



Original Article

Macrophage phenotypes and Gas6/Axl signaling in apical lesions



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KEYWORDS

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Abstract *Background/purpose:* Macrophages participate in the periapical inflammation with pro-inflammatory M1 cells and anti-inflammatory M2 cells. Gas6/Axl signal is the responsible pathway for the activation of M1 and polarization of M2. The aim of this study was to compare the number of CD16⁺ M1 cells, CD206⁺ M2 cells, and Gas6/Axl expression between apical granulomas and radicular cysts.

Materials and methods: Twenty-four cases of granuloma and twenty of cysts were submitted to immunohistochemistry using anti-CD16 and anti-CD206 antibodies for determining M1 and M2 macrophages and investigating the cells with positive Gas6 and Axl expression.

Results: There were more numerous of M1 macrophages in radicular cysts (175.9 ± 87.7) compared to apical granuloma (116.6 ± 55.8), and M2 macrophages was higher in cysts (204.0 ± 97.6) than granuloma (152.9 ± 64.6). The level of Gas6/Axl expression were similar. There was a significant different in M1 macrophage ($P = 0.014$) between two diagnosis. In patients with or without root resorption, the number of M1 were 194.6 ± 57.2 compared with 139.1 ± 79.6 . The number of M2 were 241.7 ± 81.4 and 164.6 ± 77.1 . The expression of Axl was stronger in root resorption patients (191.1 ± 43.6), but the tendency in Gas6 expression was similar. Significant differences were noted in high M2 infiltration and Axl positive lesions.

Conclusion: It appears that macrophages associated with significantly higher numbers in

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radicular cysts than apical granuloma. Meanwhile, macrophages and Axl receptor was intensively expressed in patients with root resorption, related to severe inflammation.

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Introduction

Apical periodontitis is caused by a bacterial infection of the pulp of the originating tooth. The infection source, typically caused by trauma or caries at the crown area, extends to the apex of the tooth and induces an immune response in the periapical tissue. The nonspecific immunologic reactions result in tissue destruction and bone resorption that typify apical periodontitis.¹ The majority of apical periodontitis can be classified as two types of diagnosis. One is a granuloma, which is a focal area of granulomatous inflammation characterized by the presence of activated macrophages. The other is a cyst, which is formed by the inflammatory proliferation of epithelial cells in the inflamed periodontal ligament.² Although granulomas and cysts are difficult to distinguish through clinical treatment or radiographic imaging, the diagnosis can be confirmed using a specimen from an apicectomy.

Once the endodontic infection occurs, macrophages become the predominant cell at the inflammatory site.³ They start to increase after pulp exposure,⁴ and become the primary inflammatory cells at the apical lesion.⁵ Furthermore, macrophages locate at various areas between the cysts and the granulomas.⁶ For example, Wolfgang indicated that macrophages were strictly restricted to the apex area or accumulated toward the cyst epithelium. In granuloma tissue, the number of macrophages decreases from the center of the lesion in peripheral directions.⁶ Metzger et al. determined that macrophage activation may occur in periapical granulomas through the production of cytokines and that not all macrophages were activated in an apical granuloma.⁷ Therefore, the distribution and function of macrophages may vary between cysts and granulomas.

Two major types of activated macrophages, M1 and M2 macrophages, have been reported and studied.^{8–10} M1 macrophages, which are the classically macrophage have a proinflammatory effect after bacterial infection through their mediation of the immune response. M2 macrophages, which are alternative macrophages, play important roles in immunosuppression and tissue repair.⁸ These two types of macrophages have opposite functions: one is inflammatory and the other is anti-inflammatory. However, the roles and distributions of M1 and M2 macrophages in apical periodontitis remain unclear.

