ORIGINAL RESEARCH

Immunohistochemical Quantification of Macrophage Subpopulations M1, M2 in Periapical Cyst and Paradental Cyst using CD64 and CD163

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Abstract

Background: Periapical and paradental cysts, linked to pulpal or periodontal infections, contain inflammatory cells, including macrophages that polarize into M1 or M2 subtypes. While immune responses are crucial in controlling these infections, their role in cyst pathogenesis is unclear. With limited research on macrophage polarization in these cysts, this study aims to further explore their role in lesion development.

Aim: To identify and quantify M1 and M2 macrophage subpopulations in periapical and paradental cysts using immunohistochemistry

Materials and Methods: Sixteen histopathologically confirmed cases of both periapical and paradental cysts were analyzed. Immunohistochemical staining for CD64 and CD163 identified macrophage subpopulations, with positive cells displaying brown cytoplasmic staining. Only cells morphologically compatible with macrophage were counted, and the connective tissue capsule was divided into luminal and deeper zones. Macrophage density was evaluated in ten high-power fields per section, and the mean number of CD64+ M1 and CD163+ M2 macrophages per field was calculated, expressed as mean ± SD.

Results: The study found that both cyst types predominantly contain M1 macrophages, with periapical cysts showing a higher abundance of both M1 and M2 subpopulations. Periapical cysts had significantly more CD64+ M1 and CD163+ M2 macrophages, particularly in the luminal and deeper zones, compared to paradental cysts.

Conclusion: The predominance of macrophages in both periapical and paradental cysts supports their inflammatory origin. CD64+ M1 macrophages indicate pro-inflammatory activity, while CD163+ M2 macrophages suggest anti-inflammatory responses, highlighting the chronic nature of these lesions.

Keywords: CD64, CD163, Macrophages, Periapical cyst, Paradental cyst

Introduction

Inflammatory odontogenic cysts comprise an important group of jaw cysts which includes periapical cyst, paradental cyst and residual cyst. These cysts require a source of infection for the epithelial remnants of Malassez to be stimulated and begin the spread of infection. Some of these cysts, such as periapical cysts depend on endodontic infection, while paradental cysts require pericoronal infection.[1]

Periapical cysts, also known as radicular cysts, typically develop as a result of chronic inflammation stemming from infections within the dental pulp.[2] The involved tooth is non-vital. Paradental cysts, alternatively referred to as collateral cysts, on the other hand, are associated with chronic inflammation in the tissues surrounding the tooth, such as the periodontal ligament. These cyst are often misdiagnosed or under reported, and they typically develop around partially or fully erupted third molar teeth, often in a distal or distobuccal location. Patients frequently present with a history of pericoronitis or periodontitis, reflecting the chronic inflammatory background that leads to cyst formation.[3]

While the exact role of immunity in the pathogenesis of these lesions is not fully understood, the immune response is known to play an important role in controlling and resolving infection. These cystic lesions show a lymphocyte- and macrophage-rich inflammatory infiltrate. Besides their effector functions, macrophages also act as antigen-presenting cells capable of initiating and regulating

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immune responses, thereby contributing to the initiation and progression of cystic lesions.[3] Given the chronic inflammatory nature of inflammatory odontogenic cysts, evaluation of M1 and M2 macrophage polarization is important, as it influences the balance between persistent inflammation and tissue repair and thereby affects the pathogenesis and biological behaviour of periapical and paradental cysts.

Macrophages polarize into pro-inflammatory M1 or immunomodulatory M2 types, identifiable by their morphology, marker expression, and functions. A balanced M1/M2 ratio is essential for proper inflammation; failure to shift from M1 to M2 leads to chronic inflammation. M1 macrophages typically express CD80, CD86, CD16, CD32, CD64 and other Fc receptors, while M2 macrophages express markers such as CD163, CD204, and CD206.[4]

This study uses CD64 as the M1 marker and CD163 as the M2 marker. CD64 is a specific and reliable indicator of M1 macrophages due to its role in antibody-dependent cytotoxicity and immune complex clearance. CD163, a haemoglobin scavenger receptor, is a well-established macrophage-specific marker widely used to identify M2 macrophages and reflects tissue responses to inflammation.[5 6]

The pathogenesis of inflammatory jaw cysts remains unclear, but macrophages in the cyst wall appear central to their development. Understanding macrophage activation may help identify new diagnostic or therapeutic strategies. Although macrophage activity has been studied in periapical cysts, limited information exists for paradental cysts. Hence, the present study analyzes macrophage infiltration and M1/M2 marker expression in periapical and paradental cysts to provide insights into their immunopathogenesis.

