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Simultaneous analysis of T helper subsets (Th1, Th2, Th9, Th17, Th22, Tfh, Tr1 and Tregs) markers expression in periapical lesions reveals multiple cytokine clusters accountable for lesions activity and inactivity status

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ABSTRACT

Previous studies demonstrate that the balance between pro- and anti-inflammatory mediators determines the stable or progressive nature of periapical granulomas by modulating the balance of the osteoclastogenic factor RANKL and its antagonist OPG. However, the cytokine networks operating in the development of periapical lesions are quite more complex than what the simple pro- versus anti-inflammatory mediators' paradigm suggests. Here we simultaneously investigated the patterns of Th1, Th2, Th9, Th17, Th22, Thf, Tr1 and Tregs cytokines/markers expression in human periapical granulomas. Methods: The expression of TNF-α, IFN-γ, IL-17A, IL23, IL21, IL-33, IL-10, IL-4, IL-9, IL-22, FOXp3 markers (via RealTimePCR array) was accessed in active/progressive (N=40) versus inactive/stable (N=70) periapical granulomas (as determined by RANKL/OPG expression ratio), and also to compare these samples with a panel of control specimens (N=26). A cluster analysis of 13 cytokine levels was performed to examine possible clustering between the cytokines in a total of 110 granulomas. Results: The expression of all target cytokines was higher in the granulomas than in control samples. TNF-α, IFN-γ, IL-17A and IL-21 mRNA levels were significantly higher in active granulomas, while in inactive lesions the expression levels of IL-4, IL-9, IL-10, IL-22 and FOXp3 were higher than in active granulomas. Five clusters were identified in inactive lesion groups, being the variance in the expression levels of IL-17, IL-10, FOXp3, IFN-γ, IL-9, IL-33 and IL-4 statistically significant (KW $p<0.05$). Three clusters were identified in active lesions, being the variance in the expression levels of IL-22, IL-10, IFN-γ, IL-17, IL-33, FOXp3, IL-21 and RANKL statistically significant (KW $p<0.05$). Conclusion: There is a clear dichotomy in the profile of cytokine expression in inactive and active periapical lesions. While the widespread cytokine expression seems to be a feature of chronic lesions, hierarchical cluster analysis demonstrates the association of TNF-α, IL-21, IL-17 and IFN-γ with lesions activity, and the association of FOXP3, IL-10, IL-9, IL-4 and IL-22 with lesions inactivity.

Keywords: Apical granuloma. Cytokine networks. Immunoregulation. Immunology.

INTRODUCTION

Periapical lesions triggered by bacterial infection of pulpal and endodontic environment are characterized by the destruction of mineralized tissues surrounding the root apex as a consequence of the local host response[19]. In this context, cytokines play a major role in the modulation of inflammatory immune responses within the periapical microenvironment, and, therefore, are critical determinants of lesions outcome[12,19]. Previous studies demonstrate that the balance between pro- and anti-inflammatory mediators determines the stable or progressive nature of periapical granulomas by modulating the balance of the osteoclastogenic factor RANKL and its antagonist OPG[12,29]. However, the cytokine networks operating in the development of periapical lesions are quite more complex than what the relatively simple pro- versus anti-inflammatory mediators' paradigm could suggest[5], being the pathogenesis of chronic inflammatory diseases influenced by several other cytokine classes[12,19].

In this scenario, Th1 cytokines (IFN-γ, IL-12) have been associated with bone destruction and lesion progression, while its classic Th2 antagonists (IL-4, IL-10, and the recently described IL-33) are described to limit or attenuate the tissue damage[19]. Beyond the Th1/Th2 archetype, Th17 cells emerged as a T subset with inflammatory properties involved in a series of infectious, autoimmune and osteolytic processes[41]. While the prototypical Th17 cytokine is IL-17, Th17 cells can also produce other effector cytokines with osteoclastogenic properties, such as IL-6 and IL-23, reinforcing the potential destructive role of Th17 subset in periapical lesions[41]. On the other hand, regulatory T cells (Tregs, a FOXp3+CD4+ subset) and Tr1 cells present suppressive effects on inflammatory osteolysis, thought to be mediated by cytokines such as TGF-β and IL-10[3,15,17]. Interestingly, the Th17/Tregs archetype was suggested to influence the outcome of periapical lesions[9,28].

Adding more complexity to the cytokine network in periapical lesions, Th9 and Th22 cytokines are expressed in human and experimental periapical lesions, where they are supposed to contribute to lesion stability[1]. IL-9 (the main Th9 product) and IL-22 (the Th22 signature cytokine) have been described as pleiotropic cytokines, whose pro- or anti-inflammatory activities may significantly differ depending on the overall cytokine milieu[5]. Other pleiotropic cytokines, such as IL-21 (a product of Th17 or T follicular helper [Tfh] cells), can also impact the overall immunoregulatory milieu, and also the osteoclastogenesis and bone resorption processes[25].

While previous studies describe the possible involvement of the mentioned cytokines in periapical lesions as a general rule, such mediators' expression have been investigated independently or in small sets[6,19,28], which does not provide a reasonable understanding of the whole cytokine network in periapical environment. Indeed, considering the notable interplay between the cytokines[5], only the simultaneous analysis of a broad cytokine panel can provide a picture of the overall immunoregulatory scenario operating at periapical lesions. Therefore, here we simultaneously investigated the patterns of Th1, Th2, Th9, Th17, Th22, Thf, Tr1 and Tregs cytokines/markers expression in human chronic periapical granulomas and their possible correlations with lesions activity pattern.

