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Macrophages subpopulations in chronic periapical lesions according to clinical and morphological aspects

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Abstract: The aim of this study was to evaluate macrophage M1 and M2 subpopulations in radicular cysts (RCs) and periapical granulomas (PGs) and relate them to clinical and morphological aspects. M1 macrophages were evaluated by the percentage of CD68 immunostaining associated with the inflammatory cytokine TNF-α, and M2 macrophages, by its specific CD163 antibody. The $CD68^+$/$CD163^+$ ratio was adopted to distinguish between the two macrophage subpopulations. Clinical, radiographic, symptomatology, treatment, and morphological parameters of lesions were collected and a significance level of $p = 0.05$ was adopted for statistical analysis. The results showed that the $CD68^+/CD163^+$ ratio was higher in the RCs (median = 1.22, $p = 0.002$), and the highest TNF-α immunostaining scores were found in RCs ($p = 0.018$); in PGs, the $CD68^+/CD163^+$ ratio was lower and associated with a greater $CD163^+$ immunostaining (median = 1.02, $p < 0.001$). The TNF-α in cyst epithelium had a score of 3 in 10 cases and predominance of M1 macrophages by $CD68^+/CD163^+$ (median = 2.23). In addition, $CD68^+$ cells had higher percentage of immunostaining in smaller RCs ($p = 0.034$). Our findings suggest that increased CD68 immunostaining associated with TNF-α cytokine in RCs results in a greater differentiation of the M1 phenotype. The higher CD163 immunostaining in PGs results in greater differentiation of the M2 phenotype. Therefore, the inflammatory state promoted by M1 macrophages is related to growth and progression of RCs; on the other hand, the immunomodulatory state of M2 macrophages is related to maintenance of PGs.

Keywords: Radicular Cyst; Periapical Granuloma; Macrophages.

Introduction

Radicular cysts (RCs) and periapical granulomas (PGs) are chronic lesions that develop in response to inflammatory mediators that originate from dental pulp necrosis[1]. The etiopathogenesis of RCs has three phases: initiation, formation, and growth. It is thought that RCs originate from granular tissue in the periapex, where epithelial rests of Malassez (ERM) or Hertwig epithelial sheath remnants are stimulated, resulting in the formation of a cystic lumen[2,3].

Three theories have been postulated to explain RC enlargement. The osmotic pressure theory explains progressive RC growth, which occurs through increasing exudate permeability through the cyst lumen due to the necrosis of central epithelial cells. The abscess theory, on the other hand, suggests a proliferative ability of epithelial tissue involving the periapical abscess area. Finally, the immunological theory suggests that ERM can acquire antigenic properties due to the expression of molecules not produced in their quiescent state. Thus, a cross reaction occurs between ERM and bacterial products through antibodies, complement system, natural killers cells, cytotoxic T lymphocytes, and macrophages[4].

Macrophages participate in host protective response against chronic periapical lesions, as well as in the development and perpetuation of inflammatory reactions. These cells play complex roles in phagocytosis and in the production of inflammatory mediators, activation of humoral and cellular immune responses, and lesion repair, while also acting as antigen-presenting cells (APCs). Macrophages can directly interact with the cyst epithelium through the release of certain cytokines, such as TNF-α, that regulate bone growth and resorption. Thus, macrophages could be associated to and explain the immunological theory of RC formation[5,6].

Macrophages can be classified into two different phenotypes: M1 and M2. The M1 phenotype produces potent effector molecules, such as reactive oxygen species and nitrogen intermediates, as well as inflammatory cytokines (IL-1, IL-6, and TNF-α), and polarizes Th1 lymphocyte responses, participating in proinflammatory states. M2 macrophages, on the other hand, release growth factors, such as TGF-β and immunosuppressive cytokines (IL-10 and IL-13), leading to the activation of Th2 lymphocyte responses, participating in immunomodulatory states[6,7].

The activation of the classical complement pathway can occur in the presence of IFN-γ and TNF-α mediators after recognition of pathogen-associated molecular patterns (PAMPs). Macrophages originating from this activation are called M1[8]. The activation of the alternative complement pathway leads to M2 phenotype polarization[9].

TNF-α is an inflammatory cytokine released mainly by M1 macrophages and is responsible for regulating cystic epithelium cell proliferation, differentiation, and apoptosis[10]. In addition, TNF-α is a recognized bone resorption modulator[3]. Thus, due to significant interactions with the cyst epithelium, studies investigating TNF-α are of significant interest.

CD68 is a 110-K-Da intracellular glycoprotein that recognizes M1 and M2 macrophages due to its location in the primary granules of macrophage lysosomes, playing a role in endocytosis and lysosomal movement[11]. CD163 is a 130-k-Da glycoprotein belonging to the scavenger receptor group and is present, specifically, in M2 macrophages. M2 cells usually display high levels of scavenger receptors, as its polarization occurs though the mannose-binding lectin-dependent pathway [9,12].