Materials and Methods

The study was conducted at the Department of Oral and Maxillofacial Pathology. The sample size was calculated using nMaster 2.0 software based on hypothesis testing for comparison of two means with equal allocation. Considering a significance level (α) of 5%, power ($1-\beta$) of 80%, an expected mean difference (μd) of 0.62, and standard deviations of 1.59 and 1.90 for the two groups, the required sample size was 32, with 16 samples per group.

Clinically recorded and histopathologically confirmed cases of periapical cyst (n = 16) and paradental cyst (n = 16) were obtained from the archives of Department of Oral and Maxillofacial Pathology. Biopsy specimen from both genders in the age group of 20-70 years were included in this study. Cases without clinical information including age, gender, location of lesion, size of lesion were excluded. Biopsy specimen without a lining epithelium and sufficient connective tissue for histopathological examination were excluded. All specimens were obtained with informed consent from participants. Clearance was obtained from the Institutional Ethics Committee prior to commencement of study.

Sample processing

The paraffin embedded tissue section of 4 μm thickness obtained from archival tissues were studied for the expression of CD64 and CD168 by immunohistochemistry. Sections were hydrated with increasing grades of alcohol, brought to distilled water and treated with hydrogen peroxide to eliminate endogenous peroxidase activity. Then, antigen retrieval with TRIS EDTA buffer (pH 9.0) was carried out. The tissue was incubated sequentially with primary antibody CD64 [(Clone: (EPR4624)/HAM56) PathnSitu Biotechnologies Rabbit anti-human FCGRIA(CD64) Monoclonal Antibody] and CD163 [(Rabbit anti-human CD163 Monoclonal Antibody (Clone EP324)], which binds to specific tissue antigen on mononuclear inflammatory cells. Subsequently, secondary antibody labelled with HRP (PathnSitu) was added, followed by a brown chromogen substrate solution DAB for visualization and counter stained with hematoxylin.

CD64 and CD163

All the immunostained slides were scanned and analyzed for antigen localization and distribution. Cells exhibiting brown staining in the cytoplasm were defined as positive, regardless of the staining intensity. The stained sections were initially screened under a light microscope to facilitate identification of positively stained cells exhibiting brownish cytoplasmic staining. Macrophage counts were subsequently conducted under 40x magnification equipped with an ocular grid eyepiece, focusing exclusively on cells morphologically consistent with macrophages. The analysis started at the border between the epithelium and connective tissue and continued deeper into the connective tissue capsule, following the criteria established by Tsai et al.[7] The connective tissue capsule was stratified into luminal and deeper zones, with ten high-power fields (HPFs) selected per slide based on the highest macrophage densities observed. To ensure accuracy, each slide's representative areas were meticulously scanned from left to right to prevent duplication in counting. Two independent observers assessed all immunostained slides to minimize subjective bias, with interobserver agreement quantified using Kappa statistics. Total cell counts were tabulated for each sample using Microsoft Excel 2010, and mean numbers of CD64 and CD163 cells per microscopic field (40x) were computed and expressed with their respective standard deviations. Mean values and percentages of these cells were compiled and compared across cases.

Statistical analysis:

Categorical and quantitative variables were expressed as frequency (percentage) and mean ± SD respectively. Descriptive statistics characterized both the samples and clinical data. Inter-group comparisons utilized independent t-tests, while correlation analysis employed Pearson correlation coefficients to explore relationships between variables. Statistical analyses were performed by using a statistical software package SPSS, version 22.0.

Results

In the present study, the immunohistochemical expression of CD64+M1 and CD163+M2 was analyzed in periapical cyst and paradental cyst. A total of 32 samples were included in the study. The positive staining control consisted of a sample of liver tissue for CD64 and spleen tissue for CD163. The study

group consisted of 16 samples of periapical cyst and 16 samples of paradental cyst.

