

**UNIVERSIDADE DE UBERABA**  
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**BALANÇO IMUNOREGULATÓRIO, TH17/TREG, EM  
CÍSTOS E GRANULOMAS PERIAPICAIS**

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre – Área de concentração Biopatologia, do Programa de Pós-Graduação em Odontologia da Universidade de Uberaba.

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## Resumo

**Introdução:** Cistos e granulomas são lesões periapicais crônicas que evoluem para conter uma infecção periapical que se formam sobre a interferência de um conjunto de mediadores inflamatórios. A IL-17 secretada pelas células Th17 e o TGF- $\beta$  produzido por células T reguladoras (Treg) tem efeitos opostos na modulação do processo inflamatório, embora não exerçam efeitos inibitórios mútuos. O TGF- $\beta$  na presença da IL-6 pode contribuir para o aparecimento da resposta Th17. Se o TGF- $\beta$  é um potente controlador da resposta imune, a IL-17 tem propriedade de reativar o processo inflamatório, que juntamente com as quimiocinas, poderiam estar contribuindo para a patogênese das lesões periapicais.

**Objetivo:** Foi analisado o tipo de infiltrado inflamatório, a presença de mastócitos, a expressão *in situ* das citocinas (IL-17, e TGF- $\beta$ ), das quimiocinas (CCL2 e CCL4), a expressão do fator de transcrição nuclear (FOXP3) em granulomas e cistos periapicais humano comparados com grupo controle.

**Materiais e métodos:** Foi analisado um total de 50 lesões (25 cistos e 25 granulomas periapicais). Para avaliar o tipo de infiltrado inflamatório foi realizada a coloração Hematoxilina/Eosina (HE) e para identificar os mastócitos foi realizada a coloração com o azul de toluidina. Citocinas, quimiocinas e a expressão do FoxP3 foram analisados pela técnica de imunohistoquímica indireta.

**Resultados:** O infiltrado inflamatório foi predominantemente constituído de mononuclear (MN). Nos cistos o infiltrado de MN foi significativamente maior em relação ao infiltrado misto (PMN/MN) (Teste exato de Fisher;  $p=0,04$ ) Nos casos em que o paciente apresentava fistula o infiltrado inflamatório misto foi significativamente maior (Teste exato de Fisher;  $p=0,0001$ ). O número de mastócitos foi significativamente maior nos granulomas do que nas lesões císticas (Manny Whitney;  $p=0,02$ ). O número de células positivas para IL-17 foi significativamente maior nos granulomas em relação aos cistos (Mann Whitney;  $p=0,01$ ). Ao comparar a expressão tecidual do TGF- $\beta$  entre granulomas e cistos periapicais, verificou-se que apesar dessa citocina estar mais expressa nos granulomas não houve diferença significativa em relação aos cistos (Mann Whitney;  $p=0,07$ ). Não foi observado diferença significativa ao comparar ambas as lesões (cistos e granulomas) com o número de células positivas para o FoxP3, CCL2 e CCL4.

Conclusões: Os mastócitos e a IL-17 devem participar na modulação do processo inflamatório nas lesões periapicais estabelecidas, principalmente nos granulomas periapicais. A IL-17 e o TGF- $\beta$  apresentam importantes efeitos moduladores na reativação das lesões periapicais crônicas.

Palavras chaves: citocinas, quimiocinas, mastócitos, cisto periapical, granuloma periapical.

## Abstract

**Introduction:** Cysts and granulomas constitute chronic periapical lesions which evolve to contain a periapical infection which is formed under the influence of a set of inflammatory mediators. IL-17 secreted by Th17 cells as well as TGF- $\beta$  produced by regulator T cells (Treg), do have opposite effects modulating the inflammatory process, although they do not exert reciprocal inhibitory effects. TGF- $\beta$  in the presence of IL-6 may contribute to the development of a response by Th17 cells. If TGF- $\beta$  is a potent cytokine to control the immune response, IL-17 is a powerful mediator reactivating the inflammatory process, which together with chemokines, could act contributing to the pathogenesis of periapical lesions.

**Objectives:** To evaluate and compare the type of inflammatory infiltrate, the presence of mast cells, in situ expression of cytokines (IL-17 and TGF  $\beta$ ), chemokines (CCL2 e CCL4), and the expression of nuclear transcription factor (FoxP3) in human granulomas and periapical cysts in an experimental as well as in a control group.

**Material and methods:** A sample of 50 lesions being, 25 cysts and 25 periapical granulomas were analyzed. In order to assess the type of inflammatory infiltrate, the method of hematoxylin/eosin staining was employed. On the other hand, the toluidine blue staining method was used to identify the mast cells. Cytokines, chemokines and FoxP3 expression were analyzed using an indirect immunohistochemical assay.

