas presented a mass of granulation tissue with numerous inflammatory cells and an absence of epithelial lining surrounding the tooth apex. The control group comprised eight samples of clinically healthy gingiva taken during the removal of third molars.

Tissue preparation

The samples obtained from patients were divided into two equal parts. Half of each specimen was immersed in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's instructions. Tissue samples were immersed and homogenized in 1 ml TRIzol reagent; after incubation for 10 min at room temperature in an RNase-free tube, 0.2 ml chloroform was added. After centrifugation at 12,000 ***g*** for 15 min at 4°C, the aqueous phase was transferred to a fresh RNase-free tube and RNA was precipitated by mixing it with 0.5 ml isopropyl alcohol and centrifuging at 12,000 ***g*** for 15 min at 4°C. The RNA precipitate was washed once with 1 ml 75% ethanol and centrifuged at 12,000 ***g*** for 15 min at 4°C. Finally, the sample was resuspended in 50 μl RNase-free diethylpyrocarbonate (DEPC) water.

The second half of the sample was fixed in neutral-buffered formalin, embedded by a routine technique in paraffin wax, and sectioned at 7 μm for hematoxylin & eosin staining.

Real-time polymerase chain reaction

Complementary DNA (cDNA) was synthesized using 2 μg RNA through a reverse transcription reaction (Superscript II, Invitrogen). Real-time polymerase chain reaction (PCR) quantitative messenger RNA (mRNA) analyses were performed in an ABI Prism 5700 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantification of amplicons. The standard PCR conditions were 95°C (10 min), and then 40 cycles of 94°C (1 min), 58°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of human primers were designed using the PRIMEREXPRESS software (Applied Biosystems) based on nucleotide sequences present in the GenBank database. The primer sequences,

Table 1. Sequences used in this study

Gene	Sense and antisense	At (°C)	Mt (°C)	bp
β-Actin	5′- GCT CGT CGT CGA CAA CGG CTC -3′ 5′- CAA ACA TGA TCT GGG TCA TCT TCT C -3′	56	75	195
GATA3	5′- GGCGCCGTCTTGATACTTTCA -3′ 5′- AGATTGCGTTGCTCGCTCTGT-3′	58	78	152
T-bet	5′- AACCCAGTTCATTGCCGTGAC -3′ 5′- TGGACTCAAAGTTCTCCCGGA -3′;	58	78	104
FOXP3	5′- CCCACTTACAGGCACTCCTC -3′ 5′- CTTCTCCTTCTCCAGCACCA -3′	60	85	125
IL-10	5′- AGA TCT CCG AGA TGC CTT CA -3′ 5′- ATT CTT CAC CTG CTC CAC GG -3′	58	85	307
TGF-β	5′- ATT GAG GGC TTT CGC CTT AG -3′ 5′- TGT GTT ATC CCT GCT GTC ACA -3′	60	82	111
IL-4	5′- GCG ATA TCA CCT TAC AGG AG -3′ 5′- TTG GCT TCC TTC ACA GGA CA -3	58	82	308
IFN-γ	5′- ATG CAG AGC CAA ATT GTC TCC -3′ 5′- AGG CAG GAC AAC CAT TAC TGG -3′	58	77	501
CCR-1	5′- CCT TCT GGA TCG ACT ACA AGT T -3′ 5′- GTA GCA GAT GAT CAT GAC CAA C -3′	60	81	200
SDF-1α/CXCL12	5′- AGA GAT GAA AGG GCA AAG AC -3′ 5′- CGT ATG CTA TAA ATG CAG GG -3′	60	79	118
CXCR4	5′- TCA TCT TCT TAA CTG GCA TTG T -3′ 5′- AAA GAT GAA GTC GGG AAT AGT C -3	65	85	162
CKβ8/CCL23	5′- TTG TTA CTG CCC TTG GAT CCC -3′ 5′- TGG AGC ACT CGC TGT TCG TTT -3′	60	82	186
RANKL	5′- CAG AAG ATG GCA CTC ACT GCA -3′ 5′- CACCATCGCTTTCTCTGCTCT -3′	65	73	203
OPG	5′- GGA ACC CCA GAG CGA AAT ACA -3′ 5′- CCT GAA GAA TGC CTC CTC ACA -3′	57	77	225

At, amplification temperature; Mt, melting temperature; bp, base pairs.

amplification and melting temperatures, and the predicted amplicon sizes (in base pairs) used are presented in Table 1. PCR conditions for each target were conscientiously optimized with regard to primer concentration, absence of primer dimer formation, and efficiency of amplification of target genes and housekeeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 400 nM specific primers and 2.5 ng cDNA were used in each reaction. The relative levels of gene expression were calculated according to the instructions in the User's Bulletin (P/N 4303859) from Applied Biosystems, by reference to the β-actin in the sample, using the cycle threshold (Ct) method. Negative controls without RNA and without reverse transcriptase were also performed. The results show one experiment representative of three.