MATERIAL AND METHODS

Samples

This study had institutional review board approval of Bauru School of Dentistry, University of São Paulo. Patients presenting periapical lesions were referred to endodontic surgery after conventional root canal treatment failure; periapical lesions diagnosis was performed as previously described[29,30], based on histopathological and radiographic analysis, being periapical lesions characterized radiographically as rarefaction lesions with the disappearance of the periodontal ligament space and discontinuity of the lamina dura. Treatment failure was defined as the presence of periradicular radiolucency that did not resolve, persisting as before acceptable endodontic treatment (i.e. having all canals instrumented and obturated, with no voids in the obturation mass, the apical terminus of the obturation at 1/1.5 mm from the radiographic apex), or that increased in size with evidences of continuous bone resorption[2]. Periapical granulomas (N=110) were collected from patients (N=110, aged 19-59; 51 females and 59 males) during periapical surgery and divided in two roughly similar fragments and stored in both formalin (for routine histological examination; was performed after hematoxylin-eosin staining) and RNAlater (Ambion, Austin, TX) (for molecular analysis) solutions. Test samples were limited to granulomas, histopathologically defined by the presence of capillaries, inflammatory cells, macrophages, and without the presence of an epithelial lining. Periapical cysts, where cavities were further developed and lined by stratified squamous epithelium, and partially epithelized lesions (epithelized granulomas) were excluded from the study. Patients with medical conditions which need the use of systemic modifiers of bone metabolism or other assisted drug therapy (i.e. systemic antibiotics, anti-inflammatory medicines, hormonal therapy) during the last 6 months before the study were excluded. Patients with preexisting

conditions, such as periodontal disease, and pregnant or lactating women were also excluded. Healthy periodontal ligament tissue samples (N=26) obtained from premolars extracted for orthodontic purposes (patients aged 19-24 years, 12 females and 14 males) and stored in RNA later were used as control specimens. Lesions were also categorized into putative active (A) and inactive (I), based in the molecular profile of RANKL/OPG mRNA expression, as previously described[29].

RNA extraction and RealTime-PCR reactions

Samples were submitted to molecular analyses as previously described[30]. In brief, total RNA was extracted from samples by using the RNeasy kit (Qiagen Inc, Valencia, CA) according to the manufacturers' instructions. The integrity of RNA samples was checked by analyzing 1 µg of total RNA on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions. After RNA extraction, complementary DNA was synthesized by using 3 µg of RNA through a reverse transcription reaction using QuantiTectRT kit (Qiagen Inc, Valencia, CA). All cytokines/Th markers (TNF-α, IFN-γ, IL-17A, IL-23, IL-21, IL-33, IL-10, IL-4, IL-9, IL-22, FOXp3) mRNA levels were measured by means of RealTimePCR using TaqMan chemistry (Invitrogen, Carlsbad, CA) in a Viia7 instrument (LifeTechnologies, Carlsbad, CA) using inventoried optimized primers/probes sets (Invitrogen, Carlsbad, CA), with basic reaction conditions (40 cycles) at 95°C (10'), 94°C (1'), 56°C (1') and 72°C (2'). The analysis of RANKL and OPG mRNA levels were also determined in all the lesions (also by RealTimePCR using TaqMan chemistry), in order to categorize each sample in putative active and inactive lesions based on the RANKL/OPG ratio as previously described[29]. The results are depicted as the relative level of gene expression; calculated in reference to internal controls GAPDH and β-actin expression in each sample using the $2^{-DD}Ct$ method[2,16].

Data analysis

Cytokine expression data is presented as a mean of mRNA expression, normalized by reference to the housekeeping genes from triplicate measurements in each sample. Comparisons among controls, inactive and active lesions were performed by ANOVA followed by Bonferroni post-test (performed in GraphPad Prism5.0 software, GraphPad Software Inc, San Diego, CA, USA); being $p<0.05$ was considered statistically significant. In order to examine possible clustering between the cytokines, a cluster analysis of 13 cytokine levels in 110 periapical granulomas was performed by using the Spearman rank correlation coefficient and the Kruskal-Wallis (KW) test[44]. After clusters determination, a hierarchical analysis was performed to access cytokine clusters association with activity/inactivity status, considering 1) the degree of lesion activity, ranked from inactivity-to-activity based in the RANKL/OPG ratio, ranging from 0.43 to 4.46, in accordance with the initially defined clusters; 2) the variance in individual cytokine expression levels within all 8 clusters were analyzed from the statistical viewpoint in order to generate a heat-like map representing the relative levels of expression (i.e. clusters with relative low levels are represented by the color white, clusters with relatively homogenous expression levels are represented by the color yellow, and clusters with increased gene expression are depicted in red; comprising a 6 grade scale representative of 16.66% within each sequential color/degree); 3) in the final ranking step the cytokines were ordered based in the cumulative frequency of high/intermediate/low levels along the inactive/active poles.

RESULTS

The pattern of cytokine expression in active and inactive granulomas

The mRNA levels expression of all targets investigated was found to be higher in total periapical granulomas when compared to controls (Figure 1). When lesions were compared based in the RANKL/OPG expression pattern[29], 40 samples were found to be active (RANKL>OPG), while 70 presented an inactive lesion profile (RANKL≤OPG) (Figure 1). When active and inactive lesions were compared, TNF-α, IFN-γ, IL-17A and IL-21 mRNA levels were significantly higher in active granulomas (Figure 1), while in inactive lesions the expression levels of IL-4, IL-9, IL-10, IL-22 and FOXp3 were higher than in active granulomas (Figure 1). The levels of IL-23 and IL-33 in active and inactive lesions were similar from a statistical viewpoint (Figure 1).