**Results:** Inflammatory infiltrate was predominantly constituted by mononuclear or monocytes cells (MNs). In cysts, a monocytes infiltrate (MNs) was significantly greater as related to a mixed infiltrate (PMNs/MNs) (Fisher's exact test,  $p=0.04$ , indicating a statistically significant difference). In those cases in which the tooth posses fistule, a mixed inflammatory infiltrate was significantly predominant (Fisher's exact test;  $p=0.0001$ , a extremely significant difference). The number of mast cells was significantly greater in the granulomas than in the cystic lesions (Mann-Whitney test;  $p=0.02$ , indicating a statistically significant difference). The number of positive cells for IL-17 was significantly greater in the granulomas when compared to the cysts (Mann-Whitney test;  $p=0.01$ , a statistically significant difference). When comparing tissue expression of TGF- $\beta$  between granulomas and periapical cysts, we found that although such cytokine showed a stronger expression in the granulomas, there was no statistically significant difference when comparing such expression to the cysts (Mann-



Whitney;  $p=0.07$ ). We observed no significant difference when comparing both types of periapical lesions (cysts and granulomas) with the number of cells positive for FoxP3, CCL2 e CCL4.

**Conclusions:** Both mast cells and IL-17, should have an active role in modulating the inflammatory process in chronic periapical lesions, mainly in the case of periapical granulomas. Both IL-17 and TGF-  $\beta$  present significant modulatory effects in reactivating chronic periapical lesions.

Key words: cytokines, mast cells, periapical cysts, periapical granulomas.

De acordo com o parágrafo § 42º do Regimento da Pró-reitoria de Pesquisa, Pós-graduação e Extensão da Universidade de Uberaba, a apresentação dessa Dissertação será realizada sob a forma de artigo científico. A organização do mesmo segue as normas da revista *Journal of Endodontics*.

**Th17/Treg immunoregulatory balance in human radicular cysts and periapical  
granulomas**

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## **Abstract**

**Introduction:** Cysts and granulomas are chronic periapical lesions mediated by a set of inflammatory mediators that develop in order to contain a periapical infection. This study analyzed the nature of the inflammatory infiltrate, presence of mast cells, and *in situ* expression of cytokines (IL-17 and TGF- $\beta$ ), chemokines (CCL2 e CCL4) and nuclear transcription factor (FoxP3) in human periapical granulomas and cysts compared to a control group.

**Methods:** Fifty-five lesions (25 periapical cysts, 25 periapical granulomas and 5 controls) were analyzed. The type of inflammatory infiltrate was evaluated by hematoxylin/eosin staining and the presence of mast cells was analyzed by toluidine blue staining. Indirect immunohistochemistry was used to evaluate the expression of cytokines, chemokines and FoxP3.

**Results:** The inflammatory infiltrate mainly consisted of mononuclear cells. In cysts, mononuclear infiltrates were significantly more frequent than mixed (polymorphonuclear/mononuclear) infiltrates ( $p=0.04$ ). Mixed inflammatory infiltrates were significantly more frequent in patients presenting fistulae ( $p=0.0001$ ). The number of mast cells was significantly higher in granulomas than in cystic lesions ( $p=0.02$ ). A significant difference in the expression of IL-17 ( $p=0.001$ ) and TGF- $\beta$  ( $p=0.003$ ) was observed between cysts and granulomas and the control group. Significantly higher IL-17 levels were also observed in cases of patients with fistulae ( $p=0.03$ ).

**Conclusions:** Mast cells and IL-17 probably participate in the modulation of the inflammatory process in established periapical lesions, especially periapical granulomas. IL-17 and TGF- $\beta$  exert important modulatory effects on the reactivation of chronic periapical lesions.

Key words: cytokines, chemokines, periapical cyst, periapical granuloma

## INTRODUCTION

Cysts and granulomas are chronic periapical lesions mediated by a set of inflammatory mediators that develop in order to contain a periapical infection (1,2) in response to microbial components present in the canal. The formation of chronic periapical lesions involves the activation of the immune response and bone resorption in the periapex. CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, macrophages, plasma cells, mast cells and eosinophils (3,4), as well as different cytokines (IL-1, IL-3, IL-6, IL-8, IL-10, IL-12, IL-17 IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$  and GM-CSF), have been detected in variable quantities in experimental models and in human periapical lesions (5-9). However, the role of the cellular immune response and of each of these cytokines in the modulation of the inflammatory process and in bone resorption needs to be further investigated.

Different subpopulations of immune cells are found in periapical lesions. Mast cells have been detected in inflammatory infiltrates of periapical granulomas and cysts, a finding suggesting an important role of these cells in these types of lesion (3, 10-12). However, little is known about the interaction between these cells, cytokines and other inflammatory elements in periapical lesions. Mast cells and macrophages are tissue-resident cells known to play an important role in the development of inflammatory reactions due to their capacity to release TNF, thus modulating local inflammation (13). On the other hand, some investigators believe that mast cells are part of a negative feedback mechanism in periapical granulomas and that the direct action of histamine on T lymphocytes may inhibit the production of IL-2 and IFN- $\gamma$ , thus suppressing the activity of T cells in response to antigens (11, 14).

Chemokines also participate in the inflammatory process, promoting the activation of integrins which, in turn, are involved in the adhesion of cells to blood vessels. The localized expression of chemokines in tissues generates chemotactic

gradients that are responsible for the guided migration and maintenance of cells at these sites (15). CCL2, CCL3 and CCL4, have also been described in periapical granulomas and have been associated with the modulation of human periapical lesions (16).

