

Presence of langerhans cells, regulatory T cells (Treg) and mast cells in asymptomatic apical periodontitis

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Abstract: Asymptomatic Apical Periodontitis is essentially an inflammatory disease of microbial aetiology. Association and function of the cell components involved, or specific inductive factors and growth mediators associated with development, maintenance and resolution of the periapical lesions are still unknown. The objective of this study was to evaluate the concentration of Regulatory T cells (FoxP3+; Treg), Langerhans cells (CD1a+; LC) and mast cells in asymptomatic apical periodontitis. 73 cases were selected: 30 periapical granulomas, 29 radicular cysts and 14 residual cysts. All groups were submitted to morphological analysis for classification of inflammatory infiltrate and thickness of the epithelial lining as well as to immunohistochemical analysis for detection of LC and Treg cells. Toluidine blue staining was used for detecting mast cells. Analysis showed higher mean numbers of LC (8.2 cells/0.2mm²), and Treg cells in radicular cysts (5.910 cells/0.2mm²). As for mast cells, it was found that radicular cysts had a higher mean number of these cells compared to other periapical lesions (12.68 cells/0.2mm²). The association between thickness of the epithelial lining and inflammatory cells showed that the presence of hypertrophic epithelium in radicular cysts presented higher density of LC. The number of LC and Treg cells play an important role in the control of the inflammatory micro-environment in periapical granulomas and radicular cysts, respectively. The presence of mast cells in radicular cysts may be associated with progression of the lesion. Knowledge regarding the inflammatory cell profile is therefore essential for a better understanding of the pathogenesis of asymptomatic periapical periodontitis.

Keywords: Periapical Periodontitis; T-Lymphocytes; Dendritic Cells; Mast Cells; Immunohistochemistry.

Introduction

Asymptomatic Apical Periodontitis is essentially an inflammatory disease of microbial aetiology.^{1,2} Despite the numerous experimental and clinical studies, one does not know exactly which associations and functions of the cell component are involved, nor specific inductive factors and growth mediators associated with development, maintenance and resolution of periapical lesions.^{3,4}



The persistence of the inflammatory process is associated with bone resorption, which consequently results in replacement by granulation tissue, thus forming periapical granulomas,⁵ or inducing the proliferation of epithelial rests of Malassez, an event which can lead to the development of a radicular cyst.^{5,6,7,8} Distinct sub-populations of inflammatory cells have been described in periapical lesions, such as macrophages, mast cells, T cells, neutrophils and dendritic cells.^{7,8,9,10}

The participation of mast cells in the pathogenesis of periapical lesions is not fully clarified and has been frequently related to allergic reactions (*i.e.* hypersensitivity).¹¹ The membrane of these cells contains IgE antibodies and the cytoplasm has metachromatic granules containing histamine, serotonin, heparin and proteases.¹² These mediators released after degranulation of mast cells play an important role in inflammation.^{13,14}

Dendritic cells, especially the LC, have an important function in the cell-mediated immune reactions as well as in the pathogenesis of periapical lesions.^{3,15} These cells are responsible for presenting antigens to T cells and are also extremely important in the activation response of immune T-helper (Th) cells to Th1, Th2, Th17 or Treg cells.^{16,17,18,19}

Studies investigating the presence and function of regulatory T cells in asymptomatic periapical lesions are scanty in the literature. These cells are specialised T lymphocytes as they control the immune response by acting on the immune-tolerance mechanism to inhibit both proliferation and activity of T helper and cytotoxic T cells.^{20,21}

Therefore, in the present study we have assessed the immunohistochemical expression of CD1a+ for LC and FoxP3+ Treg cells and also verified the presence of mast cells in periapical granulomas and radicular and residual cysts. In addition, we have assessed the number of FoxP3+, CD1a+ and mast cells in these lesions as well as correlated it to the intensity of inflammatory infiltrate and the thickness of the epithelial lining.