Previous studies have suggested that macrophage polarization contributes to the formation, development, and growth of both RCs and PGs[6,13]. However, information regarding the specific location of M1 and M2 macrophages, the phenotypes associated with TNF-α in RC epithelium, and the relationship of these cells with clinical aspects and lesion stage and size have not yet been investigated. Thus, the aim of this study was to quantitatively and comparatively evaluate M1 and M2 macrophages in RCs and PGs through the expression of CD68, TNF-α, and CD163. In addition, the roles of macrophages and TNF-α in inflammatory infiltrates and cyst epithelial lining were also assessed.

Material and methods

Study design and tissue samples

Sixty tissue specimens, 30 PGs and 30 RCs, archived at the Federal University of Rio Grande do Norte (UFRN) Oral Pathology Department were randomly selected. Only excisional biopsies from surgical resections in paraffin blocks containing sufficient material for histopathological analyses were included in the study. For RCs, lesions with sufficient epithelium and fibrous capsule were selected. For PGs, any specimens presenting epithelium were excluded, in order to discard epithelized granulomas, since they were not the focus of this investigation.

Data regarding gender, age, lesion location, size, radiographic data, symptomatology, and treatment were obtained from biopsy records. Cases presenting incomplete information were excluded. The study was approved by the Federal University of Rio Grande do Norte Research Ethics Committee for Human Stidues (Protocol 1,998,683).

Morphological analyses

Specimens were stained with hematoxylin and eosin and examined at 5-µm thickness under light microscopy (Olympus BX41, Olympus Japan Co., Tokyo, Japan) at 40x, 100x, and 400x magnifications. The intensity of the inflammatory infiltrates was analyzed at 400x and classified according to Tsai *et al*. (2004) criteria[14]. Nine microscopic fields divided into three consecutive fields were selected for RC analysis. The analysis began in the epithelium and extended deep into the capsule. Lesions presenting inflammatory infiltrates restricted to 1/3 of the microscopic field were classified as Grade I (low infiltrate), lesions presenting inflammatory cells in up to 2/3 of the microscopic field were defined as Grade II (moderate infiltrate), and lesions exhibiting inflammatory infiltrates in more than 2/3 of the microscopic field were categorized as Grade III (intense infiltrate). The mean of three fields was calculated and the intensity of the inflammatory infiltrates was established for each case. Tsai *et al*. (2004) criteria were also adopted for PGs, but the analysis began in the middle of lesion and extended deep into the connective tissue wall. The epithelial lining thickness of RCs was analyzed according to methodology proposed by Moreira *et al*. (2000)[15], where cyst epithelium consisting of 2 to 10 cell layers were considered atrophic, while epithelium with over 10 cell layers were considered hyperplastic. Finally, RCs capsule thickness was classified as thin (below 1.5 mm) or thick (above 1.5 mm), according to Jurisic *et al*. (2008) criteria[16].

Immunohistochemical analyses

CD68 monoclonal antibody staining (DAKO, Carpinteria, CA, USA) was performed to confirm the macrophage nature. In addition, monoclonal TNF-α (Abcam; Cambridge, MA, USA) and CD163 antibody staining (Abcam; Cambridge, MA, USA) were used to verify the polarization profiles for M1 and M2, respectively. The specifications for each antibody are displayed in Table 1.

For all antibodies, 3-µm thick sections were obtained from paraffin blocks, dewaxed, and submitted to antigenic retrieval. After, the specimens were submitted to the Trilogy process (Cell Marque, CA, USA) at a 1:100 ratio of distilled water in a Pascal vessel for 10 minutes. Subsequently, the samples were blocked by endogenous peroxidase with hydrogen peroxide and then incubated with protein block (Thermo Scientific, Runcorn, UK) for 5 minutes, in order to prevent non-specific binding. Two washes were performed with a Tween 20 solution at 1% in TRIS-HCl (tris-hydroxymethyl-aminomethane, Sigma Chemical Co., St. Louis, MO, USA), pH 7.4 with two changes at 5 minutes of incubation to allow for anti-CD68 primary antibody (1:1000, for 60 minutes), anti-CD163 (Flex for 60 minutes), and anti-TNF-α (1:500, Hidef for 60 minutes) staining. The process was completed by sample dehydration, diaphanization, and slide assembly. Lung histological sections were used as positive controls for the anti-CD68 and anti-CD163 antibodies. For negative controls, the primary antibody was replaced with bovine serum albumin (BSA) at 1% in buffer solution. Cells presenting a brownish staining in the cytoplasm and on the cell surface were classified as macrophages.

Two previously calibrated examiners analyzed each case by light microscopy (Olympus BX41, Olympus Japan Co., Tokyo, Japan). After the analysis, the slides were scanned (Panoramic MIDI, 1.15 SPI, 3D HISTECH, Budapest, Hungary) and images were obtained through

Table 1. Manufacturer, clone, antigen retrieval, dilution, and incubation period of primary antibodies.