Axl is an immune factor relating to infection¹¹ that can also be a macrophage marker in an inflamed area.¹² According to a report, Axl can be considered as a marker of M1 activation, which is elevated by IL-4.¹³ However, the influence of Axl depends on the microenvironment. For example, Axl signaling in oral squamous cell carcinoma induces M2 polarization.¹⁴ Moreover, Gas6, a receptor of

Axl, activates the host-cell-like macrophages.¹¹ According to Toshifumi, Axl ligated to Gas6 and induced macrophages in a mouse airway, but it could not active the interstitial macrophages and other lung leukocytes.¹² The relationship between Axl and Gas6 depends on the tissue and disease.

Many studies have reported on the roles of M1 and M2 macrophages and Axl and Gas6 in inflammatory diseases, but their roles in apical periodontitis remain unknown. In this study, we used immunohistochemistry (IHC) to examine the distributions of M1 and M2 macrophages and Axl and Gas6 in apical granulomas and cysts. The association of the distributions of M1 and M2 macrophages and Axl and Gas6 expressions with the clinical and pathological features of apical cysts and granulomas were also analyzed.

Material and methods

Sample collection

Samples were obtained and selected from patients who underwent apicectomy in the Endodontic Department of Dentistry, National Defense Medical Center, Taipei, Taiwan. Patients with records missing from their charts or pathology results and those who had not received a differential diagnosis were excluded, and 44 patients remained. The study design was approved by the Institutional Review Board of the Tri-Service General Hospital (TSGHIRB 1-106-05-00). In this study, the diagnosis of root resorption is adapted from the previous study.¹⁵ Brief, the periapical film taken before surgery were used to evaluate if there is root resorption or not. Radiography was available in thirty-eight cases, in which radiolucent indentation with shortening of root-tip is diagnosed as root resorption. A total of 24 pathologic diagnosis cases of apical granulomas and 20 cases of periapical cysts were examined in our study. Cysts presented as fully developed cavities lined by epithelia of variable thicknesses, and periapical granulomas exhibited a severe infiltration of inflammatory cells without epithelial lining. Samples were selected and subjected to analysis using IHC.

IHC assay

A series of 4- μ m sections were cut from each tissue block. Sections were dewaxed and subjected to microwave antigen retrieval. The tissue sections were then incubated at 4°C overnight with the following primary antibodies following the manufacturers' recommendations: CD16 antibody ([1:500 dilution]; Abcam, Cambridge, UK), CD206 antibody ([1:500 dilution]; Abcam, Cambridge, UK), Axl

antibody ([1:500 dilution]; Abcam, Cambridge, UK), and Gas6 antibody ([1:500 dilution]; BIOWORLD, USA). After washing three times, a secondary biotinylated antibody was added for 30 min at 25 °C, followed by diaminobenzidine as a chromogen. These tissue sections were lightly counterstained with hematoxylin and examined under an optical microscope. Positive cell counts were quantitatively determined as described previously.¹⁴ Macrophages were counted in the three most confluent microscopic fields ("hot spots"). The mean count was the average of the positive cell counts from the three areas.

Statistical analysis

Positive cells are presented as mean \pm standard deviation (SD), and IHC results were analyzed using the t test. Statistical analysis was performed using SPSS 22.0 (SPSS, Chicago, USA). A significance level of $P < 0.05$ was established for all statistical analyses.

Results

Patient characteristics

A total of 44 patients were included in our study, consisting of 19 male and 25 female patients. Among the male patients, 10 were diagnosed as having cysts and 9 were diagnosed as having granulomas. Among the female patients, 10 were diagnosed as having cysts and 15 were diagnosed as having granulomas. No significant difference was exhibited between diagnosis and gender (Table 1). For all patients, the average age was 45 years. Most of the patients were aged between 50 and 59 years (34%), followed by 40–49 years (27%).

Incisors were the teeth most frequently subjected to apicectomy with 26 cases (59.1%), followed by 11 cases

concerning premolars (25%), 4 cases concerning molars (9.1%), and 3 cases concerning canines (6.8%). Furthermore, nine (20%) patients experienced an external resorption at the root area before surgical treatment.