The regulation of cellular immune response in the development and progression of dental periapical lesions is still not well understood. However, the balance between different proinflammatory and immunoregulatory cytokines is known to be of crucial importance for these processes (17). The most variable immune response patterns are observed in periodontal and periapical inflammation. Helper T cells type 1 (Th1) and type 2 (Th2), regulatory T cells (Treg) and Th17 cells have been described in periodontal diseases (18, 19).

IL-17 secreted by Th17 cells (20) and TGF- $\beta$  produced by Treg cells (21), among others, have opposite modulatory effects on the inflammatory process, although they do not exert mutual inhibitory effects. In the presence of IL-6, TGF- $\beta$  contributes to the development of a Th17 response (22). Whereas TGF- $\beta$  is a potent modulator of the immune response, IL-17 is able to reactivate the inflammatory process, including the induction of inflammation characterized by the presence of neutrophils. Thus, the amount of TGF- $\beta$ , as well as the presence or absence of proinflammatory cytokines, determines the balance between the expression of transcription factors such as ROR $\gamma$ t and FoxP3 and, consequently, whether the immune response profile will be Th17 or Treg (21).

Different Th1/Th2 cytokines and chemokines have been demonstrated in periapical lesions, but the correlation between these mediators and the Th17/Treg profile is still not clear. The objective of the present study was to evaluate the presence of IL-17- and TGF- $\beta$ -producing cells, chemokines (CCL2 e CCL4), FoxP3, and mast cells in human periapical granulomas and cyst capsules compared to control tissues.

## **MATERIAL AND METHODS**

### **Tissue samples**

Twenty-five cases of human radicular cysts and 25 cases of human periapical granulomas were studied. Samples were collected during surgery at the Dental Clinic of UNIUBE, Uberaba, Minas Gerais, Brazil, and fixed in 10% buffered formalin for 24 hours. The fragments were then embedded in paraffin and the blocks were cut into histological sections of approximately 5  $\mu$ m. The control group consisted of pulp tissues obtained from five patients with a surgical indication for extraction of impacted healthy third molars. The study was approved by the Ethics Committee of UNIUBE (CAAE – 0003.0.227.000-06). The periapical lesions were diagnosed based on clinical, radiographic and histopathological findings. Radicular cysts were defined as follows: 1) a lytic radiolucent lesion seated on the periapical region of a non-vital tooth, 2) a cavity with a fluid or semi-solid content detected during surgery, and 3) histological evidence of stratified non-keratinizing squamous epithelium completely or partially lining a cystic cavity or tissue fragments. For dental granulomas, in addition to the clinical and radiographic features mentioned above, the observation of a chronic inflammatory reaction in the biopsy specimen detected upon histopathological analysis was used. The following data were obtained from the patients' records: age, sex, location of the lesions. Radiographs were evaluated for evidence of previous endodontic treatment and the size of the periapical radiolucency. Furthermore, the radiographic limits of the lesions were analyzed and classified as defined (with or without a radiopaque line around the lesion and no visible bone resorption) or diffuse (no distinct limits and clearly visible bone resorption). Dental pulp samples were obtained from a group of impacted third molars



recommended for extraction. These tissues did not show any inflammation and were used as control samples.

### **Histochemistry for the detection of mast cells**

The slides were deparaffinized, washed in distilled water, stained with fuchsin-orange G, and rapidly immersed in 60% alcohol. Next, the slides were rapidly immersed in toluidine blue stain and then quickly washed in 60% alcohol. Mast cells were differentiated in 95% alcohol by the development of a red color, followed by dehydration in absolute alcohol and xylene. Finally, the slides were mounted and observed under a common light microscope.

### **Immunohistochemistry**

For immunohistochemistry, deparaffinized sections were treated with 3% hydrogen peroxide in methanol for 10 min and incubated for 30 min at 90°C for antigen detection. The sections were incubated in 2% bovine serum albumin for 30 min at room temperature to reduce nonspecific binding. Next, the specimens were individually incubated with anti-cytokine monoclonal antibodies specific for human FoxP3 (1:20) (AF3240; R&D Systems, Minneapolis, MN, USA), IL-17 (1:20) (AF317-NA; R&D), TGF- $\beta$  (1:20) (MAB240; R&D), CCL2 (1:50) (SC649; R&D), and CCL4 (1:50) (SC649; R&D), diluted in 2% bovine serum albumin prior to use, for 2 hours at 37°C. The specimens were then incubated with secondary biotinylated anti-mouse Ig, anti-rabbit Ig and anti-goat Ig antibodies using the Link System 002488 (Dako, Carpinteria, CA, USA) for 30 min at 37°C. The sections were washed and then incubated with the streptavidin-peroxidase conjugate (Dako) for 30 min. The reaction was developed by

incubation with diaminobenzidine (Sigma, St Louis, MO, USA). The sections were counterstained with hematoxylin.