Methodology

This is a retrospective study in which 73 cases of asymptomatic apical periodontitis were

selected, being 30 of periapical granulomas, 29 of radicular cysts and 14 of residual cysts. All cases were obtained from the archives of the Oral and Maxillofacial Surgical Pathology Service, Department of Stomatology, School of Dentistry, University of São Paulo. All procedures performed in our study were in accordance with human research ethical standards set by institutional and/or national research committees and with the 1964 Helsinki Declaration, including later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. This study was approved by the research ethics committee of the School of Dentistry University of Sao Paulo, according to protocol number 2.135.164.

For morphological analysis, the haematoxylin/eosin stained slides of each case were histologically re-evaluated by means of light microscopy to describe the major morphological aspects of each lesion. The inflammatory infiltrate was classified as mild, moderate or intense depending on the method adapted from Tsai et al.²² The thickness of the epithelial lining of radicular and residual cysts was defined as being atrophic (2 to 10 layers of cells and flat epithelial/capsular border) or hyperplastic (> 10 layers of cells and corrugated epithelial/capsular border, often arranged in proliferative arches) considering the cyst's lining as a whole.²³

Sections of 3 mm were cut and placed on glass slides before being stained in a solution of 1% toluidine blue solution (Dinâmica, São Paulo, São Paulo, Brazil) for 20 seconds and then washed in distilled water until the excess has been removed. The slides were mounted onto a SCA coverslipper apparatus (Tissue-Tel®, Sakura, Japan) for analysis with a light microscope (AxioScope®, Carl Zeiss, Germany).

Immunohistochemical reactions were performed in 3 mm sections according to standardised procedures and by using the En-Vision Dual Link System kit (HRP®, Dako Cytomation®, Carpinteria, USA) and diaminobenzidine as chromogenic agent. Antigens were recovered by using Tris-EDTA buffer solution at pH 8 for FoxP3 and at pH 9 for CD1a (Dako Cytomation®, Carpinteria, USA). The primary antibody anti-FoxP3 (Clone 236/E7, Abcam, Cambridge, UK) was diluted at 1:200 ratio and

incubated in humid chamber for 18 hours at 4°C, whereas the anti-CD1a antibody (Flex Monoclonal Mouse Anti-Human, clone 010, ready-to-use [Link], DAKO Corporation, Carpinteria, USA) was diluted at 1:50 and incubated in humid chamber for 60 minutes at room temperature. Negative controls consisted of slides incubated with bovine serum albumin in place of the primary antibody. Sections of reactive lymph nodes were used as positive controls for immune-histochemical markers of inflammatory cells.

The slides were viewed with a light microscope (AxioScope®, Carl Zeiss, Germany) by a single observer and cells positively immunolabelled for CD1a and FoxP3 antibodies, and mast cells stained with toluidine blue (Merck Sigma-Aldrich, Saint Louis, USA) were counted by using the *hot spot* technique.²⁴ This technique consists in selecting the three most significant fields of the entire section and in counting the cells within these three fields at a 400x magnification. The final value is the average of these three counts and is expressed in number of cells/0.2 mm².

The results were analysed by using the SPSS software version 17.0 (SPSS, Inc. Chicago, USA). As the quantitative variables of this study had no normal distribution (Kolmogorov-Smirnov, $p < 0.05$),

the Kruskal-Wallis test was used to compare the lesions regarding counts of cells marked by CD1a, FoxP3 and mast cells, time of evolution and size. Pearson's chi-square test was also used to analyse the qualitative variables (i.e. gender, symptoms, inflammatory infiltrate and thickness of the epithelial lining) of the lesions. A significance level of 5% was adopted for all statistical tests.

Results

The patients' demographic and clinical data were obtained from surgical pathology records, thus some variables were missing and the overall value of each variable was specified in their descriptive analysis (Table 1). Radicular cysts were mostly observed among patients who were male (55.2%, 16/29), and with mean age of 46.64 ± 15.16 years old, mean evolution time of 15 ± 7.74 months and 73.1% (19/26) of the cases being asymptomatic and measuring, on average, 16.45 ± 11.87 mm.

The majority of the residual cysts were mostly observed among patients who were male (58.3%, 7/12), with half of the cases (50%, 5/10) being asymptomatic. The mean age was 53.36 ± 8.66 years old and the mean evolution time was 16.88 ± 19.65 months, with the lesions measuring 28.10 ± 19.80 mm, on average.