Statistical analysis

Data were analyzed using the Kruskal–Wallis test followed by Dunn's test and by multiple and simple regression analysis.

Results

Osteoclast chemotaxis and activation

Regarding osteoclastic regulators, the expression of all evaluated factors was higher in both lesion types compared to control samples ($P < 0.01$). The expression of RANKL was significantly higher in granulomas than in cysts ($P = 0.006$). On the other hand, the expression of SDF-1α/CXCL12 ($P = 0.04$) and CCR1 ($P = 0.014$) was higher in cysts. Both types of lesion exhibited a similar expression of CXCR4, CKβ8/CCL23, and OPG (Fig. 1). The RANKL : OPG ratio was slightly higher in granulomas (1.27) in comparison with cysts (0.91) but no statistical significance was reached ($P = 0.177$).

Expression of Th1 and Th2 regulators

The assessment of mRNA expression revealed significant levels of Th1 (IFN-γ) and Th2 (GATA-3 and IL-4) regulators in periapical cysts and granulomas in relation to control samples. However, the expression of the Th1 regulator, T-bet, in periapical cysts was similar to that in controls (Fig. 2). Periapical cysts exhibited a greater expression of GATA-3 ($P = 0.001$), while a greater expression of T-bet ($P = 0.05$) was seen in granulomas. Accordingly, the T-bet : GATA-3 ratio was significantly higher in granulomas (0.96) than cysts (0.50) ($P = 0.05$). The expression of IFN-γ and IL-4 was similar in both lesions (Fig. 2).

T regulatory cells

Greater expression of Foxp3 was detected in granulomas in comparison with cysts ($P = 0.012$). The expression of IL-10 was also higher in granulomas than cysts ($P = 0.009$). However, a similar expression of TGF-β was observed in both lesions (Fig. 3).

Discussion

Apical periodontitis is characterized by a chronic inflammatory infiltrate that can result in destruction of the periapical tissue of the affected teeth. The alveolar bone resorption around the tooth apex involves production of direct regulators of osteoclastic activity (RANKL and OPG) and osteoclastic chemotactic factors and receptors (5, 19, 35, 51). We found that periapical lesions display significantly high levels of RANKL and OPG compared with clinically healthy periodontal tissues, in accordance with previous studies (30, 43, 44). Furthermore, when comparing cysts and granulomas, we observed a higher expression of RANKL than OPG in granulomas, resulting in a greater RANKL : OPG ratio in granulomas compared with cysts. In contrast, previous results using immunohistochemistry showed higher numbers of OPG-positive than RANKL-positive cells in granulomas but a similar RANKL : OPG ratio in both type of lesions (25). Considering the RANKL : OPG balance, our results suggest a greater resorptive activity in granulomas. On the other hand, after analyzing the expression of osteoclast chemotactic factors, we verified a higher expression of CCR1 and SDF-1α/CXCL12 in periapical cysts, although no difference was observed in CKβ8/CCL23 and CXCR4 expression when both type of lesions were compared. We previously demonstrated a more prominent expression of CCR5 and CCR2 in cysts, while the expression of macrophage inflammatory proteins 1α and 1β, RANTES, and monocyte chemoattractant protein-1 were similarly expressed in both lesion types (36, 37). These results suggest that different osteoclastic chemotactic pathways may be activated in cysts and granulomas. Despite the role of chemokines in osteoclast differentiation, their activation seems to occur only in the presence of RANKL.