Antibody	Manufacturer	Clone	Antigen retrieval	Dilution	Incubation
CD68	DAKO	KP1	Trilogy	1:1000	60 min
CD163	Abcam	ab87099	Trilogy	Flex	60 min
TNF-α	Abcam	ab6671	Trilogy	1:500	60 min

the Panoramic Viewer 1.15.2 program (3D HISTECH, Budapest, Hungary). For CD68 and CD163 antibodies, five representative and random fields were selected after identification of the highest immunoreactivity areas at a 100-μm scale. $CD68^+$ and $CD163^+$ cell counts were performed using the Image J® program and the positivity index (PI) was adopted according to Pontes-Santos *et al*. (2017)[17]; data are reported as the percentage of positive cells presenting brownish cytoplasmic staining in each sample. The PI was calculated by applying the formula (PI) = (number of immunopositive cells)/(number of counted cells) x 100. For TNF-α, a semi-quantitative analysis was performed for each lesion using scores according to Andrade *et al*. (2016)[18], as follows: score 1 (≤25% positive cells), score 2 (>25% and ≤50% positive cells), score 3 (>50% and ≤75 % positive cells), and score 4 (>75% of positive cells).

Statistical analyses

The results were analyzed using the freeware program IBM SPSS Statistics (version 20.0; IBM Cop., Armonk, NY, USA). Descriptive statistics were used to characterize the samples. Inflammatory infiltrates grades I and II were combined into one group (low/moderate) and compared to grade III (intense). Data distribution was analyzed by the Shapiro-Wilk test. The chi-square test or Fisher's exact test were used to determine possible associations between clinical and morphological features. The non-parametric Mann-Whitney test and Spearman's correlation test were applied to assess possible differences and correlations between CD68, CD163, and TNF-α immunostaining with clinical and morphological data. The $CD68^+/CD163^+$ ratio was adopted to differentiate between M1 and M2 macrophages. A significance level of $p = 0.05$ was considered for all statistical tests.

Results

Clinical data

The periapical lesions assessed herein were more frequent in women, corresponding to a female-to-male ratio of 1.85:1, with mean age of 41.31±14.49 years. The most frequent site of RCs was the anterior maxilla (n = 18.60%) while PGs were most frequent in the posterior maxilla (n = 10, 33.3%) and mandible (n = 10, 33.3%). Regarding radiographic features, well-circumscribed radiolucency was the most important aspect for both RCs and PGs. However, two RC cases presented cortical bone resorption and two PG cases presented periodontal ligament widening (Table 2).

Symptomatic cases were slightly more frequent in RCs compared to PGs. Pain was related to exacerbation with purulent secretions (n = 7) and palpation sensitivity (n = 3) in RCs, whereas pulsatile pain (n = 5) was reported in PGs, although no association was found between symptoms and type of lesion. Most patients with RC (80%) and with PG (96%) had no previous endodontic treatment, and treatments were not associated with the appearance of lesions (Table 3). In addition, all RC patients that received endodontic treatment had lesion recurrence, with the time interval varying from 5 to 10 years.

Morphological analyses

In RC samples, atrophic epithelium (n = 17; 56.7%) was more frequent than hyperplastic epithelium (n = 13, 43.3%), and thick capsules were more frequent (n = 18; 60%) than thin capsules (n = 12.40%). Mean RC size was 1.79 cm. Regarding PGs, thick connective tissue walls were more frequent (n = 20; 66.7%) than thin connective tissue walls (n = 10; 33.3%), with mean PG size of 1.08 cm.

Both RCs and PGs presented intense inflammatory infiltrates (Grade 3) (73.3% and 93.3%, respectively, p=0.038, Table 4).

Table 2. Site and radiographic features of periapical lesions.

Clinical and radiographic features		n (%)
Gender	Female	39 (65.0)
	Male	21 (35.0)
Site	Anterior region of maxilla	27 (45.0)
	Posterior region of maxilla	17 (28.3)
	Anterior region of mandible	3 (5.0)
	Posterior region of mandible	13 (21.7)
Radiographic features	Cortical bone resorption	2 (3.3)
	Well circumscribed radiolucency	56 (93.4)
	Periodontal ligament widening	2 (3.3)

Table 3. Rates of symptomatology and endodontic treatment in chronic periapical lesions.

Periapical lesions	Clinical features							
	Symptomatology			P.R. (95% CI)	Endodontic treatment			P.R. (95% CI)
	Yes n (%)	No n (%)	*p*		Yes n (%)	No n (%)	*p*	
RCs	17 (56.7)	13 (43.3)	0.121[a]	1.30 (0.87-2.71)	6 (20.0)	24 (80.0)	0.103[b]	0.82 (0.68-1.0)
PGs	11 (36.7)	19 (63.3)			1 (3.3)	29 (96.6)		
Total	28 (46.7)	32 (53.3)			7 (11.7)	53 (88.3)		

CI: 95% Confidence interval; P.R.: prevalence ratio; p: p-value; [a]Chi-squared test; [b]Fisher's Exact Test.

Table 4. Intensity of the inflammatory infiltrate in RCs and PGs.