Table 1. Demographic and clinical data.

Variable	Radicular cyst	Granuloma	Residual cyst
	n(%)	n(%)	n(%)
Sex			
Male	16(55.2)	12(46.2)	7(58.3)
Female	13(44.8)	14(53.8)	5(41.7)
Total	29(100.0)	26(100.0)*	12(100.0)*
Color skin			
White	23(85.2)	23(88.5)	4(44.4)
Black	4(14.8)	3(11.5)	5(55.6)
Total	27(100.0)*	26(100.0)*	9(100.0)*
Symptomatology			
Asymptomatic	19(73.1)	17(77.3)	5(50.0)
Symptomatic	7(26.9)	5(22.7)	5(50.0)
Total	26(100.0)*	22(100.0)*	10(100.0)*
Total	29(100.0)	30(100.0)	14(100.0)

*missing data.

Periapical granulomas were mostly observed among patients who were female (53.8%, 14/26), and asymptomatic (77.3%, 17/22). The mean age was 47.32 ± 20.71 years old and the mean evolution time was 38.31 ± 40.18 months, with the lesions measuring 11.71 ± 6.63 mm, on average.

Morphological assessment

Morphological analysis showed that grade-III inflammatory infiltrate was commonly present in radicular cysts (58.6%, 17/29) and periapical granulomas (66.7%, 18/27). In residual cysts, grade-II inflammatory infiltrate was more frequent (42.9%, 6/14). There were no statistically significant differences (Pearson's chi-square test, $p = 0.104$).

Morphological analysis of the thickness of the epithelial lining revealed that radicular cysts mostly presented hypertrophic epithelium (65.5%, 19/29), whereas residual cysts had a higher percentage of atrophic epithelium (64.3%, 9/14). No statistically significant differences were found (Pearson's chi-square test $p = 0.065$).

Assessment of the number of CD1a+, FoxP3+ and Mast cells

Analysis of the immunohistochemical expression of anti-CD1a molecules showed that the mean numbers of CD1a+ cells were 8.16 ± 2.60 cells/0.2 mm² in radicular cysts, 0.85 ± 2.06

cells/0.2 mm² in periapical granulomas and 6.47 ± 2.21 cells/0.2 mm² in residual cysts (Figure 1). Periapical granulomas had values lower than those of residual and radicular cysts (Kruskal-Wallis test, $p < 0.001$) (Table 2).

As for anti-Fox-P3 antibody, on the other hand, analysis of the immunohistochemical analysis showed that the mean numbers of FoxP3+ cells were 5.91 ± 2.58 cells/0.2 mm² in radicular cysts, 4.11 ± 1.24 cells/0.2 mm² in periapical granulomas and 5.30 ± 2.46 cells/0.2 mm² (n = 14) in residual cysts (Figure 2). Periapical granulomas had values lower than those of residual and radicular cysts (Kruskal-Wallis test, $p < 0.044$) (Table 2).

Analysis of toluidine blue staining revealed that the mean numbers of stained mast cells were 12.68 ± 6.78 cells/0.2 mm² in radicular cysts, 7.31 ± 4.23 cells/0.2 mm² in periapical granulomas and 5.42 ± 3.55 cells/0.2 mm² in residual cysts (Figure 3). Radicular cysts had values higher than those of the other lesions ($p < 0.001$) (Table 2).

Assessment of the association between Inflammatory Infiltrate and Epithelial Lining Thickness and between Numbers of CD1a+, FoxP3+ and Mast Cells

Assessment of the association between inflammatory infiltrate and immunolabelling revealed that there was statistically significant difference