### **Morphometric analysis**

Morphometric analysis consisted of the quantification of the number of immunopositive cells using images of the histological sections captured with a digital system and analyzed with the Image J software (National Institutes of Health, USA). For this purpose, each field to be quantified was captured with a camera coupled to the microscope and to a computer for digitalization of the image. The number of cells in each field was determined, as well as the area of each field (0.091575). The density of positive cells is expressed as the number of cells per mm<sup>2</sup>.

### **Statistical analysis**

The data were analyzed using the Statview software (Abacus Concepts, Berkeley, CA, USA). After determination of the normality of the data, the Fisher, Mann-Whitney and Kruskal-Wallis tests were applied. P values <0.05 were considered to be statistically significant.

## **RESULTS**

The number of mast cells and expression of cytokines, chemokines and FoxP3 were evaluated according to the type of periapical lesion (cyst and granuloma), type of inflammatory infiltrate and presence or absence of a fistula.

Fifty-five cases, including 32 females and 23 males, with a mean age of 34.81 years, were studied. Twenty-five cases were diagnosed as periapical cyst and 25 cases

as granuloma. Five samples were pulp tissues from third molars, which served as the control group. Morphological analysis of the third molar pulp tissue specimens showed no pathological process.

The inflammatory infiltrate mainly consisted of mononuclear cells in 28 cases, whereas a mixed (polymorphonuclear/mononuclear) infiltrate was observed in 22 cases. In cysts, mononuclear infiltrates were significantly more frequent than mixed infiltrates ( $p=0.04$ ) (Table 1). Evaluation of the presence of fistulae and the type of inflammatory infiltrate showed that mixed inflammatory infiltrates were significantly more frequent in lesions with fistulae ( $p=0.0001$ ) (Table 1). Representative images of the mononuclear and mixed infiltrates are shown in Figure 1A and 1B, respectively.

The accumulation of mast cells was significantly higher in granulomas compared to cystic lesions ( $p=0.02$ ) (Figure 2) (Figure 1C and 1D). No significant difference in the number of mast cells was observed between the two types of inflammatory infiltrate or between patients with and without fistulae (data not show).

Next, the number of immunostained cells in the lesions was determined (Figure 1E - N). With respect to chemokine expression, a significant increase of CCL2 ( $p=0.001$ ) and CCL4 ( $p=0.0012$ ) was observed in cysts and granulomas when compared to the control group (third molar pulp tissue), but there was no difference between these two lesions (Figure 3). Analysis of cytokine expression showed a significant difference in IL-17 ( $p=0.001$ ) and TGF- $\beta$  ( $p=0.0003$ ) expression between cysts and granulomas and the control group (Figure 3). Similarly, a significant difference in FoxP3 expression was observed between cysts and granulomas and the control group ( $p=0.0009$ ). An important finding was that IL-17 levels were significantly higher in cases of patients presenting fistulae ( $p=0.03$ ). On the other hand, expression of TGF- $\beta$  was significantly higher in cases in which the infiltrate consisted of polymorphonuclear/mononuclear

cells ( $p=0.001$ ). Furthermore, a positive correlation between FoxP3 and TGF- $\beta$  was observed in periapical lesions (cysts and granulomas) (Spearman correlation:  $p=0.018$ ,  $z=3.12$ ) (data not show).

## DISCUSSION

Inflammatory cells are essentially protective cells, but may cause severe damage to host tissues. The cytoplasmic granules of these cells contain several enzymes that, when released, degrade the structural elements of cells and extracellular matrix. In the present study, the inflammatory infiltrate mainly consisted of mononuclear cells and the number of mast cells was significantly higher in granulomas than in cystic lesions. This finding is in contrast to another comparative study, which demonstrated a larger number of mast cells in cysts compared to granulomas (12). This difference might be attributed to the fact that the latter authors used immunohistochemistry for the detection of mast cells. In the present study, mast cells were observed close to the periphery of cystic lesions, in agreement with other studies suggesting that the presence of tryptase-positive mast cells at the periphery of these lesions may contribute to both lesion expansion and bone resorption (4, 12).

Although CCL2 levels were apparently higher in granulomas and CCL4 levels were higher in cysts, the expression of both chemokines was significantly higher in these lesions when compared to healthy pulp tissues. Chemokines are important mediators during the inflammatory process and elevated expression of CCL2 has been associated with greater recruitment of cells to the inflammatory focus (23). In addition, CCL4, CCL3 and IP-10 are involved in the recruitment of CD8<sup>+</sup> cells (16, 24, 25).

In the present study, *in situ* expression of IL-17 was significantly higher in granulomas when compared to cysts. We found no studies in the literature comparing

IL-17-positive cells between periapical cysts and granulomas. However, the presence of IL-17 was associated with an exacerbation of inflammatory response, an elevated number of neutrophils and bone resorption in both experimental models (26) and humans (27). This is in agreement with the present results showing a larger number of IL-17-positive cells in cases of lesions that presented fistulae and lesions with a predominance of polymorphonuclear cells. In chronic periodontal diseases, significantly elevated IL-17 levels were observed in both gingival sulcus fluid and cell culture supernatants when compared to the control group (28). In a recent study, were demonstrated not only the expression of IL-17 in human periodontal disease but also the presence of Th17 cells, and suggested the existence of strong evidence of the presence of these cells in these chronic inflammatory lesions (19).