When the dental pulp is invaded by bacteria, the root canal provides the habitat for a mixed microbiota that leads to an inflammatory response at the periapex. This response largely prevents microbial

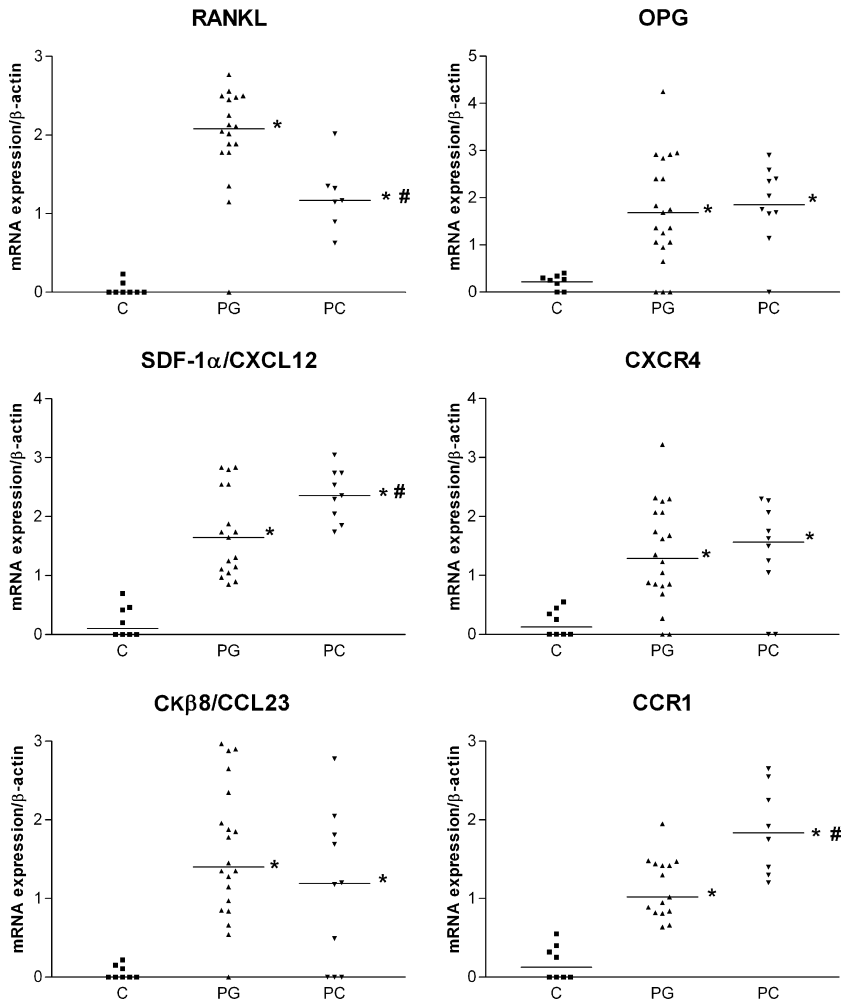

Fig. 1. Expression of osteoclastic regulators in human periapical cysts and granulomas. Total RNA from healthy gingival tissues (C), periapical granulomas (PG), and cysts (PC) was extracted and the expression of RANKL, OPG, SDF-1α/CXCL12, CXCR4, CKβ8/CCL23, CCR1 was analyzed by real-time polymerase chain reaction. The messenger RNA expression was quantified as its ratio to β-actin. $^{*}P < 0.05$ compared to controls. $^{\#}P < 0.05$ comparing two lesion types.

invasion into the periapical tissues and T-cell populations take an important part in this process (27). Th1 and Th2 cells both differentiate from common T precursor cells, with transcription factors T-bet and GATA-3, key regulators of Th1 and Th2 differentiation, respectively (20, 21, 26, 40, 54). The impact of the Th1 and Th2 responses in bone resorption associated with periapical lesions is not fully understood and controversial results have been reported. Inflammatory bone resorption may be upregulated *in vivo* by Th1-type mediators, such as IFN-γ and downregulated by Th2-type mediators, such as IL-10 and IL-4 (14). On the other hand, it has been shown that both Th1 and Th2 may have inhibitory effects on bone loss (1). Recently a new T-cell polarization state (Th17) distinct from Th1 and Th2 was described. It has been reported that IL-17, a Th17-cell-derived cytokine is detectable in gingival crevicular fluid (45) and in the supernatants of inflammatory cells isolated from periapical lesions (8), suggesting that Th17 cells also regulate osteoclastogenesis, possibly through IL-17-mediated induction of RANKL on osteoclastogenesis-supporting cells (18, 34).