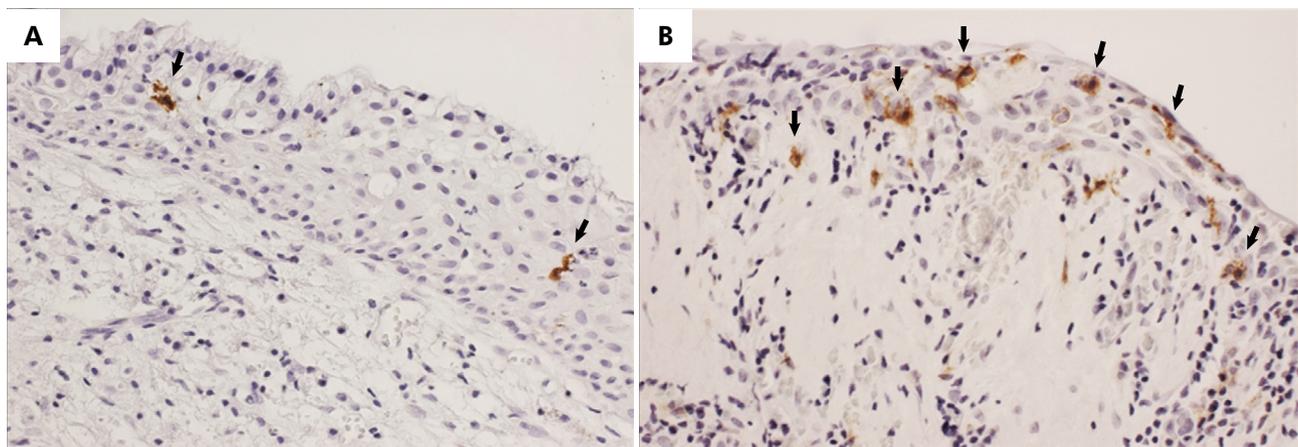


Figure 1. Langerhans Cells - Positive immunostaining for CD1a (black arrows) in radicular cyst (A) and residual cyst (B) (original magnification: 400x).

Table 2. Distribution of Langerhans cells (CD1a), Treg cells (FoxP3) and mast cells in chronic apical periodontitis.

Variables	n	Mean Rank	p-value*
CD1a			
Radicular cyst	29	53.71	
Periapical granuloma	30	17.0	< 0.001**
Residual cyst	14	45.25	
Total	73		
FoxP3			
Radicular cyst	29	42.81	
Periapical granuloma	29	29.33	0.044**
Residual cyst	14	38.39	
Total	72		
Mast cells			
Radicular cyst	29	49.07	
Periapical granuloma	29	31.09	< 0.001**
Residual cyst	14	21.68	
Total	72		

*Kruskal-Wallis test; **Statistically significant.

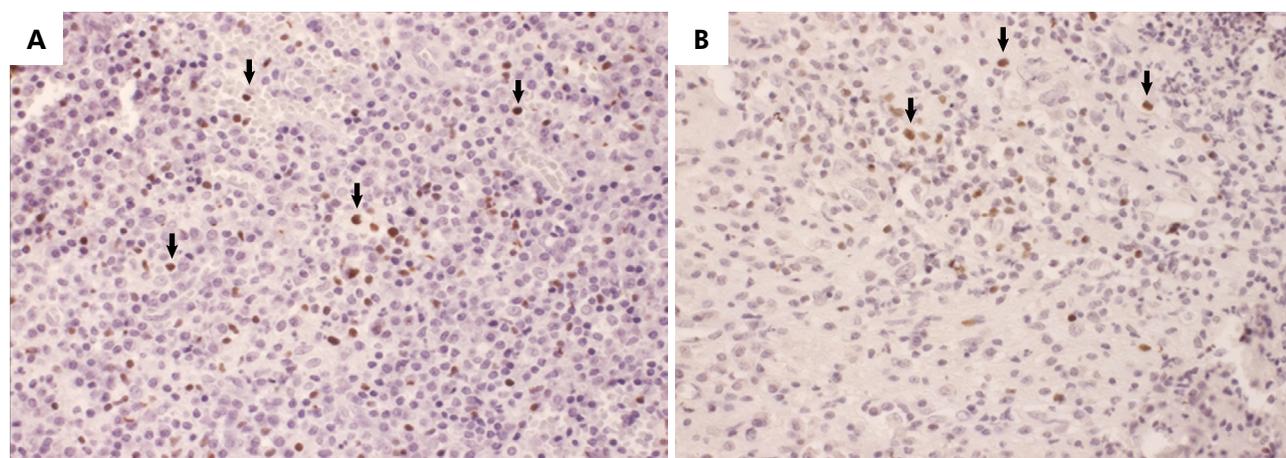


Figure 2. Regulatory T cells (Treg) - Positive immunostaining for FoxP3 (green arrows) in radicular cyst (A) and periapical granuloma (B) (original magnification: 400x).

regarding CD1a+ cells in periapical granulomas (Kruskal Wallis test, $p < 0.001$), since the number of CD1a+ cells decreased as inflammatory infiltrate was more intense (Table 3).