The Th17 or Treg profile has not been established for chronic periapical lesions. Treg lymphocytes are able to produce IL-10 (21, 29), TGF- $\beta$ , and IL-35 (21). Local expression of TGF- $\beta$  in cysts and granulomas has been documented and this cytokine induces the expression of FoxP3 (21). Continuous expression of this factor is important to maintain the suppressor activity of Treg cells (30).

In the present study, higher expression of IL-17 in granulomas was associated with the presence of fistulae. Chronic periapical lesions may progress to fistulae in the presence of an antigen stimulus and the consequent stimulation of IL-1, IL-6 or TNF- $\alpha$  production, together with TGF- $\beta$ , may contribute to the production of IL-17. IL-17, in turn, mediates the attraction of neutrophils, a characteristic observed in inflammatory processes, as demonstrated in the present study by the higher expression of IL-17 in periapical lesions. The expression of IL-17 and reactivation of inflammation play a role in the elimination of microorganisms as well as in the enlargement of lesions. Thus there is strong evidence that the presence of IL-17 in these chronic inflammatory lesions

may induce the production of RANKL, activating osteoclasts, with consequent bone resorption phenomena observed in the pathogenesis of periapical lesions.

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## Figures Legends

**Figure 1:** (A and B) Illustrations of inflammatory infiltrate in radicular cysts and granuloma, respectively (HE) (100x); (C and D) Mast cell (Toluidine blue) in radicular cysts and granuloma, respectively (100x); (E and F) *in situ* IL-17 expression by immunohistochemical in radicular cysts and granuloma, respectively. (G and H) *in situ* TGF- $\beta$  expression by immunohistochemical in radicular cysts and granuloma, respectively (40x); (I and J) *in situ* FOXP3 expression by immunohistochemical in radicular cysts and granuloma, respectively (40x); (K and L) *in situ* CCL2 expression by immunohistochemical in radicular cysts and granuloma, respectively; (M and N) *in situ* CCL4 expression by immunohistochemical in radicular cysts and granuloma, respectively (O) Dental pulp samples were used as control specimens stained with anti-IL-17 antibody (40x); P) radicular cysts stained without primary antibody and with LINK-SYSTEM DAKO (40X).

**Figure 2:** Number of mast cells/mm<sup>2</sup> on periapical lesions grouped according to their histopathological characteristics on cysts and granulomas. The horizontal lines represent the median, bars indicate the 25% a 75% percentile distribution, and the vertical lines indicate the 10% a 90% percentile.  $p=0.02$  (Kruskal-Wallis test).

**Figure 3:** *In situ* expression of cytokines (IL-17, TGF- $\beta$ ), transduction factors (FoxP3) and chemokines (CCL2 e CCL4) on cysts and periapical granulomas and control tissue (3A) (Kruskal Wallis;  $p=0.001$ ;  $p=0.0003$ ;  $p=0.0009$ ;  $p=0.001$ ;  $p=0.0012$  respectively). and on lesions grouped according to the characteristics on cellular infiltrate on mono nuclear and polymorphonuclear/mono nuclear cells(3B) (Mann Whitney test,  $p=0.001$

and  $p=0.03$  respectively). Results are expressed on number of positive cells/mm<sup>2</sup>. The horizontal lines represent the median, bars indicate the 25% a 75% percentile distribution, and the vertical lines indicate the 10% a 90% percentile.