Lesion	Intensity of the inflammatory infiltrate		Total n (%)	*p*	P.R. (95% CI)
	Intense (Grade 3) n (%)	Low/moderate (Grade 1+2) n (%)			
PGs	28 (93.3)	2 (6.7)	30 (100.0)	0.038*	1.27 (1.00-1.61)
RCs	22 (73.3)	8 (26.7)	30 (100.0)		

CI: 95% Confidence interval; P.R: prevalence ratio; *p*: p-value; *Chi-squared test.

Immunohistochemical analyses

Macrophage immunostaining was more evident in subepithelial regions in RCs and middle regions in PGs (Figure 1). The CD68 antibody was expressed both on lysosomal granules and macrophage surfaces, whereas the CD163 antibody was expressed mainly on M2 macrophages membranes (Figure 2).

The results indicate that the more intense the inflammatory infiltrate, the greater the amount of macrophages. A statistically significant difference was found between $CD68^+$ (median= 50.13, $p = 0.017$) and $CD163^+$ (median= 37.96, $p = 0.005$) cells, but no difference was found between TNF-α scores and inflammatory infiltrate intensity (median= 2.0, $p = 0.56$).

CD68 and CD163 staining was assessed in each lesion by the $CD68^+/CD163^+$ ratio. RCs presented a significantly higher $CD68^+/CD163^+$ ratio (median = 1.22) compared to PGs (p=0.002) (Table 5).

No statistically significant difference was observed between RCs and PGs in relation to CD68 (p = 0.143), while a higher median was verified for CD163 in PGs (p <0.001). In addition, a higher median for TNF-α was observed in RCs ($p = 0.018$).

In RCs, M1 macrophage polarization was verified, due to a higher $CD68^+/CD163^+$ ratio associated with higher TNF-α score. In contrast, a lower $CD68^+/CD163^+$ ratio and a higher percentage of $CD163^+$ cells was observed in PGs, indicating M2 macrophage polarization in these lesions.

Concerning macrophage polarization and TNF-α expression in RCs epithelium, 10 cases presented score 3 (33%), eight (27%) had score 2, eight score 1 (27%), and four presented score 4 (13%). No significant difference was observed between TNF-α immunoexpression and epithelial thickness (median = 2.0, $p = 0.632$). In addition, no difference was noted between CD68 and CD163 immunoexpression and atrophic or hyperplastic epithelial thickness (median = 30.60, $p = 0.738$, median = 13.53, $p = 0.818$, respectively). However, $CD68^+$ cells were more often observed in the epithelium. The $CD68^+/CD163^+$ ratio (median = 2.23) in RC epithelium indicates a greater predominance of M1 macrophages, similar to what was observed in RC capsules. This could be related to TNF-α release, which could then play a role in RC enlargement (Figure 3).

Larger RC lesions had lower percentages of $CD68^+$ cells, while smaller lesions had higher percentages, resulting in a moderate negative correlation ($p = 0.034$, r_s <0). This was also observed for TNF-α score, although no significant correlation was detected. On the other hand, these findings were not observed in PG samples (Table 6).