The age range for samples in the study groups were 40-88 years and 35-70 years with a mean age of 35.12 ± 8.37 years and 33.68 ± 14.44 years in periapical cyst and paradental cyst respectively. With reference to location, periapical cysts were mostly located in the maxilla (81%) and in the anterior region (75 %), while fewer in the posterior region (6% cases). On the other hand, all the paradental cysts were in the mandible region, 68% in the left posterior mandibular region and 31% in the right posterior mandibular region. Pathological features of periapical cyst and paradental cyst regarding size (grossly), inflammation, cyst wall thickness, and vascularization were included.[8] Most periapical cysts (68%) were less than 2 cm in size, with a smaller

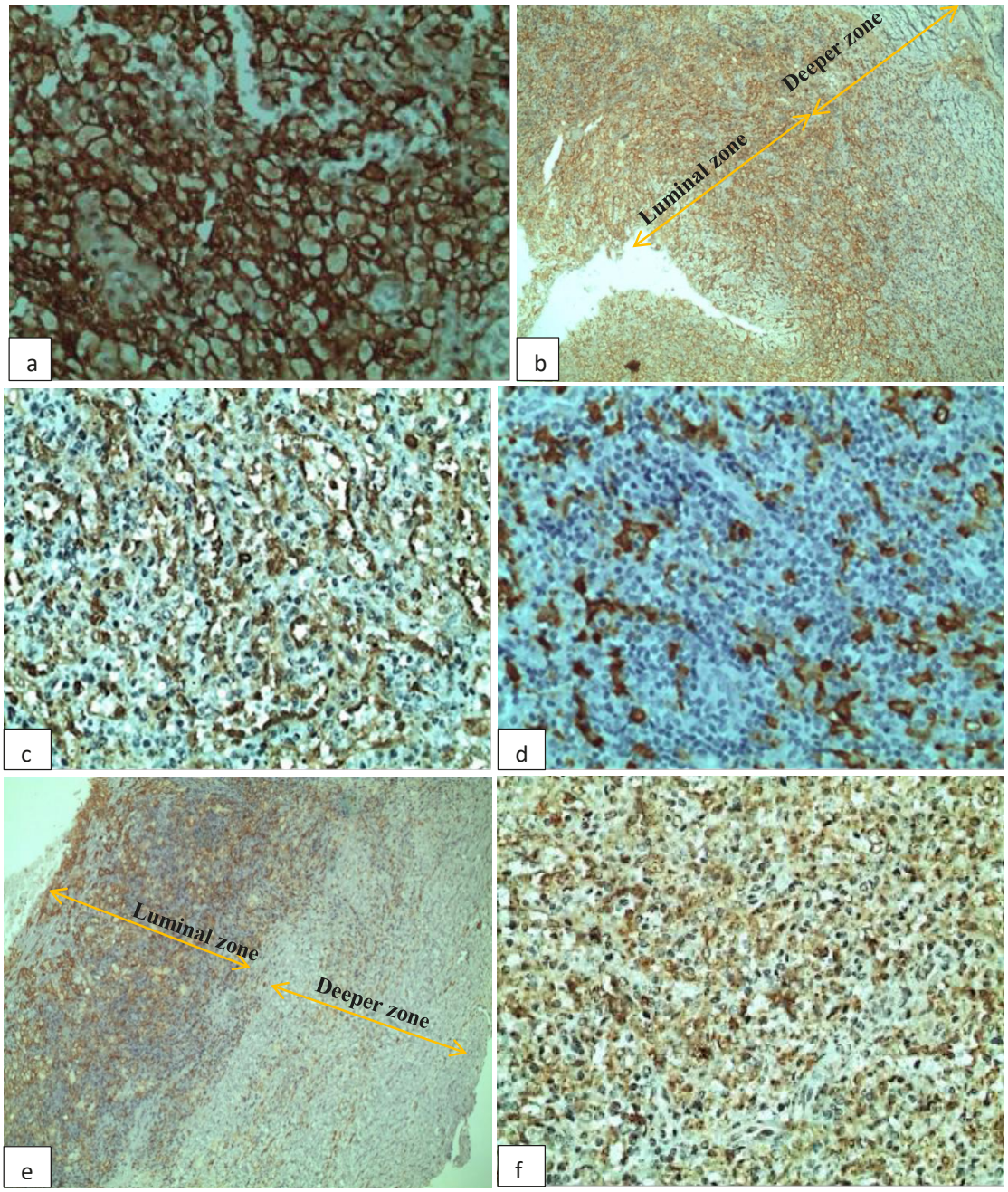

Fig. 1: a) Immunocytoplasmic staining of CD64 in periapical cyst (40x) **b)** Immunocytoplasmic staining of CD64 in luminal zone & deeper zone of connective tissue capsule (10x) of periapical cyst **c)** Immunocytoplasmic staining of CD64 in control, Liver (40x) **d)** Immunocytoplasmic staining of CD163 in periapical cyst (40x) **e)** Immunocytoplasmic staining of CD163 in luminal zone & deeper zone of connective tissue capsule (10x) **f)** Immunocytoplasmic staining of control, spleen tissue (40x)

percentage between 2-3 cm (25%) and only (6%) over 3 cm, while the majority of paradental cysts (75%) were also under 2 cm, with (12.5%) between 2-3 cm and none exceeding 3 cm. Both types of cysts exhibited intense inflammatory infiltrates, being highly inflamed (Grade III) based on the presence