There is a common agreement on the Th2 cytokine hierarchy at the sites of experimental apical inflammation (3, 32, 33). However, the Th1/Th2 balance and mechanisms of T-cell regulation in the human periapical cysts and granulomas remain to be clarified. We showed that periapical cysts exhibit a greater expression of GATA-3, suggesting a predominance of the Th2 response in these lesions. Despite high levels of GATA-3 in cysts, the expression of the Th2 marker response, such as IL-4, was similar in both cysts and granulomas. Recent studies indicate that the overexpression of GATA-3 enhances the development of fibrosis (16). In agreement with this, the fibrogenic activity might be important for the development of the collagenous capsule seen in periapical cysts.

In the present study, the increased expression of RANKL and T-bet, a marker of the Th1 response, suggests that the Th1 response could be modulating RANKL expression and osteoclastogenesis in human granulomas. Intriguingly, levels of IFN-γ were similar in both lesions. Regarding the Th1/Th2 balance in human apical periodontitis, it has been previously shown that mononuclear cells from periapical tissues produce relatively high levels of IFN-γ, indicating the predominance of the Th1 immune response whereas the expression of Th2 cytokines (IL-4 and IL-10) is not detected (7). In agreement with this, the presence of IFN-γ-positive cells was observed in periapical granulation tissue while IL-4-positive cells were only detected in apical regenerating tissue (13). The reasons for this discrepancy are not known. However, it is possible that the different methodologies used to quantify cytokine levels could be an underlying reason. In our study, the use of mRNA analysis to quantify the expression of these molecules clearly showed that T-bet and IL-10 are increased in granulomas and that IL-4 and IFN-γ expression is similar in both lesions.

The increased expression of IL-10 and the Treg cell marker Foxp3 in granulomas compared with cysts suggests that this T-cell population is greater in granulomas. In line with this, we can hypothesize that IL-10 might be produced by Treg cells and take part in the mechanism of suppression by Treg cells of the Th1 response in granulomas, as previously demonstrated in others diseases (28). Furthermore, IL-10 decreases the activity of $CD4^{+}$ Th1-cell-associated alveolar bone loss *in vivo* (53), and may also play an opposite role to high levels of RANKL seen in granulomas. Although it has been shown that TGF-β and IL-4 have a role in Foxp3 expression (6, 10), in the present study, despite the high levels of Foxp3 in granulomas in relation to cysts, no difference regarding these molecules was observed. These discrepancies may be explained because the temporal requirement is difficult to fulfill with tissue specimens originating from human patients.

In contrast to other granulomatous diseases, in the periapical diseases there is a continuous polymicrobial antigenic source