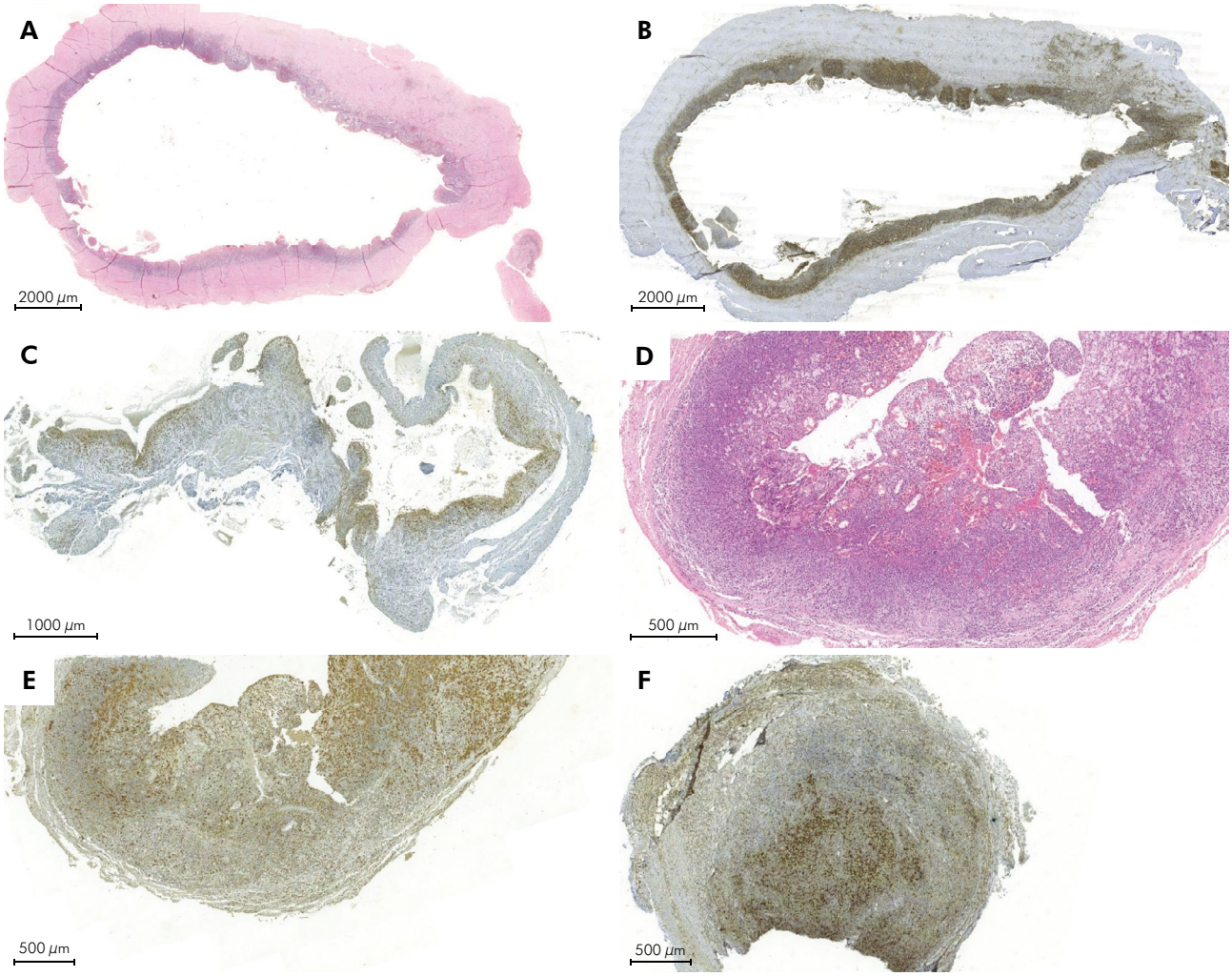

Figure 1. (A) Hematoxylin and eosin immunostaining of RCs (Scale bar: 2000µm); (B and C) Intense inflammation and subepithelial immunostaining areas in RCs (Scale bar: 2000µm, 1000µm); (D) Hematoxylin and eosin immunostaining of PGs (Scale bar: 500 µm); (E. F) Middle region PG immunostaining (Scale bar: 500µm).

Discussion

Macrophages phagocyte almost any foreign agent and their infiltration lasts a relatively long time, since they present a half-life of several months to years, acting as sentinels in teeth periapices[7]. They also act as APCs in essential initial steps of acquired immunity, i.e., antigen processing and introduction to antigen-specific T-helper lymphocyte clones, through a process involving lymphocyte recognition of a MHC class II molecule in macrophages[19].

Macrophages can be polarized into two different phenotypes: M1 and M2. The microbiota of an infected tooth canal may influence this polarization and RC development. Interferon-γ (IFN-γ) and bacterial lipopolysaccharides (LPS) are important stimuli regarding M1 macrophage polarization producing inflammatory cytokines like TNF-α, found mainly in RCs. Interleukins IL-4, IL-10, and IL-13 allow for the activation of M2 subpopulations. Therefore, bacterial flora composition could modulate the polarization of periapical macrophages, thus influencing RC development [6,20,21]. M1 macrophages are classically considered potent effector cells that participate in proinflammatory processes. M2 macrophages, on the other hand, are characterized by their ability to inhibit the cytotoxic and inflammatory functions