of more than 50 inflammatory cells per high power field. Histologically, these cysts demonstrated thick cystic walls (>1.5 mm), though there was no significant correlation between wall thickness and lesion type. Vascularization was predominantly low, with 68% of cases showing fewer than 10 blood vessels per mm^2 and 31.2% exhibiting moderate vascularization (11-15 blood vessels per mm^2). No cases exhibited high vascularization.

In this study, immunohistochemical analysis revealed notable differences in macrophage subsets between periapical and paradental cysts. (Fig 1, 2) In periapical cysts, CD64+ M1 macrophages were more abundant than CD163+ M2 macrophages. The overall count of M1 macrophages was 544.96 ± 214.76 cells, significantly higher than the M2 macrophage count of 312.93 ± 145.52 cells ($p<0.001$). Similarly, in paradental cysts, M1 macrophages outnumbered M2 macrophages, with an overall count of 234.34 ± 105.37 cells for M1 compared to 168.12 ± 72.14 cells for M2 ($p<0.001$).

When comparing the distribution of macrophages within the luminal and deeper zones, M1 macrophages were consistently more concentrated in the luminal zone than in the deeper zone for both types of cysts. (Fig 1, 2) In periapical cysts, the density of M1 macrophages was 752.50 ± 51.65 cells in the luminal zone, compared to 337.43 ± 27.76 cells in the deeper zone ($p<0.001$). In paradental cysts, the pattern was similar, with 325.43 ± 62.20 cells in the luminal zone and 143.25 ± 37.07 cells in the deeper zone ($p<0.001$). A similar trend was observed for M2 macrophages, though their overall numbers