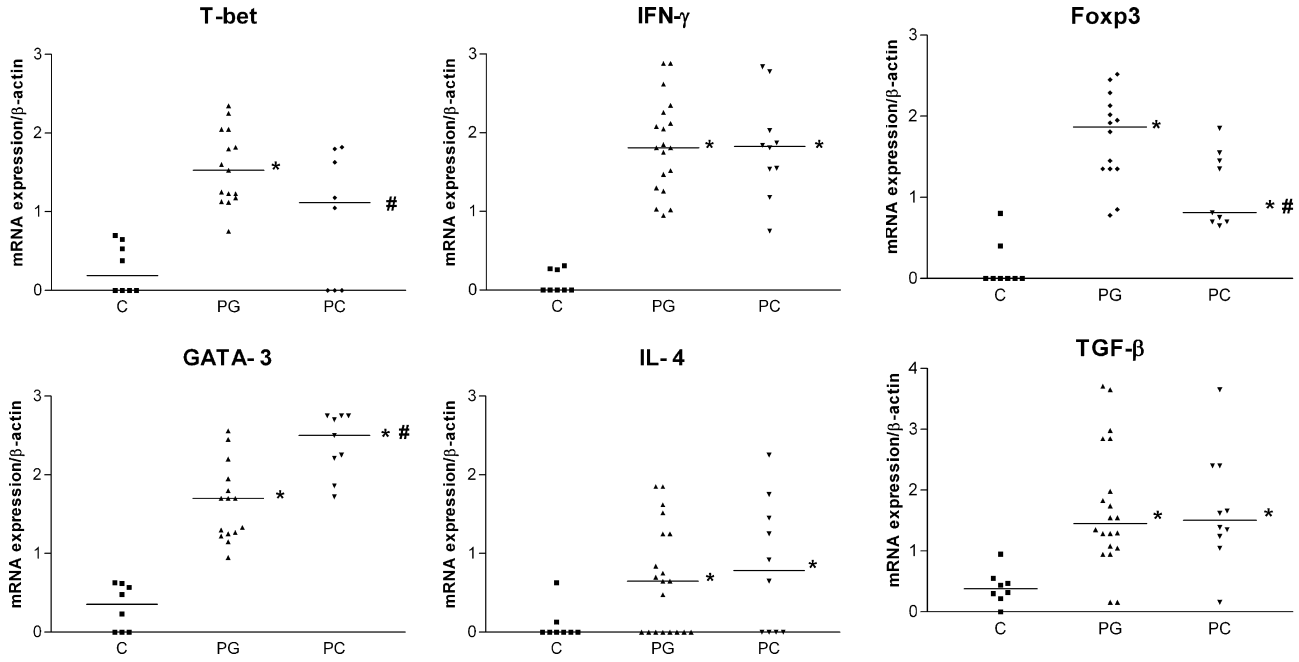

Fig. 2. Expression of T helper type 1 (Th1) and Th2 markers in human periapical cysts and granulomas. Total RNA from healthy gingival tissues (C), periapical granulomas (PG), and cysts (PC) was extracted and the expression of T-bet, interferon-γ (IFN-γ), GATA3, and interleukin-4 (IL-4) was analyzed by real-time polymerase chain reaction. The messenger RNA expression was quantified as its ratio to β-actin. $^{*}P < 0.05$ compared to controls. $^{\#}P < 0.05$ comparing two lesion types.

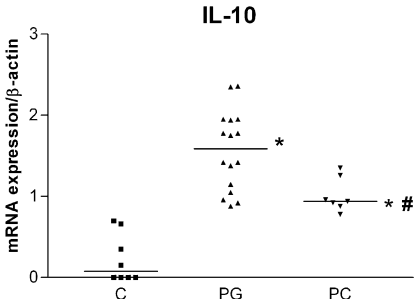

Fig. 3. Expression of regulatory T (Treg) cell markers in human periapical cysts and granulomas. Total RNA from healthy gingival tissues (C), periapical granulomas (PG) and cysts (PC) was extracted and the expression of Foxp3, transforming growth factor β (TGF-β) and interleukin-10 (IL-10) was analyzed by real-time polymerase chain reaction. The messenger RNA expression was quantified as its ratio to β-actin. $^{*}P < 0.05$ compared to controls. $^{\#}P < 0.05$ comparing two lesion types.

from the apex in the periapical environment that may shift the response. In this setting, even established human Th2 responses can be shifted, at least *in vitro*, to a Th1 profile by antigen stimulation in the presence of IL-12 (2). Conversely, the presence of IL-4 may shift Th1 responses to a less polarized phenotype, even in established Th1 responses, which seem to be less susceptible to redirection or immune deviation compared to Th2 responses (38). Furthermore, independent of the expression of individual cytokines, we believe that the balance between opposing factors, Th1 (IFN-γ) vs. Th2/Treg (IL-10), may determine the overall biological effect in the lesions. In accordance with this, a recent study found that the balance between RANKL and OPG, and not their individual expression values (24), was associated with the progressive or stable nature of periapical granulomas. Therefore, in scenarios with similar levels of IFN-γ, the increased expression of IL-10 in granulomas could account for the differential behavior of this lesion when compared to cysts.

Despite the role of the RANK–RANKL system in osteoclast differentiation and activity, it can also influence the development of the immune response. Indeed, RANKL expression is induced on activated T cells, and RANK expression can be found on dendritic cells (42, 48). Notably, dendritic cells can induce Treg cell proliferation and expansion as well as their development (49) with an important role displayed by RANKL (22). Interestingly, the greater levels of Foxp3 and RANKL were verified in granulomas. Moreover, RANKL suppresses the production of proinflammatory cytokines both *in vivo* and *in vitro* in response to stimulation by bacteria and their components (23).

Although the expression chemotatic factors for osteoclast precursor cells were higher in cysts, the expression of RANKL was significantly higher in granulomas. Our results showed a predominance of osteoclast activity in granulomas that was correlated with the Th1 response. The concomitant expression of Treg cell markers suggests a possible suppression of the Th1 response in granulomas. On the other hand, Th2 activity is augmented in cysts. The mechanisms of development of these periradicular lesions are still not fully understood, but the imbalance of immune and osteoclastic cell activity in cysts and granulomas seems to be critically regulated by Treg cells.

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