Cluster and hierarchical analyzes of cytokine association with active and inactive lesions

In the view of the distinctly divergent cytokine profile between controls and lesions, cluster analysis was only performed with the lesion data in order to avoid biased associations. Initial cluster analysis performed with all (active + inactive) the lesions (Figure 2) resulted in the identification of 2 major clusters, comprised by 41 and 69 samples, presenting a 98% match with the clustering based in the RANKL/OPG patterns[29]. Considering such clear dichotomy, we performed additional cluster analysis within active and inactive lesions groups. In inactive lesions group (Table 1), 5 clusters were identified, being the variance in the expression levels of IL-17, IL-10, FOXp3, IFN-γ, IL-9, IL-33 and

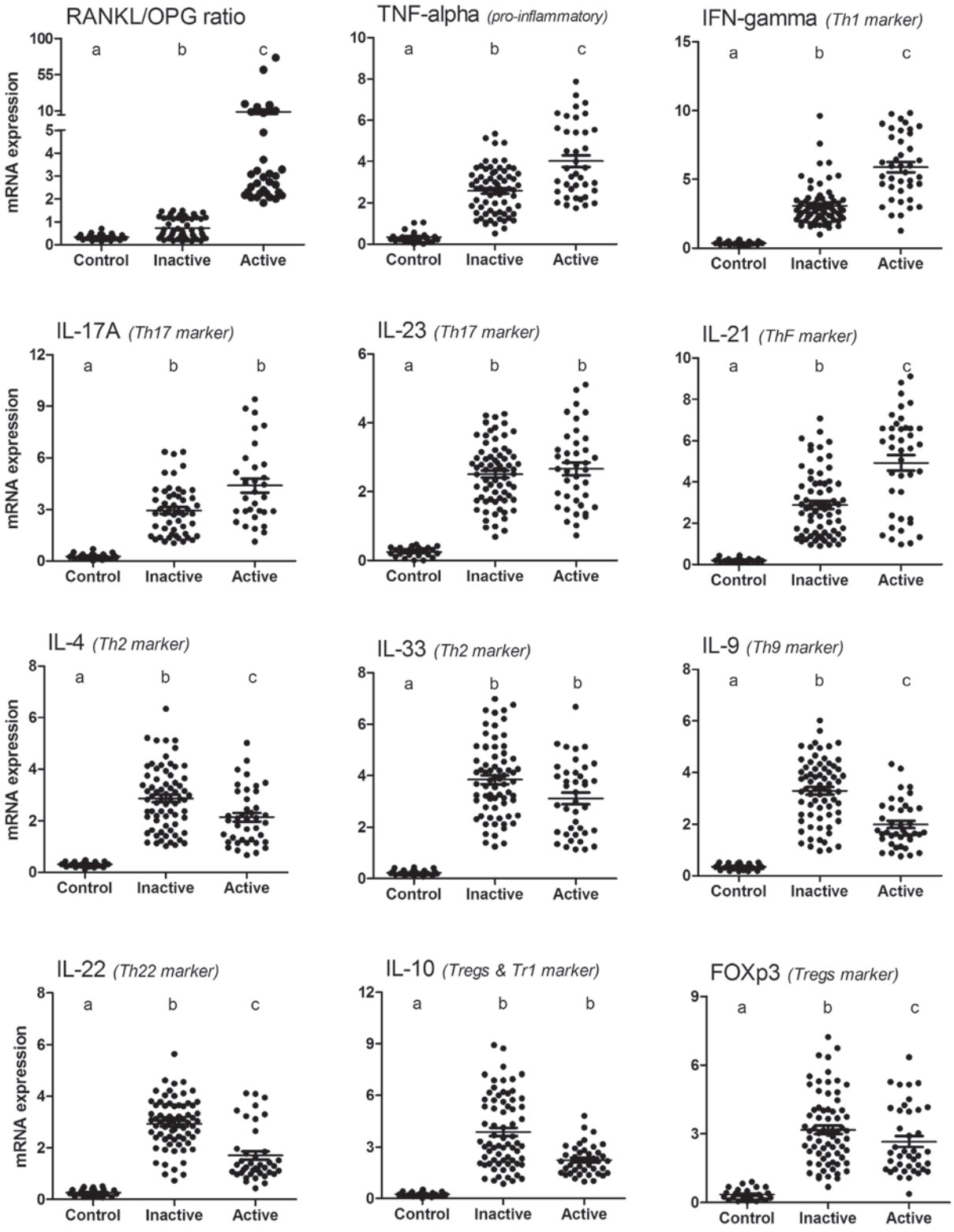

Figure 1 - Expression of individual mRNAs, with normalization to housekeeping genes, in periapical granulomas. Total RNA was extracted from periapical granulomas (experimental groups, N=110) and periodontal ligament (control group, N=26), and levels of RANKL and OPG mRNA were measured quantitatively by RealTimePCR using TaqMan chemistry. Based in profile of RANKL/OPG expression29 the lesions were then categorized into active (RANKL>OPG) or inactive (RANKL»OPG and RANKL<OPG). Different letters (a, b, c) represent statistically significant differences among the respective groups ($P<0.05$; One-way ANOVA, Bonferroni post-test); *$P<0.05$ (unpaired t-test) represent statistically significant differences between controls and periapical lesions (active + inactive)

IL-4 statistically significant (KW $p<0.05$) among the clusters of inactive lesions, while IL-22, OPG, IL-23, TNF-α, RANKL and IL-21 levels presented KW p values higher than 0.05. When active lesions were analyzed (Table 2), 3 clusters were identified, being the variance in the expression levels of IL-22, IL-10, IFN-γ, IL-17, IL-33, FOXp3, IL-21 and RANKL statistically significant (KW $p<0.05$) among the active lesions clusters, while IL-9, IL-4, TNF-α, OPG and IL-23 levels presented KW p values higher than 0.05.