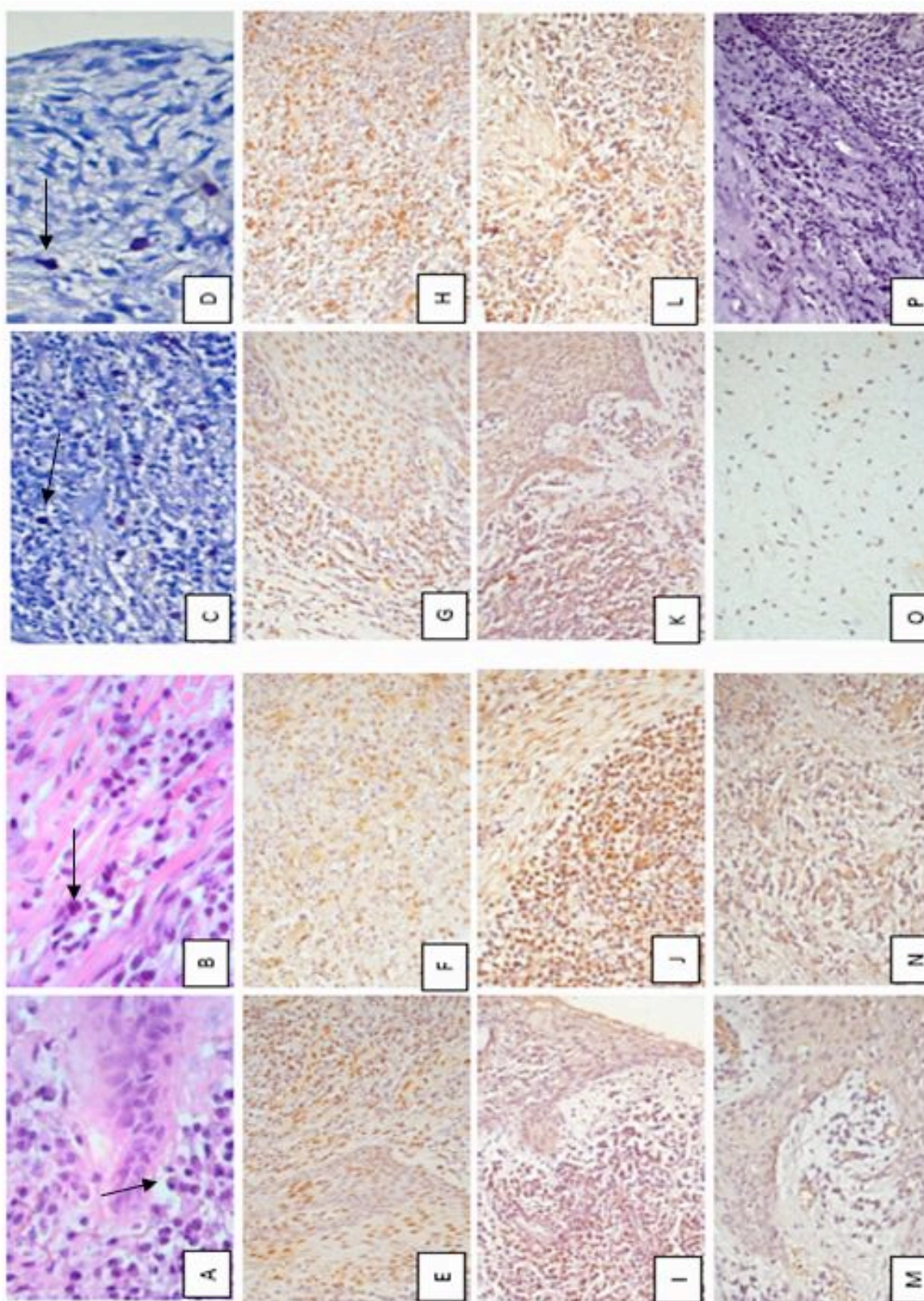
**Table 1:** characteristics of inflammatory reaction according to lesion (cysts or granuloma) with the presence or absence of fistulae.

\*(Fisher Test; p=0,04) \*\* (Fisher test; p=0,0001)

PMN/MN (polymorfonuclear / mononuclear) e MN (mononuclear)

Inflammation	Lesion Periapical		Fístulae	
	cysts	Granulomas	absent	Present
MN	18 (72%)*	10(40%)	24(80%)	4(20%)
PMN/MN	7(28%)	15(60%)	6(20%)	16(80%)**