Distribution of M1 and M2 macrophages in cysts and granulomas

To classify the distributions of M1 and M2 macrophages in cysts and granulomas, we used CD16 and CD206 to determine the presence of macrophages. Fig. 1 depicts the positive cell expressions of different markers. Positive cell numbers (mean \pm SD) of CD16 + in cysts and granulomas were 175.9 ± 87.7 and 116.6 ± 55.8 , respectively. The CD16 positive cells were present at a higher level in periapical cysts than in apical granulomas ($P = 0.014$; Table 2). Also, the numbers of CD206 positive cells in cysts and granulomas were 204 ± 97.6 and 152.9 ± 64.6 , respectively. The CD206 positive cells were present at higher levels in periapical cysts than in apical granulomas ($P = 0.054$; Table 2) (and Fig. 2). These results indicate that cysts exhibited more macrophages in both M1 and M2 phenotypes. Next, we also assessed the expressions of Axl and Gas6 in cysts and granulomas. The numbers (mean \pm SD) of Axl positive cells in cysts and granulomas were 153.1 ± 62.2 and 151.1 ± 58.7 , and the numbers of cells marked for Gas6 were 145.0 ± 67.8 and 145.0 ± 69.8 . However, no statistically significant differences were identified with respect to the presence of Axl and Gas6 (see Table 2 and Fig. 2).

Root resorption patients express more Axl and M1 and M2 macrophages

We further analyzed the association of the numbers of M1 and M2 macrophages and Axl and Gas6 expression with root resorption. The average numbers of CD16 positive cells in patients with or without root resorption were 194.6 ± 57.2 and 139.1 ± 79.6 ($P = 0.061$), respectively (Fig. 3 and Table 3). The average numbers of CD206 positive cells in patients with and without root resorption were 241.67 ± 81.4 and 164.6 ± 77.1 ($P = 0.014$, Table 3), respectively. The average numbers of Axl positive cells with or without root resorption were 191.1 ± 43.6 and 147.2 ± 61.5 ($P = 0.028$, Table 3), respectively. The numbers with respect to Gas6 expression in groups with or without root resorption were 163.1 ± 88.4 and 140.8 ± 63.1 ($P = 0.406$), respectively. Significant higher number of CD206 and Axl positive cells was noted in the root resorption group (Fig. 3).

Discussion

Periapical periodontitis is the occurrence of an inflammatory lesion around the apex of a tooth. According to the study of Manal et al., 33% of radiolucent jaw lesions were apical cysts and 40% were apical granulomas.¹⁶ Apical cysts are more common at the posterior teeth of the mandible, whereas granulomas usually occur at the posterior teeth of the maxilla.¹⁷

Table 1 Clinical and pathology characteristics of patients.

	Total patients (N = 44)
Gender, n (%)	
Male	19 (43.2%)
Female	25 (57.1%)
Age, n (%)	
< 50 years	24 (54.5%)
\geq 50 years	20 (45.5%)
Teeth, n (%)	
Incisor	26 (59.1%)
Canine	3 (6.8%)
Premolar	11 (25%)
Molar	4 (9.1%)
Diagnosis, n (%)	
Cyst	20 (45.5%)
Granuloma	24 (54.5%)
Root resorption	
Y	9 (20.5%)
N	35 (79.5%)

General data: Gender, age, tooth, diagnosis at apical lesion, root resorption.