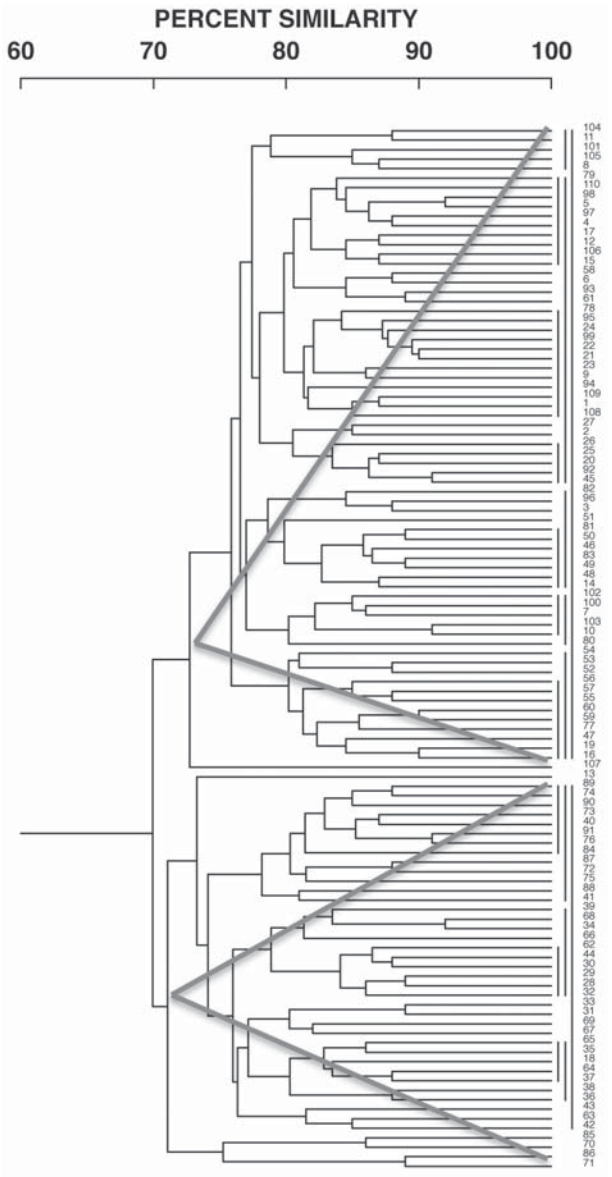

Figure 2- Patterns of cytokine expression in active and inactive periapical granulomas. Total RNA was extracted from periapical granulomas (N=110) and periodontal ligament control samples (N=26), and levels of TNF-α(classic pro-inflammmatory cytokine), IL-10 (Treg and Tr1 marker), IFN-γ(Th1 marker), IL-4 (Th2 marker), FOXp3 (Treg marker), CTLA4 (Treg marker), TGF-β (Treg and Th3 marker), IL-9 (Th9 marker), IL-17A (Th17 marker), IL-17F (Th17 marker), IL-21 (Th17 or Tfh marker), IL-23 (Th17 marker) and IL-22 (Th22 marker) were measured quantitatively by RealTimePCR using TaqMan chemistry. Based on the profile of RANKL/OPG expression29, the lesions were then categorized into active (RANKL>OPG) or inactive (RANKL≈OPG and RANKL<OPG)

The subsequent hierarchical analysis (Table 3 and Figure 3) ordered the samples regarding the activity level, from inactivity to activity ends, and demonstrated that inactivity pole was characterized by the highest OPG levels, sequentially followed in a downward way by FOXp3, IL-10, IL-9, IL-4 and IL-22. OPG and IL-22 expression profiles were relatively stable within inactive clusters; FOXP3 and IL-10 levels prevail in the clusters located in the inactivity pole edge, while a significant variation in the expression of IL-4 and IL-9 was verified in specific clusters (Table 3 and Figure 3). On the other hand, the lesion activity pole was characterized by the highest expression of TNF-α, downward followed by RANKL, IL-21, IL-17 and IFN-γ (Table 3 and Figure 3). High levels of TNF-α expression were a hallmark of all active clusters, RANKL expression prevails in the clusters located in the activity pole edge; also, a fairly specificity in the IFN-γ, IL-17 and IL-21 expression peaks was verified in definite clusters within active lesions (Table 3 and Figure 3). Interestingly, one cluster from inactive lesions subset presented a relatively high expression of IL-17. In an intermediate level within inactivity and activity poles, the cytokines IL-23 and IL-33 were not significantly associated with lesions' status (Table 3 and Figure 3).

DISCUSSION

Regulatory molecules such as cytokines play a key role in the pathogenesis periapical lesions[12,19]. Since the fragmented analysis of Th1, Th17, Th2, Th9, Th22 and Tregs related cytokines/markers expression suggests its involvement in periapical lesion development, but do not allow the analysis of the overall cytokine network operating in periapical environment; in this study we simultaneously investigated the patterns of such factors expression in active and inactive periapical lesions, as well the possible existence of cytokine clusters that could account for lesions outcome.

When the global cytokine expression profile in periapical lesions was compared with healthy control tissues, the expression of all cytokines/markers investigated was found to be significantly augmented in the lesions, in accordance with previous studies[1,6,9,10,19]. In a general context, the widespread cytokine expression in the lesions is interpreted as a reflex of the chronic host response to the unremitting infection in the root canal and periapical area[12,19]. While the dichotomous

comparison between health and disease conditions can be fairly revealing, it does not provide a disease severity and activity gradient, limiting the strength of such data to support more robust hypotheses. However, the comparison between active and inactive lesions[29,48] provides a better picture of clinical variance, and therefore provide the support necessary to more robust analyses.