FIGURE 1



**FIGURE 2**

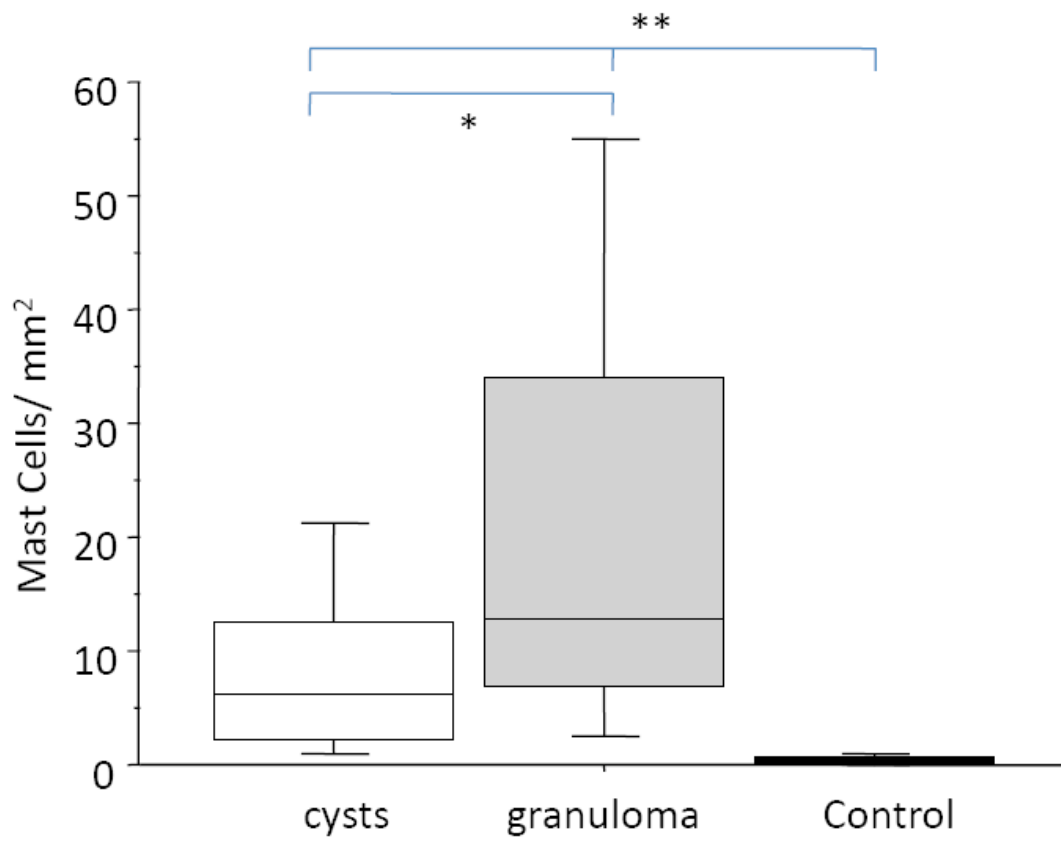
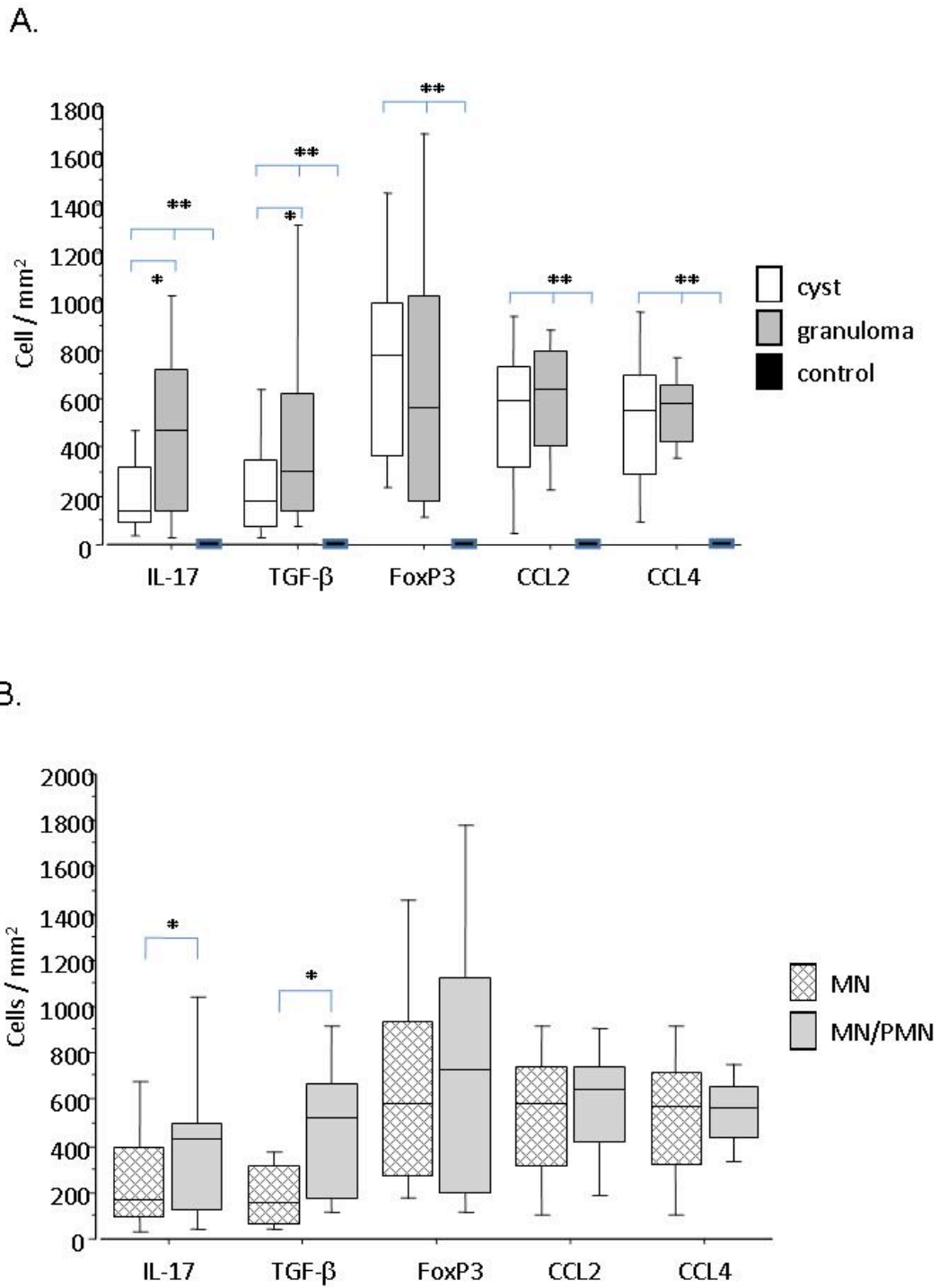