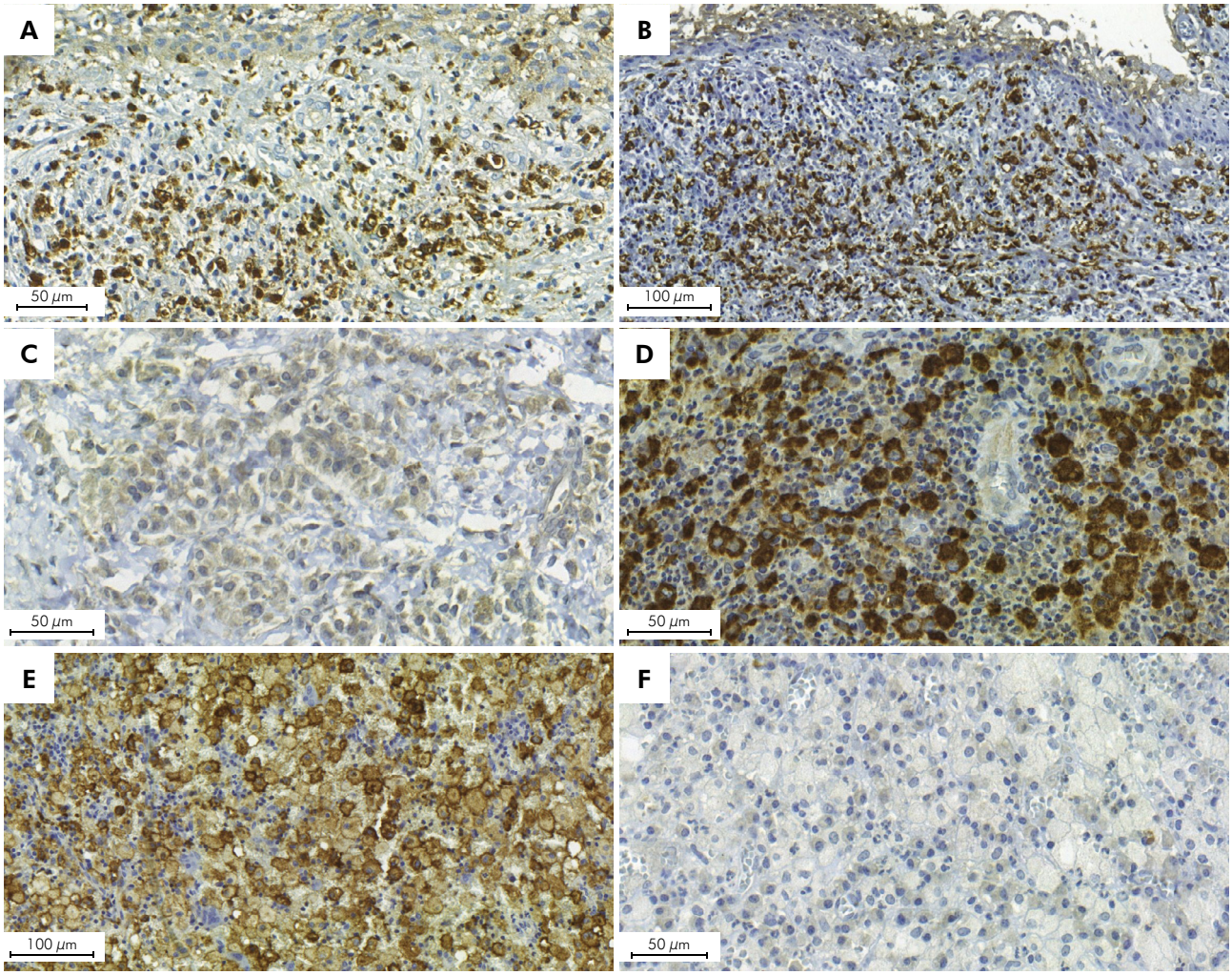

Figure 2. (A) CD68 immunostaining in lysosomal granules and macrophage surfaces in RCs (Scale bar 50µm); (B) CD163 immunostaining indicates M2 macrophages in RCs (Scale bar 100µm); (C) Score 3 RC capsule TNF-α immunostaining (Scale bar 50µm); (D) PG CD68 immunostaining and intense inflammatory infiltrate (Scale bar 50 µm); (E) High percentage of CD163 membrane immunostaining in PG histiocytes (Scale bar 100µm); (F) Score 1 TNF-α PG immunostaining (Scale bar 50µm).

Table 5. Percentages of $CD68^+$, $CD163^+$, and $CD68^+/CD163^+$ ratio in RCs and PGs.

Lesion	$CD68^+$			$CD163^+$		TNF-α scores		$CD68^+/CD163^+$	
	n	Median	Q25-Q75	Median	Q25-Q75	Median	Q25-Q75	Median	Q25-Q75
RCs	30	49.2	30.4 – 62.2	30.6	24.2 – 38.8	2.0	1.0-3.0	1.22	1.03 – 1.73
PGs	30	51.0	43.0 – 66.1	48.4	37.7 – 64.5	1.5	1.0-2.0	1.02	0.97 – 1.14
p value		$p = 0.143$		< 0.001		0.018		0.002*	

Q25-Q75: 25-75% quartiles; * Mann-Whitney non-parametric test.

of M1 macrophages, while also being involved in angiogenesis, anti-inflammatory effects, tissue repair and remodeling, and fibrosis[11].

When macrophages are activated, they present increased cell size and lysosomal enzyme content, as well as a more active metabolism and greater ability to destroy endocytosed organisms[7]. This morphological aspect is often observed in M1 macrophages, which are the effector cells concerning microorganism destruction[22].