Initially, considering the active lesions scenario, the expression of TNF-α, IFN-γ, IL-17 and IL-21 prevail in these lesions. TNF-α is classically described as a pro-inflammatory and osteoclastogenic cytokine[13,18]. Indeed, TNF-α was one of the first bone resorptive mediators identified in human periapical lesions, being its involvement in experimental periapical lesions progression clearly demonstrated in a cause-effect experiments[18]. Our data also demonstrated that the Th1-signature cytokine IFN-g was highly expressed in active lesions. While IFN-g is described to inhibit osteoclastogenesis *in*

Table 1- Five clusters (C1-C5) were identified in the inactive lesions group, being the variance in the expression levels of IL-17, IL-10, FOXp3, IFN-γ, IL-9, IL-33 and IL-4 statistically significant (KW p<0.05) among the clusters, while IL-22, OPG, IL-23, TNF-α, RANKL and IL-21 levels presented KW p values higher than 0.05

	inactive C1	inactive C2	inactive C3	inactive C4	inactive C5	KW p	ANOVA p
IL-17	4.10±1.34	2.60±1.00	4.98±0.91	2.70±1.11	1.75±0.79	0.00000	0.00000
IL-10	3.07±1.90	5.79±1.60	3.82±1.30	2.12±0.74	2.16±1.11	0.00000	0.00000
FOXp3	3.46±2.47	4.36±1.32	2.42±0.66	2.10±0.83	2.53±1.07	0.00000	0.00000
IFN	2.75±1.48	2.76±0.95	1.93±0.68	3.03±0.52	5.58±1.96	0.00000	0.00000
IL-9	3.03±1.88	3.45±0.80	2.69±1.15	4.21±0.97	1.84±0.86	0.00011	0.00000
IL-33	2.24±0.52	3.70±1.32	3.54±1.48	4.84±1.26	3.53±1.30	0.00157	0.00146
IL4	2.03±0.71	3.13±1.31	1.97±0.75	2.76±1.12	3.81±0.84	0.00252	0.00244
IL-22	4.18±1.11	2.94±0.88	3.07±0.60	2.58±1.02	2.78±0.92	0.09754	0.01919
OPG	1.07±0.54	0.78±0.41	0.93±0.49	0.80±0.41	0.60±0.26	0.40770	0.27883
IL-23	2.15±0.97	2.64±0.93	2.12±0.74	2.68±0.85	2.48±1.01	0.42409	0.40102
TNFα	1.91±0.55	2.59±0.98	2.92±0.68	2.56±1.49	2.61±1.42	0.49012	0.60985
RANKL	0.47±0.39	0.38±0.18	0.47±0.29	0.43±0.18	0.57±0.34	0.76167	0.36040
IL21	2.31±0.96	3.03±1.88	2.90±1.35	3.07±1.57	2.39±1.10	0.89569	0.73603
N	5	27	11	18	9	nd	nd

Table 2- Three clusters (C1-C3) where identified in the active lesions group, being the variance in the expression levels of IL-22, IL-10, IFN-γ, IL-17, IL-33, FOXp3, IL-21 and RANKL statistically significant (KW p<0.05) among the clusters, while IL-9, IL-4, TNF-α, OPG and IL-23 levels presented KW p values higher than 0.05

	active C1	active C2	active C3	KW p	ANOVA p
IL-22	1.13±0.30	3.30±0.79	1.45±0.77	0.00007	0.00000
IL-10	1.71±0.44	2.36±0.61	3.10±0.98	0.00022	0.00001
IFN	5.72±1.43	3.62±2.41	8.02±1.84	0.00029	0.00002
IL-17	4.74±1.74	5.54±0.75	3.14±0.92	0.00073	0.00111
IL-33	3.85±1.18	1.88±1.25	2.80±1.19	0.00135	0.00069
FOXp3	1.98±1.04	3.08±1.51	3.55±1.68	0.01007	0.00902
IL21	3.88±2.48	6.07±2.49	5.91±1.09	0.01426	0.01684
RANKL	1.43±0.65	1.16±0.44	0.86±0.35	0.03475	0.02563
IL-9	1.73±0.77	1.80±0.58	2.60±1.12	0.05429	0.02585
IL4	2.31±1.14	1.48±0.47	2.36±1.16	0.15877	0.10883
TNFα	4.34±1.80	3.76±1.97	3.63±1.56	0.40797	0.50789
OPG	0.32±0.23	0.36±0.16	0.30±0.19	0.55426	0.77663
IL-23	2.74±0.88	2.57±1.52	2.60±1.27	0.68514	0.91416
N	20	9	11	nd	nd

vitro, a clear association with increased bone loss is described *in vivo*, where the upregulation of TNF-α, IL-1β and RANKL overcome the direct anti-osteoclastogenic effect described *in vitro*[11,14,22,34,35]. Regarding periapical lesions, IFN-γ positive cells are found in both periapical granulomas and cysts, where they are supposed to be involved in periapical lesion development[6]. Additionally, the Th17-prototytical cytokine IL-17A, described as a potent inflammatory and osteoclastogenic factor, was also found in higher levels in active periapical lesions where it is supposed to exacerbate the inflammatory osteolytic process[7,28,33,41]. The last cytokine found to be overexpressed in active lesions is IL-21, a cytokine produced by Th17 and Tfh cells previously implicated in osteoclastogenesis and bone destruction[8,24]. Tfh, a CD4+ Tcell subset found in the B-cell follicles of secondary (and feasibly tertiary) lymphoid organs[27], is described as a major contributor to B cell-mediated antibody responses and an important source of IL-21[43]. Considering the chronic nature of periapical lesions and the abundant presence of B cells in such environment[26], it would be possible to suggest that IL-21 in periapical area contributes to a Thf-B cell response axis, similarly to what was described in tertiary lymphoid tissues associated with chronic infection sites[20,47]. Since B cells are described as a potential RANKL source[12,19], Thf-B cell axis can directly drive lesions activity via RANKL production.