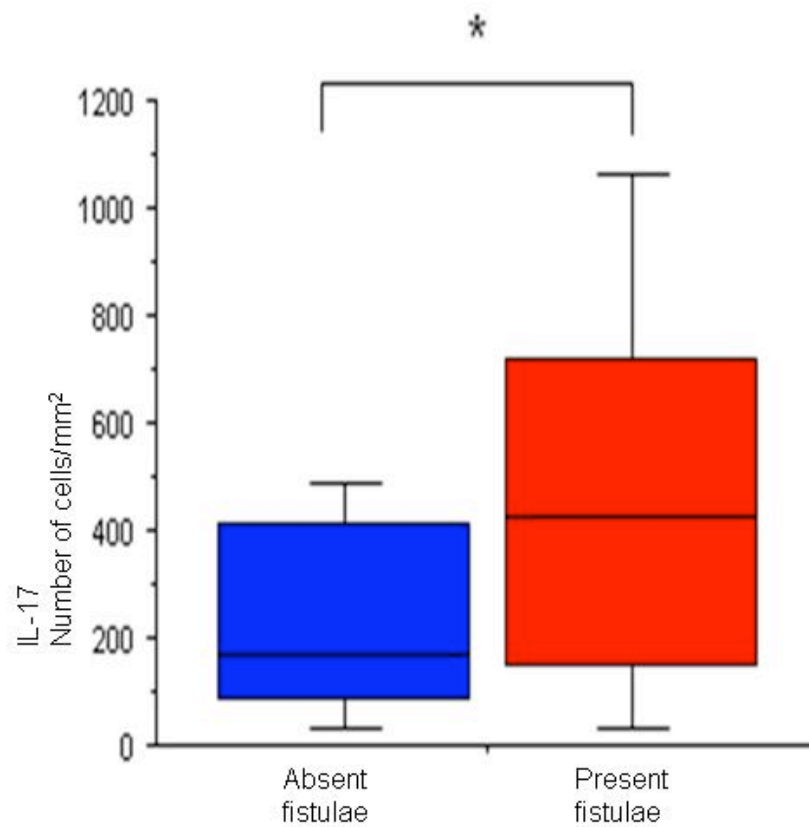
FIGURE 3



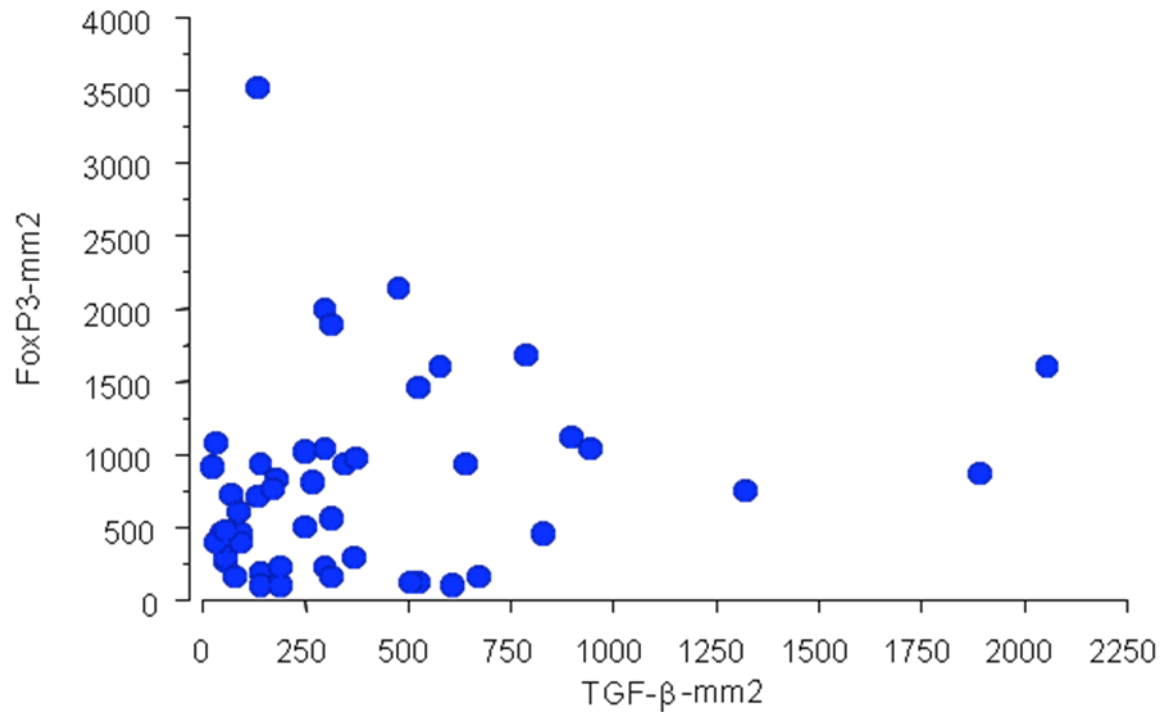


## ANEXOS

**Figure 4:** Number of IL-17 cells/mm<sup>2</sup> on periapical lesions grouped according to a absent or present the fistulae. The horizontal lines represent the median, bars indicate the 25% a 75% percentile distribution, and the vertical lines indicate the 10% a 90% percentile. \*(Mann-Whitney; p=0,03 ).



**Figure 5:** Positive correlation in periapical lesions (cysts and granulomas) between the transcription factor FoxP3 and cytokine TGF- $\beta$ . (Spearman correlation,  $P = 0.018$ ,  $z = 3.12$ )



**Figure 6.** Positive correlation in periapical lesions (cysts and granulomas) between the chemokine CCL2 and cytokine IL-17 (Spearman correlation,  $p=0.018$ ,  $z= 3.12$ )