The association between thickness of the epithelial lining and immunolabelling of these cells showed that the hypertrophic epithelium in radicular cysts had a higher density of CD1a+ cells compared to an atrophic epithelium in the same lesion (Mann-Whitney's test, $p < 0.05$) (Table 4).

Discussion

Asymptomatic apical periodontitis lesions develop from a chronic infection in the dental pulp²⁵ and are histologically characterised by the presence of dense connective and granulation tissue infiltrated by different inflammatory cells.²⁶ Among them, there are dendritic cells, T regulatory lymphocytes and mast cells, all antigen-presenting cells (APCs) playing a key role in the development and resolution of these lesions.⁴

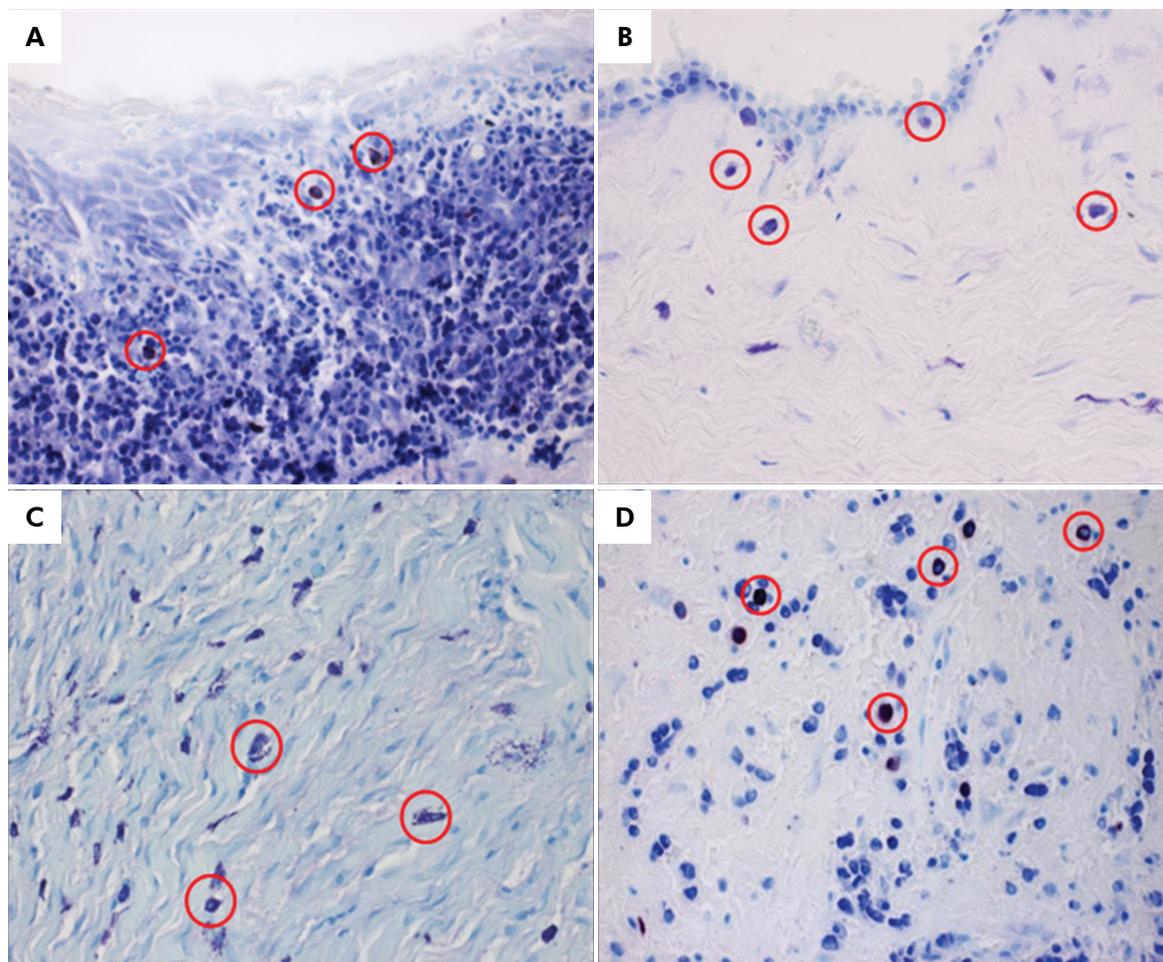


Figure 3. Mast cells - Toluidine blue staining for mast cells (red circles) in radicular cyst (A) (original magnification: 100x), residual cyst (B) and periapical granuloma (C e D) (original magnification: 400x)

Table 3. Correlation between inflammatory infiltrate and Langerhans cells (CD1a), Treg cells (FoxP3) and mast cells in chronic apical periodontitis.

Inflammation	Residual cyst			Radicular cyst			Periapical granuloma		
	n	Mean rank	p-value*	n	Mean rank	p-value*	n	Mean rank	p-value*
CD1a									
I	4	7.25		4	13.25		1	26.0	
II	6	8.83	0.506	8	16.19	0.845	8	19.25	< 0.001**
III	4	5.75		17	14.85		18	11.00	
FoxP3									
I	4	6.25		4	8.38		1	26.0	
II	6	7.58	0.718	8	19.75	0.079	8	12.31	0.257
III	4	8.63		17	14.32		18	14.08	
Mast cells									
I	4	7.13		4	9.63		1	22.0	
II	6	7.83	0.963	8	13.44	0.243	8	11.06	0.311
III	4	7.38		17	17.0		18	14.86	

*Kruskal-Wallis test; **Statistically significant.

Table 4. Correlation between the thickness of the epithelial lining and Langerhans cells (CD1a), Treg cells (FoxP3) and mast cells in residual and radicular cysts.

Inflammation	Residual cyst			Radicular cyst		
	n	Mean Rank	p-value*	n	Mean Rank	p-value*
CD1a						