Taken together, the discussed evidences suggest a role of TNF-α, IFN-γ, IL-17A and IL-21 in the periapical lesions progression. In order to clarify the possible interplays between such cytokines, cluster hierarchical analyses were performed and 3 distinct cytokine clusters were identified in active lesions subset. The maximum activity (i.e. higher RANKL/OPG ratio) cluster (active cluster 1) was characterized by the highest TNF-α and RANKL levels among active lesions, and relatively high IL-21, IL-17 and IFN-γ expression. In this high activity scenario, it is possible to suggest that local TNF-α and IL-21 activity favors the infiltration and activity of both Th1 and Th17 cells, as previously described in experimental arthritis[32]. Accordingly, when mice strains with opposing bone resorption susceptibility phenotypes were compared, parallel high levels of TNF-α and IFN-γ were associated with increased bone resorption activity[45,46]. However, a negative correlation between the relatively high levels of both IFN-γ and IL-17 was observed in all the active lesion clusters. Indeed, active cluster 2 was characterized by the highest levels of IL-21 and IL-17, while the active cluster 3 presented the highest IFN-g levels and a high IL-21 expression. Therefore, Th1- (active cluster 3) and Th17- (active cluster 2) biased clusters were evidenced within active clusters. Accordingly, previous studies demonstrate a reciprocal Th1/Th17 inhibition, suggesting that Th1 and Th17 mediators may be independently associated to the progression of inflammatory osteolytic lesions[4]. It is essential to consider that the hierarchical analysis points to Th1-biased cluster (active cluster 3) as lower activity cluster than the clusters with high Th17 activity (active clusters 1 and 2). Accordingly, while literature seems to present a relative consensus regarding the osteoclastogenic role of IL-17/Th17, the association of IFN-γ/Th1 with bone resorption process remains quite controversial[12,19]. Regarding IL-21, the levels of such cytokine were found to be elevated in both Th1- and Th17-biased clusters, but no direct positive/negative correlations were observed, suggesting that Tfh cells may operate in parallel (or even cooperatively) with both Th1 and Th17 mediators.

Table 3- Hierarchical analysis ordering the samples regarding the activity level, from inactivity to activity ends based on the RANKL/OPG profile

lesion status	inactivity							activity
RANKL/OPG	0.43	0.48	0.50	0.53	0.95	2.8	3.22	4.46
TNFα	1.91±0.55	2.59±0.98	2.92±0.68	2.56±1.49	2.61±1.42	3.63±1.56	3.76±1.97	4.34±1.80
RANKL	0.47±0.39	0.38±0.18	0.47±0.29	0.43±0.18	0.57±0.34	0.86±0.35	1.16±0.44	1.43±0.65
IL21	2.31±0.96	3.03±1.88	2.90±1.35	3.07±1.57	2.39±1.10	5.91±1.09	6.07±2.49	3.88±2.48
IL-17	4.10±1.34	2.60±1.00	4.98±0.91	2.70±1.11	1.75±0.79	3.14±0.92	5.54±0.75	4.74±1.74
IFN	2.75±1.48	2.76±0.95	1.93±0.68	3.03±0.52	5.58±1.96	8.02±1.84	3.62±2.41	5.72±1.43
IL-33	2.24±0.52	3.70±1.32	3.54±1.48	4.84±1.26	3.53±1.30	2.80±1.19	1.88±1.25	3.85±1.18
IL-23	2.15±0.97	2.64±0.93	2.12±0.74	2.68±0.85	2.48±1.01	2.60±1.27	2.57±1.52	2.74±0.88
IL-22	4.18±1.11	2.94±0.88	3.07±0.60	2.58±1.02	2.78±0.92	1.45±0.77	3.30±0.79	1.13±0.30
IL4	2.03±0.71	3.13±1.31	1.97±0.75	2.76±1.12	3.81±0.84	2.36±1.16	1.48±0.47	2.31±1.14
IL-9	3.03±1.88	3.45±0.80	2.69±1.15	4.21±0.97	1.84±0.86	2.60±1.12	1.80±0.58	1.73±0.77
IL-10	3.07±1.90	5.79±1.60	3.82±1.30	2.12±0.74	2.16±1.11	3.10±0.98	2.36±0.61	1.71±0.44
FOXp3	3.46±2.47	4.36±1.32	2.42±0.66	2.10±0.83	2.53±1.07	3.55±1.68	3.08±1.51	1.98±1.04
OPG	1.07±0.54	0.78±0.41	0.93±0.49	0.80±0.41	0.60±0.26	0.30±0.19	0.36±0.16	0.32±0.23
relative gene levels	low							high