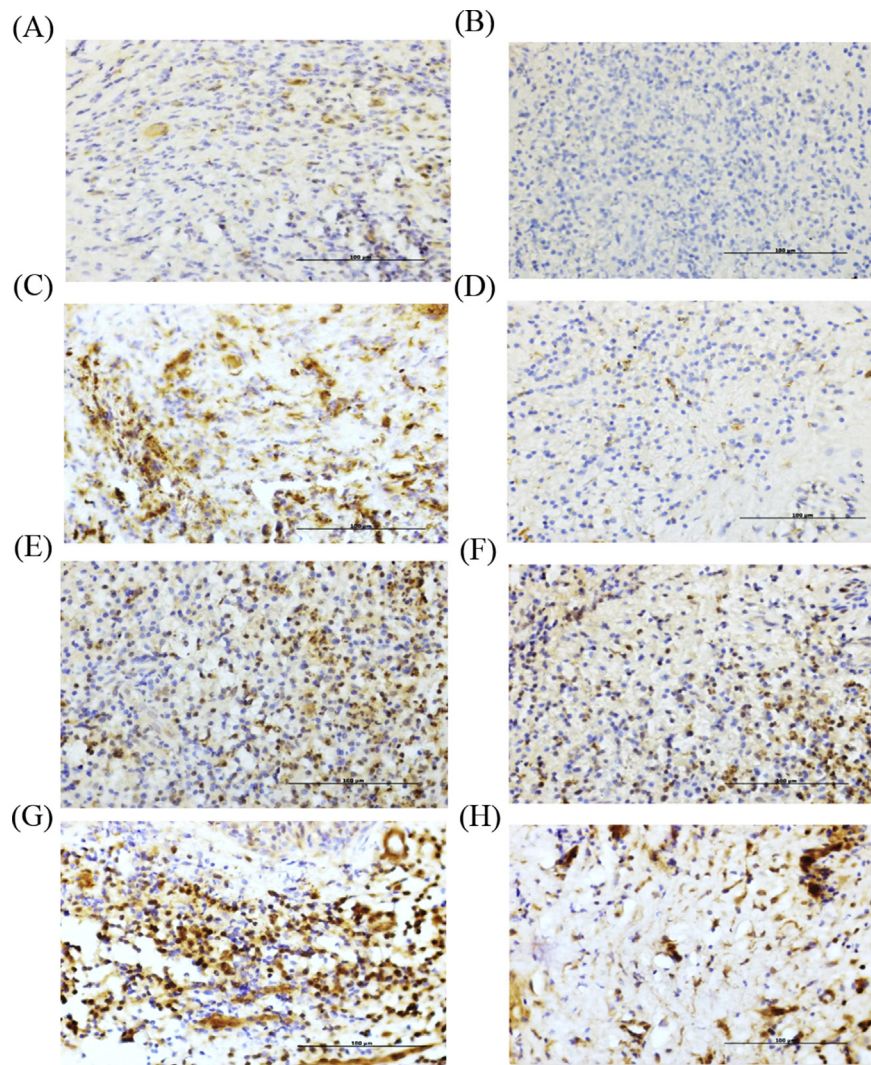


Figure 1 Immunochemistry stain results in periapical cysts and granulomas. Immunohistochemical staining periapical periodontitis. By immunohistochemistry, we evaluated the comparison of immunohistochemistry in apical cysts and granulomas. Representative case of (A, B) CD16 positive cells in cyst and granuloma (C, D) CD206 positive cells in cyst and granuloma (E, F) Axl positive cells in cyst and granuloma (G, H) Gas6 positive cells in cyst and granuloma All images were $400\times$, Scale bar: 100 nm.

Periapical lesions (included cyst and granuloma) are inflammatory reactive disease, in which macrophages play an indispensable role in disease process and the distribution of macrophages in apical lesions are different. Many studies

have reported on the roles of M1 and M2 macrophages in inflammatory diseases, but their roles in apical periodontitis remain unknown. According to previous study, the distribution of macrophages in apical lesions are different.^{6,18,19} For example, the role of different phenotypes macrophages M1 and M2 could be opposite in periapical root resorption.¹⁸ M1 macrophages promote inflammation through the secretion of proinflammatory cytokines such as $\text{TNF-}\alpha$ and NO, which leads to the activation of osteoclasts. In contrast, M2 macrophages have an inhibitory effect on inflammation mediated by interleukin-10, leads to the activation of cementoblast.¹⁸

Some studies have focused on cells at periapical lesion sites. According to Carlos, $\text{IFN-}\gamma$ protein levels are high in radicular cysts, whereas interleukin 4 (IL-4) expression is strong in samples of periapical granulomas.⁹ Ihan determined that periapical granulomas contained a higher proportion of activated T-cells than cysts, and that T-helper 2 cells and IL-4 play important roles in cyst formation.²⁰

Table 2 Relationships between various diagnoses and IHC positive cells.