Atrophic	9	8.06	0.499	10	9.9	0.018**
Hypertrophic	5	6.50		19	17.68	
FoxP3						
Atrophic	9	8.72	0.138	10	13.35	0.446
Hypertrophic	5	5.30		19	15.87	
Mast cells						
Atrophic	9	6.94	0.502	10	12.35	0.222
Hypertrophic	5	8.50		19	16.39	

*Mann-Whitney's test; **Statistically significant.

The mean numbers of CD1a+ cells in radicular cysts, periapical granulomas and residual cysts in the present study were, respectively, 8.16, 0.853 and 6.471 cells/0.2 mm², thus showing a significant association between LC and type of lesion. Santos et al.³ found similar results by reporting that 69.2% of the radicular cysts had CD1a+ cells. On the other hand, only 11.1% of the periapical granulomas were positive for CD1a.³ However, LC count was not significant in the cases of periapical granulomas, which is contrary to the results reported by Suzuki et al.¹⁶

LC were found in all cases of radicular cysts, a finding also reported in the studies by Suzuki et al.¹⁶ and Carrillo et al.²⁷ On the other hand, Piattelli et al.²⁸ and Santos et al.³ detected no such cells in radicular cysts. This lower number of CD1a+ cells may be related to the migration of these cells to regional lymph nodes after their activation after contact with antigens²⁹ or even by cellular apoptosis process after presentation of the antigen to CD4+ T cells.³

The association between inflammatory infiltrate and LC showed that the grade of inflammatory infiltration increases as the number of CD1a+ cells decreases in the periapical granulomas. This result suggests that immature dendritic cells play an important role in controlling the inflammatory process in periapical granulomas, since they are directly related to the process of immunological tolerance.^{30,31} For Santos et al.,³ the intensity of inflammatory infiltrate is not only related to an

increase in the amount of antigens in the lesion, but also to an exacerbated response of the individual's defence mechanism.