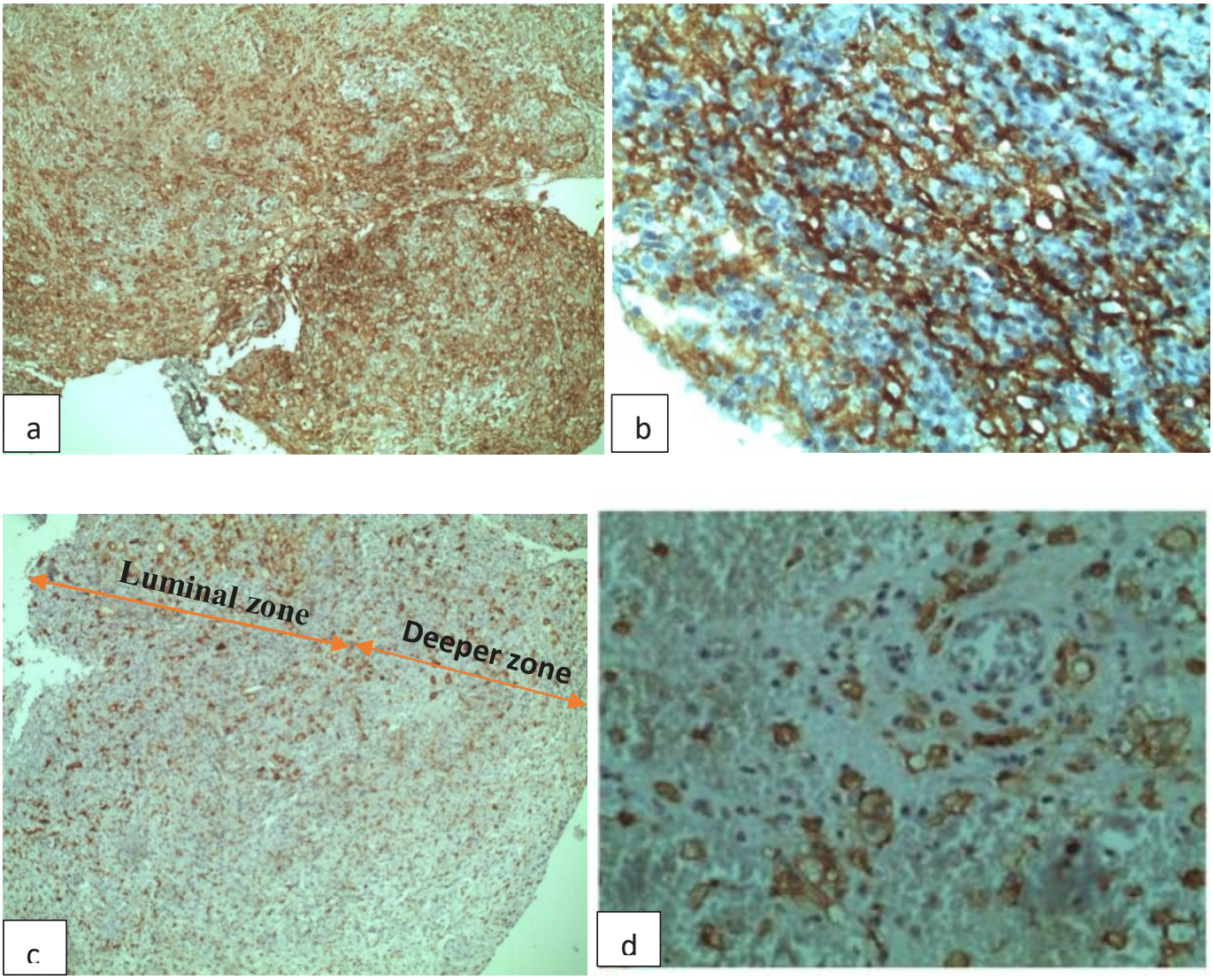

Fig. 2: a) Immunocytoplasmic staining of CD64 in paradental cyst (10x) **b)** Immunocytoplasmic staining of CD64 in paradental cyst (40x) **c)** Immunocytoplasmic staining of CD163 in paradental cyst (10x) **d)** Immunocytoplasmic staining of CD163 in paradental cyst (40x)

were lower than M1 macrophages in both zones. In periapical cysts, M2 macrophages were 453.37 ± 33.28 cells in the luminal zone and 337.43 ± 27.76 cells in the deeper zone ($p<0.001$), while in paradental cysts, their densities were 229.31 ± 39.80 cells in the luminal zone and 106.93 ± 34.44 cells in the deeper zone ($p<0.001$). These findings demonstrate a clear predominance of M1 macrophages over M2 macrophages, particularly in the luminal zone, in both periapical and paradental cysts. The results underscore a significantly higher presence of both M1 and M2 macrophages in periapical cysts, reflecting a more pronounced inflammatory response in these lesions compared to paradental cysts. (Graph 1)