Marker	Number of positive cells Mean \pm SD		P
	Cyst (N = 20)	Granuloma (N = 24)	
CD16	175.9 \pm 87.7	116.6 \pm 55.8	0.014*
CD206	204.0 \pm 97.6	152.9 \pm 64.6	0.054*
Axl	153.1 \pm 62.2	151.1 \pm 58.7	0.914
Gas6	145.0 \pm 67.8	145.0 \pm 69.8	0.998

Numbers of positive cells: mean \pm SD; * indicates a significant difference ($P < 0.05$, t-test analysis using SPSS 22.0).

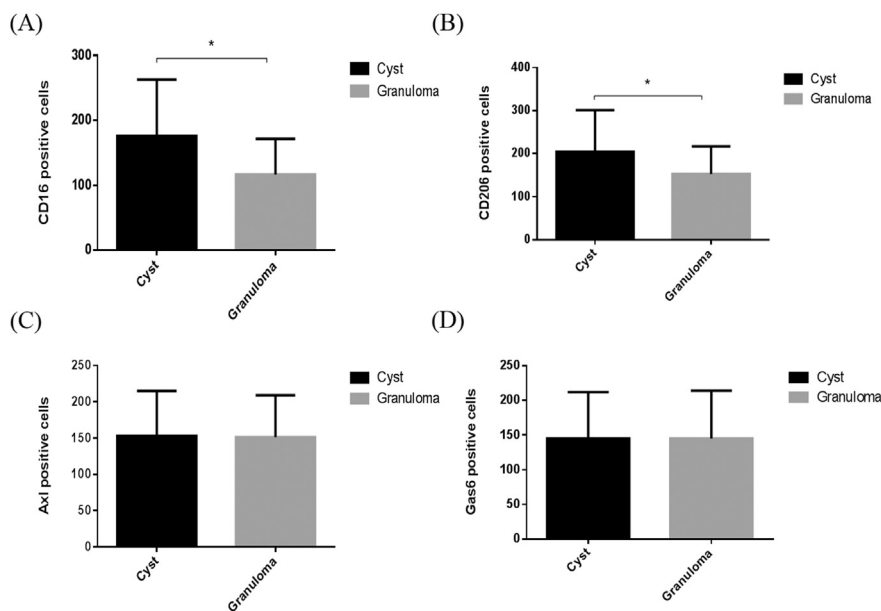


Figure 2 Quantitation of CD16, CD206, Axl, and Gas6 in cysts and granulomas. (A) CD16 positive cells in cyst and granuloma (B) CD206 positive cells in cyst and granuloma (C) Axl positive cells in cyst and granuloma (D) Gas6 positive cells in cyst and granuloma. Positive cells were counted in the three most confluent microscopic fields. Data are expressed as mean ± SD. * indicates a significant difference ($P < 0.05$).

In our study, we demonstrated that periapical cysts have high levels of M1 and M2 macrophages. According to another study, $IFN-\gamma$ is more present in radicular cysts, which induces the polarization of M1 phenotypes and might explain why M1 cells are present in a higher numbers in cysts.^{13,20} In addition, researchers determined that M2 macrophages are stimulated by IL-4. Although IL-4 is more

typically expressed in relation to granuloma diagnosis, it could still be related to the disease progression of cysts.^{13,20}

We also explored the mechanism through which macrophages appear in apical lesions through cytokines. According to relevant research, Axl can be deemed a marker of M1 activation, which is elevated by IL-4 and $IFN-\gamma$.¹³