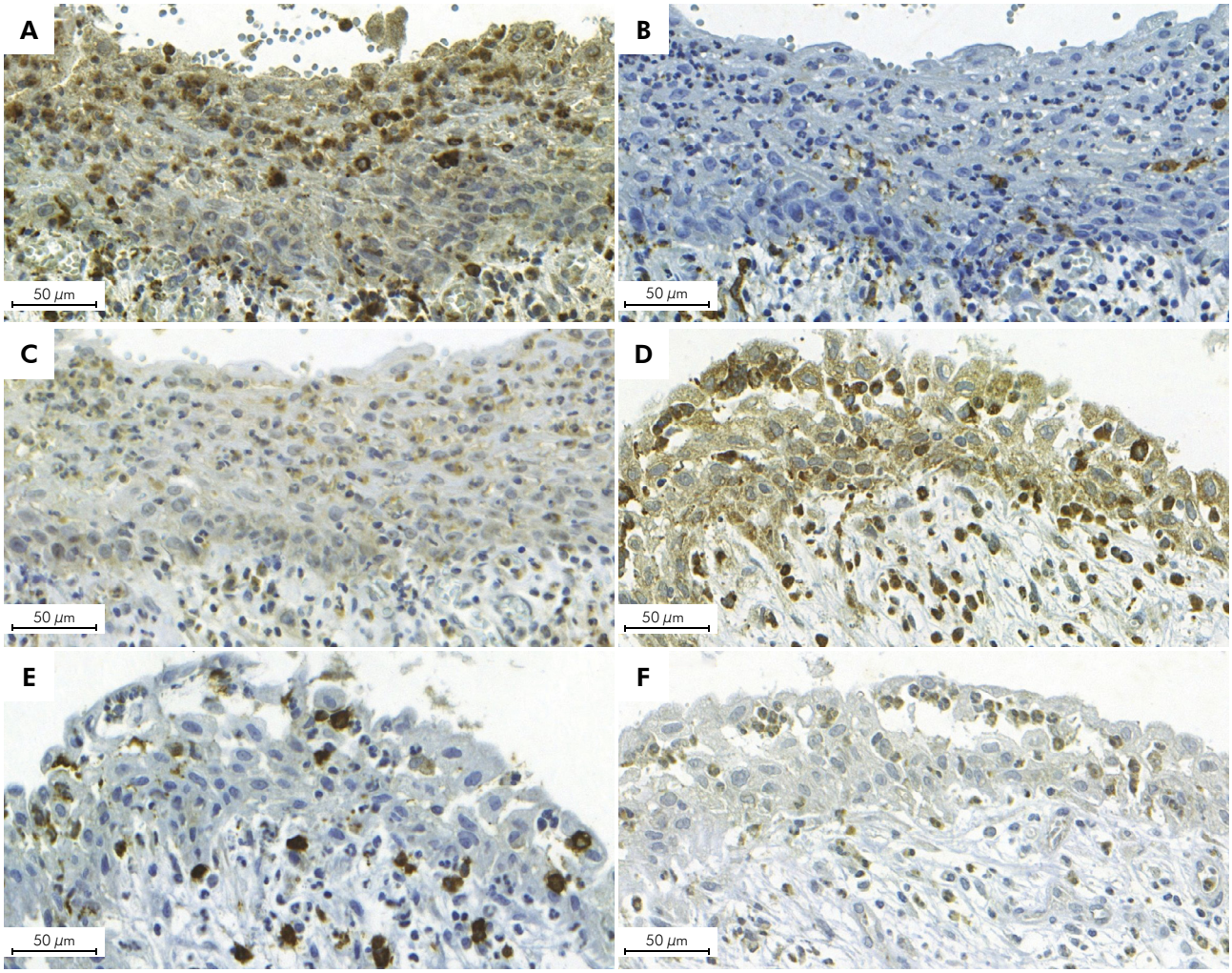

Figure 3. A, B, and C are from the same case. (A) High percentage of CD68 immunostaining in the RC hyperplastic epithelium (Scale bar 50µm); (B) Low percentage of CD163 immunostaining in the RC hyperplastic epithelium (Scale bar 50µm); (C) Score 4 TNF-α immunostaining in the hyperplastic RC epithelium (Scale bar 50µm); D, E and F are from the same case. (D) High percentage of CD68 immunostaining in the RC atrophic epithelium (Scale bar 50µm); (E) Low percentage of CD163 immunostaining in the RC atrophic epithelium (Scale bar 50µm); (F) Score 3 TNF-α immunostaining in the RC atrophic epithelium (Scale bar 50µm).

Table 6. Correlation between size and immunoexpression scores of CD68 and TNF-α in RCs and PGs.

	n	Size (cm) Median	Q25-Q75	CD68+		TNF-α scores	
				r_s	*p*	r_s	*p*
RCs	30	1.7	1.0-2.25	-0.38	0.034	-0.19	0.30
PGs	30	1.0	0.5-1.3	0.18	0.32	-0.15	0.42

Q25-Q75: 25-75% quartiles; r_s: Spearman's correlation coefficient; *p*: p-value.

In the present study, TNF-α was chosen due to its presence in the cyst epithelium, verified by immunostaining. Previous investigations have suggested that this cytokine could stimulate bone resorption and regulate RC epithelium cell proliferation, differentiation, and apoptosis. Thus, TNF-α analyses are considered a vital tool to explain cyst growth. Several studies have reported that basal layer keratinocytes in RCs express TNF-α. This cytokine is reported to interact with RC epithelium

leading to RC growth since it regulates the proliferation and differentiation of ERM present in the periapex [10,23]. In the present study, 10 RC cases had a score 3 and four cases had a score 4 regarding TNF-α in the epithelium, indicating the possible role of this cytokine in cyst growth.

Most lesions presented a well-circumscribed radiolucency and only two RC cases had cortical bone resorption. These cases had a score 3 regarding TNF-α and had an intense inflammatory infiltrate. This cytokine is reported to stimulate bone resorption in RCs[23,24] and its presence was higher in cysts than in granulomas.