Suzuki et al.¹⁶ found a significant association between density of CD1a+ cells and epithelial proliferative potential in radicular cysts, residual cysts and epithelised granulomas. These cells were detected in epithelial components and sub-epithelial layers of the lining epithelium, with the majority of the cells being located in the intra-epithelial areas of the lesions. These results suggest that LC can reside in epithelial components, acting as a front-line agent against root canal antigens.^{16,32}

In the present study, the association between the thickness of the epithelial lining and LC CD1a+ revealed that radicular cysts with hypertrophic epithelium presented higher cell density. The epithelial proliferative potential in residual cysts was much lower than in radicular cysts, which might be due to the lesion's chronicity and consequently to milder inflammatory changes. According to some authors, the inflammatory stimulus provoked by growth factors and cytokines unchain the epithelial proliferation.^{3,16,23}

Analysis of the number of Treg cells (FoxP3+) showed a significantly greater amount of these cells in radicular cysts compared to periapical granulomas and residual cysts. Marçal et al.³³ assessed the number of FoxP3+ cells in residual cysts and periapical granulomas and found no significant differences between these lesions. On the other hand,

Peixoto et al.³⁴ and Fukuda et al.¹⁷ found a higher number of FoxP3+ cells in periapical granulomas compared to radicular cysts. The authors suggest that Treg cells play an important role in modulating the inflammatory micro-environment in periapical lesions.^{17,34} These cells act as effector cells of the disease after receiving activation signals of stimulated LC.^{16,35}

The results of the present study showed evidence of the relevance of Treg cells as they are an important factor in controlling the inflammatory micro-environment in radicular cysts. Different from other diseases, the root canal is a continuous antigenic source of several microorganisms in the periapical lesions.¹⁷ Therefore, in radicular cysts, it is suggested that epithelium plays a key role in the immune tolerance because it seems to express some cytokines on its layers, including dendritic cells, which are responsible for controlling the inflammatory process.¹⁶

The presence of mast cells was assessed by using the toluidine blue staining technique, which was shown to be very reliable for identification of granules of mast cells in periapical lesions.^{33,36,37} However, one limitation of this staining technique is its low sensitivity in detecting partially degranulated cells.^{11,37} In this way, the number of mast cells may have been under-estimated in the present study.

Microscopic analysis of the number of mast cells revealed that these cells were more present in radicular cysts than in periapical granulomas and residual cysts. This finding is consistent with those reported elsewhere, in which tryptase-positive cells were detected by using toluidine blue staining.^{11,37,38} These observations suggest that mast cells may play a key role in the pathogenesis of asymptomatic apical periodontitis, more notably in cysts. The presence of mast cells in radicular cysts may be associated with the lesion progression in terms of production of proteoglycans, angiogenic response, vasodilatation, synthesis of collagen, bone resorption, inflammatory regulation and increased cystic fluid.^{36,37,38}

The recruitment of mast cells and consequent release of prostaglandins during degranulation may have a role in the bone resorption, thus promoting cyst growth.³⁸ In addition, mast cells in asymptomatic periapical inflammatory lesions can act as antigen-presenting cells (APCs) for T cells. The activation of T cells would lead to the stimulation of mast cells and consequently to degranulation and release of cytokines (e.g. TNF- α), resulting in pro-inflammatory and pro-secretory effects on these cells and other cellular types.³⁸

The main limitation of this retrospective study was the lack or the quality of clinical data of the lesions in part of the surgical pathology records.

Conclusions

Periapical granulomas with mild-moderate inflammatory infiltrate had a higher mean number of CD1a+ LC. This result suggests that LC play an important role in the control of inflammatory process in periapical granulomas, since these cells are directly related to the process of immune tolerance. Radicular cysts had a higher mean number of Treg cells (FoxP3+), thus evidencing the relevance of these cells as an important factor in the control of inflammatory micro-environment in radicular cysts. Mast cells were more commonly found in radicular cysts, indicating that these cells play a key role in their pathogenesis. The greater presence of mast cells in radicular cysts may be associated with the progression of the lesion.

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