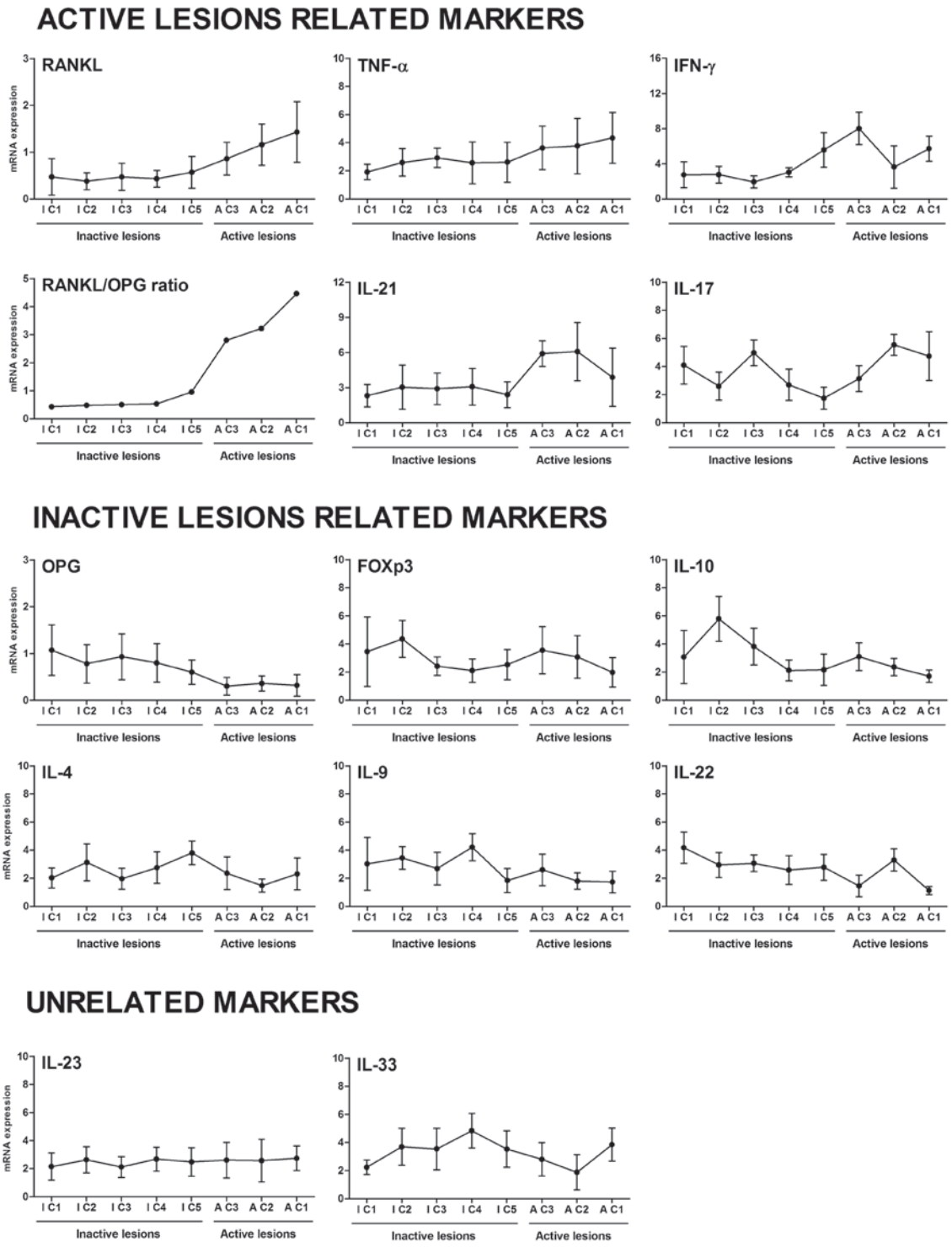

Figure 3- Patterns of cytokine expression in the clusters associated with active and inactive periapical granulomas nature. Hierarchical analysis demonstrated that the inactivity pole was characterized by the highest OPG levels, sequentially followed in a downward way by FOXp3, IL-10, IL-9, IL-4 and IL-22. OPG and IL-22 expression profiles were relatively stable within inactive clusters; FOXP3 and IL-10 levels prevail in the clusters located in the inactivity pole edge, while a significant variation in expression of IL-4 and IL-9 was verified in specific clusters. On the other hand, the lesions activity pole was characterized by the highest expression of TNF-α, downward followed by RANKL, IL-21, IL-17 and IFN-γ. High levels of TNF-α expression were a hallmark of all active clusters, and RANKL expression prevail in the clusters located in the activity pole edge; also, a fairly specificity in the IFN-γ, IL-17 and IL-21 expression peaks was verified in definite clusters within active lesions. Interestingly, one cluster from inactive lesions subset, presented a relatively high expression of IL-17. The cytokines IL-23 and IL-33 were not significantly associated with lesions' status

Moving the focus towards the putative determinants of lesions inactivity, IL-4, FOXp3, IL-10, IL-9 and IL-22 levels in inactive lesions overcome the levels observed in active sites. IL-4 represents the prototypic Th2 cytokine, being a potential protective mediator due its ability to upregulate OPG levels and suppress pro-inflammatory responses[40]. Besides IL-4, IL-33 also has been associated with Th2 responses and presents similar properties towards bone protective action, such as the inhibition of osteoclast differentiation[39]. Along Th2 responses, Tregs (characterized by the expression of FOXp3) and Tr1 cells are supposed to attenuate periapical lesions development[6,9]. Indeed, IL-10 (a characteristic product of both Tregs and Tr1 subsets) was previously detected in periapical lesions, where it inhibits inflammatory cells influx and bone resorption[30,38]. While the positive correlations observed between IL-10 and FOXp3 levels in inactive lesions reinforce that Tregs can be a significant source of IL-10 in periapical area, the lack of definitive Tr1 markers does not allow stronger assumptions regarding its possible involvement in this system. Besides IL-4 and IL-10, IL-9 and IL-22 were found to be overexpressed in inactive lesions, in accordance with previous data[1]. Th9 cells have been described to present an interesting plasticity, acting together with Th2 in some inflammatory processes or exerting immunosuppressive actions via IL-10 production[42]. IL-22 is also highly pleiotropic, since it can cooperate with IL-10 in a regulatory network that reduces the severity of experimental arthritis[37] or exerts pro-inflammatory effects by a synergistic action with classic pro-inflammatory mediators such as TNF-α of IL-17[49].