The Pearson correlation analysis revealed varying relationships between M1 and M2 macrophage subsets in periapical and paradental cysts. In the luminal zone, weak negative correlations were observed: the correlation coefficient for M1 and M2 macrophages in periapical cysts was -0.072, while in paradental cysts, it was -0.101. These values suggest that as the density of M1 macrophages increases, the density of M2 macrophages decreases, albeit weakly. Table 1 provides a detailed summary of these correlation values for both zones and cyst types. Similarly, in the deeper zone, the correlations were weak and negative, with coefficients of -0.084 for periapical cysts and -0.143 for paradental cysts, indicating a slight inverse relationship between M1 and M2 macrophages. When examining the correlation between M1 and M2 macrophages within the same zone, periapical cysts in the luminal zone showed a weak positive correlation with a coefficient of 0.030, while paradental cysts had a slightly stronger weak positive correlation of 0.237. In contrast, in the deeper zone, periapical cysts displayed a perfect positive correlation between M1 and M2 macrophages with a coefficient of 1.000, while paradental cysts showed a moderate positive correlation with a coefficient of 0.538. These are summarized in Table 2. The findings indicate that M1 and M2 macrophages in the deeper zone of periapical cysts are perfectly positively correlated, while in the luminal zones of both types of cysts, the correlations are weakly positive.

Discussion

Periapical and paradental cysts are inflammatory odontogenic cysts arising in relation to teeth. Both contain inflammatory infiltrates, including macrophages, which play key roles in innate immunity through their dual phenotypes , M1 and M2, which play distinct functions in inflammation and tissue repair.

Several studies have evaluated immune cells in periapical lesions. An ex vivo study reported a higher $CD68^+/CD163^+$ ratio in radicular cysts than in periapical granulomas (median 2.05 vs 1.26), indicating predominant M1 polarization.[9] Another study similarly found a higher $CD68^+/CD163^+$ ratio and increased TNF-α expression in radicular cysts, supporting an M1-skewed inflammatory state driving cyst progression.[10] Further work has shown dense macrophage, lymphocyte, and plasma cell infiltration, with elevated CD68 and TGF-β1 expression in chronic periapical lesions, underscoring the

Graph 1: Overall density of CD64+M1 and CD163+M2 macrophages in luminal and deeper zone of periapical and paradental cyst

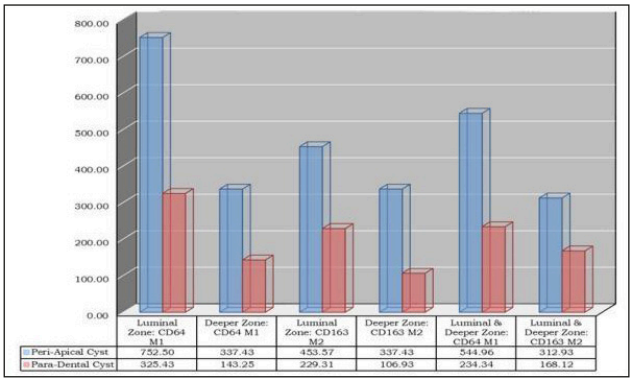

Table 1: Pearson correlation statistics among a particular zone between periapical cyst and paradental cyst

		Pearson's Correlation Coefficient
Luminal Zone: CD64 M1	Periapical Cyst	– 0.101
	Paradental Cyst	
Deeper Zone: CD64 M1	Periapical Cyst	– 0.143
	Paradental Cyst	
Luminal Zone: CD163 M2	Periapical Cyst	– 0.072
	Paradental Cyst	
Deeper Zone: CD163 M2	Periapical Cyst	– 0.084
	Paradental Cyst	

Table 2: Pearson correlation statistics among luminal zone and deeper zone of periapical cyst and paradental cyst

		Pearson's Correlation Coefficient
Periapical Cyst	Luminal Zone: CD64 M1	0.030
	Luminal Zone: CD163 M2	
	Deeper Zone: CD64 M1	1.000
	Deeper Zone: CD163 M2	
Paradental Cyst	Luminal Zone: CD64 M1	0.237
	Luminal Zone: CD163 M2	
	Deeper Zone: CD64 M1	0.538
	Deeper Zone: CD163 M2	

central role of macrophages in lesion maintenance.[11] Thus, whereas periapical cysts are fairly well studied with regards to immune-cell composition — particularly macrophage M1/M2 polarization — there is a striking lack of comparable data for paradental cysts, representing a notable gap in the literature on these cysts.