Most PGs and RCs presented intense inflammatory infiltrate. A significantly higher number of PGs had a Grade 3 inflammatory infiltrates, 27-fold higher than in RCs. These results are similar to those reported by Andrade *et al*. (2016)[18], who detected intense inflammatory infiltrates in both lesions, explaining their inflammatory nature.

Some authors have analyzed periapical lesion sites with high prevalence of macrophages and observed that most $CD68^+$ cells were located in active inflammation areas, mainly in the middle of PGs and in subepithelial regions of RCs, with lower amounts in the periphery[25]. These lesions are preferably exudative, and macrophages are recruited in the cyst lumen due their proximity to antigenic stimuli from root canal microorganisms. In addition, these cells release cytokines, which then interact with epithelial remnants in periapical tissue, leading to proliferation[4]. The present study found similar results, suggesting that macrophages are located in areas containing higher amounts of microorganisms.

The higher medians for CD68 and CD163 in cases with intense inflammatory infiltrate demonstrate that more intense inflammation leads to higher amounts of macrophages, corroborating Santos *et al*. (2017)[25].

Higher $CD68^+/CD163^+$ ratios, alongside higher TNF-α immunoexpression scores in RCs indicate greater polarization of the M1 phenotype, with the release of TNF-α and its participation in a proinflammatory state mediated by Th1 responses. These results were similar to those observed in RCs epithelium, with a higher predominance of the M1 phenotype. A higher amount of $CD163^+$ cells indicates polarization of the M2 phenotype with Th2 activation responses in PGs. These results are in agreement with those reported by Weber *et al*. (2017)[6], who analyzed RCs, PGs, and inflamed dentigerous cysts specimens. The authors observed increased M1 macrophage polarization in RCs, whereas M2 polarization was more frequent in PGs, concluding that the periapical tissue microenvironment may define the type of lesion to be developed, leading to a specific macrophage subpopulation.

Pain may also be related to macrophage subpopulations and Th1 response. In the present study, more RC cases were symptomatic compared to PGs, perhaps due to the proinflammatory profile of cysts, in which M1 macrophages play an important role[6]. The release of IL-1 and IL-6, in addition to COX-2 products, such as prostaglandins E_2 (PGE_2) and Th1 response products, is responsible for painful symptoms[9]. Increased COX-2 levels may perpetuate the inflammatory response due to the high expression of inflammatory mediators related to the activation and function of immunocompetent cells in PGs and RCs. Their involvement is mediated by macrophages and cytokine-induced growth factors, such as TGF-ß1 and TNF-α. A previous study revealed that <50% of cells in both types of lesions displayed staining for COX-2, and concluded that TNF-α and COX-2 are similarly expressed in PGs and RCs[26]. On the other hand, the present study found higher TNF-α scores in RCs. The pulsatile pain reported in PGs can be due to higher inflammatory infiltrate, as well as higher amounts of $CD68^+$ cells[18].

CD68 immunoexpression was moderately correlated with lesion size. Higher CD68 immunoexpression was noted in smaller cysts, while lower CD68 immunoexpression was observed in larger cysts. TNF-α immunoexpression was also inversely proportional to lesion size, albeit not significantly. These findings indicate that macrophages act in etiopathogenesis, explaining RC formation, development, and enlargement. Previous studies have reported higher amounts of macrophages and TNF-α in the initial phase of cysts, participating in exudate and lumen formation[16,25].

High TNF-α levels have been detected in the connective tissue walls of lesions, due to the

high amounts of macrophages in PGs and RCs[16]. However, no significant correlation was found between TNF-α scores and the percentage of macrophages in lesions presenting thick fibrous connective tissue walls.

This study presents new and relevant information concerning macrophage locations near the dental apex, in the middle region of PGs, and subepithelial region of RCs. In addition, novel data is reported concerning M1 macrophage polarization and TNF-α immunoexpression in cyst epithelium, which stimulates epithelium proliferation, leading to RC development and growth. Lesions with cortical bone resorption presented higher TNF-α scores indicating that this cytokine plays an active inflammatory role mediated by the Th1 phenotype and M1 macrophages. This study found high levels of macrophages in small and initial lesions due to active antigenic stimuli. Macrophage subpopulations have been previously described[6,13], but associations between these cells and TNF-α, as well as comparisons with clinical information, were not available until now.

Conclusions

The association of $CD68^+/CD163^+$ ratios >1 with greater TNF-α immunoexpression suggest M1 phenotype polarization in both RC capsule and epithelium. The TNF-α scores 3 and 4 and high $CD68^+$ immunoexpression in the cyst epithelium confirms the role of this cytokine in epithelial proliferation and, consequently, in the increase of cyst size mediated by a proinflammatory Th1 response state. This cytokine also presented high scores in cases with cortical bone resorption. In contrast, the association of $CD68^+/CD163^+$ ratios <1 with higher $CD163^+$ immunoexpression suggests M2 phenotype polarization in PGs. PG lesions also had lower TNF-α scores. Thus, the immunomodulatory effect of Th2 allow for PG maintenance without the release of cytokines that directly stimulate epithelial proliferation.

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