When potentially protective mediators associated with periapical lesions inactivity are scrutinized by the cluster hierarchical analysis, 5 distinct clusters were observed. The lowest activity pole is comprised by a cluster (inactive cluster 1) presenting the highest levels of OPG and IL-22, in parallel with relatively high levels of IL-10 and FOXP3 (the Tregs hallmark), comprising therefore a Th22/Tregs-biased cluster. While IL-22 can be a product of Th17 cells and operates in concert with IL-17 in inflammatory and autoimmune diseases[51], recent evidences demonstrate that when produced by Th22 cells in an milieu with low IL-17 levels (such as this specific cluster), IL-22 induce IL-10-mediated immunosuppressive effects[31]. Also, since Tregs are able to suppress Th17/IL-17-mediated responses[50], it is possible to suggest that Tregs favor IL-22/IL-10 axis via Th17 suppression. The subsequent cluster in a presumed ascending activity level (inactive cluster 2) is characterized by the highest levels of FOXp3 and IL-10, along relatively high levels of both IL-4 and IL-9; typifying a Treg/Tr1-biased cluster. The classic description of Tregs and Tr1 cells as significant sources of IL-10 was previously discussed, and supports the dominance of Tregs and Tr1 in determining such cluster inactivity via IL-10 production. Interestingly, it was recently demonstrated that the presence of FOXP3 is sufficient to suppress the expression of IL-22[21], which could account for the relatively low levels of IL-22 observed in this cluster. While such association contradicts the hypothesis of the Th22/Tregs- cluster previously discussed, we can consider that the relatively high IL-4 and IL-9 levels may be a reflex of a distinct T cell polarization in this cluster. Indeed, both Th2 responses (via Th2-chemokine CCL22 mediated Tregs chemoattraction) and IL-10 can contribute to the immunosuppressive response via IL-10[17,42].

The next inactive lesions cluster (inactive cluster 3) is characterized by a high IL-10 expression, relatively low FOXp3, IL-4 and IL-9 levels, and a singular high IL-17 expression. Initially considering the lack of a direct correlation/association between FOXp3 levels and IL-10, it is possible to hypothesize a dominant role for Tr1 instead of classic FOXp3+ Tregs in this cluster. Indeed, the high levels of IL-17 in parallel with the low levels of FOXp3 may be representative of a plastic behavior of Th17/Tregs cells, where environmental signals can limit Tregs suppressive activity[52]. Following the clusters ascending activity level, the subsequent cluster (inactive cluster 4) is characterized by the highest IL-9 and IL-33 levels. Since no evidences of collaborative actions between IL-33 and IL-9 are reported, it is reasonable to consider that these T cell subsets may exert independent roles in the determination of lesions inactivity. While the data regarding IL-9 and bone lytic process is scarce as previously discussed, its possible association with lesions inactivity relies on the possible association with IL-10 production[1,42]. Considering the potential protective role of IL-33, while its anti-osteoclastogenic action was recently described[23], IL-33 levels are similar in overall inactive/active lesions, weakening the hypothesis that IL-33 plays a major role as a determinant of periapical lesions inactivity.

Finally, in the edge between inactive and active lesion clusters, a Th2-biased cluster (inactive cluster 5) is characterized by the highest IL-4 levels within inactive lesions. IL-4 is usually described to limit or attenuate the tissue damage due its anti-inflammatory properties, which include the inhibition of RANKL, concomitantly with OPG upregulation[36], and the suppression of pro-inflammatory and Th1-type responses[6,19,35]. Recently, it was described that the Th2-type chemokine CCL22 (which can be induced by IL-4) attenuates periodontal lesions severity though Tregs chemoattraction[17]. However,

the relatively low levels of FOXp3 and IL-10 in this Th2 biased cluster, as well as the lack of negative correlations between IL-4 and IFN-g does not support such hypotheses. These results suggest that a dominant IL-4 response, in parallel to low levels of other potentially protective cytokines, may not be highly effective in determining lesions inactivity. Indeed, this IL-4-biased cluster is located in the frontier between inactive and active lesions, being its RANKL/OPG ratio roughly 2 times higher than the other 4 inactive clusters.

Taken together, our results demonstrate distinct patterns of cytokine expression in active and inactive periapical granulomas. In active lesions, pro-inflammatory Th1 and Th17 skewed clusters in concert with IL-21 are supposed to independently drive lesions progression. Conversely, inactive lesions present a more complex scenario, were Th22/Tregs, Tregs/Tr1, Tr1, Th9 and Th2 biased clusters can account for lesion inactivity status. However, further cause-and-effect studies are required to fully dissect the cytokine network involved in the pathogenesis of periapical lesions, aiming to unravel the protective and destructive pathways and therefore contribute to improve the diagnosis and treatment of these pathologies. However, further studies are required to support our hypothesis.

CONCLUSION

A clear dichotomy exists in the profile of cytokine expression in inactive and active periapical lesions. While the widespread cytokine expression seems to be a feature of such chronic lesions, hierarchical cluster analysis demonstrates the association of TNF-α, IL-21, IL-17 and IFN-γ (ordered by their supposed destructive potential) with lesions' activity, and the association of FOXp3, IL-10, IL-9, IL-4 and IL-22 (ordered in its supposed protective potential) with lesions' inactivity.

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