Gross examination revealed that the majority of cases were less than 2 cm in size. Microscopic analysis showed that all cystic samples had thick capsules. Furthermore, most cases displayed grade III inflammation and a low level of vascularization.

In this study, CD64 was used as the M1 marker and CD163 as the M2 marker to evaluate macrophage subpopulations in periapical and paradental cysts. CD64, or HAM56 is a transmembrane glycoprotein involved in antibody-dependent cytotoxicity and immune complex clearance—functions characteristic of M1 macrophages—and shows high specificity for M1 polarization with minimal expression in other immune cells.[12] CD163, a hemoglobin scavenger receptor specific to the monocyte–macrophage system, reflects tissue inflammatory responses and helps distinguish macrophages from dendritic cells. Its high expression is strongly associated with M2 macrophages, making it a well-established and widely used M2 marker.[13]

Previous literatures related to periapical cyst macrophage quantification, used the pan macrophage marker CD68. To the best of our knowledge, this is the first report comparing the expression and presence of these macrophage phenotypes in periapical cyst and paradental cyst. Moreover the present study also evaluated the macrophage populations in the luminal and deeper zones of connective tissue capsule.

Our findings demonstrate significant macrophage infiltration and polarization into M1 and M2 subtypes within these cysts. Importantly, the M1 macrophage subpopulation was identified as the dominant type in both periapical and paradental cysts. In periapical cysts, CD64+ M1 macrophages were more prevalent than CD163+ M2 macrophages. This pattern was also observed in paradental cysts, where M1 macrophages outnumbered M2 macrophages. Both CD64+ M1 and CD163+ M2 macrophages were predominantly concentrated in the luminal zone of the cystic regions, with fewer in the deeper zones. Notably, periapical cysts exhibited a higher density of M1 macrophages in both the luminal and deeper zones compared to paradental cysts, indicating a more pronounced pro-inflammatory response. These observations align with previous research showing elevated levels of interferon-gamma and tumor necrosis factor-alpha, cytokines associated with M1 macrophages, in periapical cysts.[14,15] Our findings in paradental cyst provide information in this lacuna. The findings underscores the dynamic interplay between inflammatory and reparative phases within cystic lesions, mediated by macrophage polarization and cytokine profiles. These findings provide valuable insights into the immune response and inflammatory processes in these cystic lesions.

Pearson correlation analysis identified different patterns of association between M1 and M2 macrophages in periapical and paradental cysts. In the luminal zone, weak negative correlations were observed, indicating that as M1 macrophages increase, M2 macrophages decrease slightly. In the deeper zone, correlations were also weak and negative. Within the same zone, periapical cysts showed weak positive correlations between M1 and M2 macrophages in the luminal zone, while paradental cysts had a slightly stronger positive correlation. In the deeper zone, periapical cysts displayed a perfect positive correlation between M1 and M2 macrophages, while paradental cysts showed a moderate positive correlation. Overall, M1 and M2 macrophages are perfectly correlated in the deeper zone of periapical cysts and weakly positively correlated in the luminal zones of both cyst types.

Based on the findings of our study, the predominance of macrophages in periapical cyst and paradental cyst indicates and supports an inflammatory origin. In addition, a significant difference in macrophage subpopulation existed between these two lesions.

The study is limited by its small sample size and constraints of immunohistochemical analysis, affecting result interpretation. Future research with larger sample sizes and enhanced cell quantification method is needed to validate findings. Further investigation could explore tissue destruction mechanisms and potential immune-modulating agents for disease resolution and tissue repair.

Conclusion

Based on the results, it can be concluded that both proinflammatory (M1) and anti-inflammatory (M2) macrophages are present in periapical and paradental cysts, with a higher prevalence of M1 macrophages, potentially influencing disease progression. These macrophages play a significant role in the biological behaviour of these cysts, particularly in the luminal zone. This investigation contributes valuable insights into the immunopathogenesis of periapical and paradental cysts. Future research directions could explore therapeutic strategies targeting macrophage polarization to modulate inflammatory responses and promote tissue regeneration in odontogenic lesions.

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