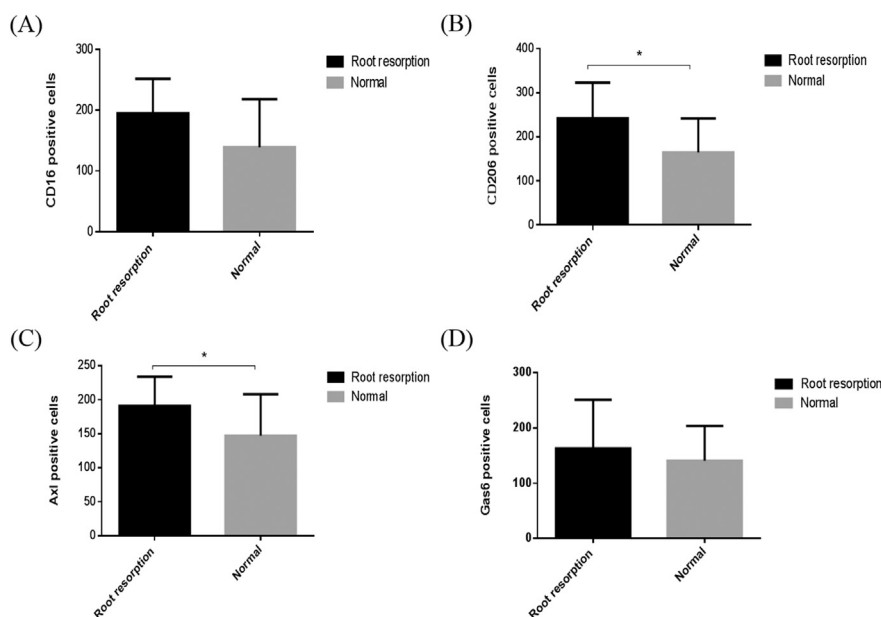


Figure 3 Quantitation of CD16, CD206, Axl, and Gas6 with and without root resorption. (A) CD16 positive cells in patients with/without root resorption (B) CD206 positive cells in patients with/without root resorption (C) Axl positive cells in patients with/without root resorption (D) Gas6 positive cells in patients with/without root resorption. Positive cells were counted in the three most confluent microscopic fields. Data are expressed as mean ± SD. * indicates a significant difference ($P < 0.05$).

Table 3 Relationship between root resorption status and IHC positive cells.

Marker	Number of positive cells Mean \pm SD		P
	With root resorption (N = 9)	Without root resorption (N = 29)	
CD16	194.6 \pm 57.2	139.1 \pm 79.6	0.061
CD206	241.7 \pm 81.4	164.6 \pm 77.1	0.014*
Axl	191.1 \pm 43.6	147.2 \pm 61.5	0.028*
Gas6	163.1 \pm 88.4	140.8 \pm 63.1	0.406

Numbers of positive cells: mean \pm SD; * indicates a significant difference ($P < 0.05$, t-test analysis using SPSS 22.0).

However, Takehiko discovered that anti-Axl treatment significantly increased M1 macrophage levels.²¹ Activation of the Axl receptors in macrophages also leads to a switch from an M1 to an M2 phenotype in tumors.²² In our study, a similar number of Axl positive cells occurred in both cysts and granulomas (Table 2). Overall, the influence of Axl on macrophages depends on the microenvironment. Therefore, it is possible that the role of the Axl signal in macrophage phenotypes is tissue or disease dependent.

Pulp infection is the most common factor in root resorption.¹⁰ The infected pulp may stimulate the inflammatory process and osteoclastic activity, thus destroying the root structure. Radiolucency is evident in the external root surface of the dentin walls and adjacent bone.¹⁰ Clinically, external apical root resorption is associated with severe apical destruction, and it is related to a poor prognosis after endodontic treatment. According to Leslie, root resorption was present more often with a diagnosis of pulp necrosis than with normal pulp, reversible pulpitis, or irreversible pulpitis, but no association was apparent between root resorption and the diagnosis of the periapical lesions.²³ We did not discover an association between periapical lesions and root resorption, but further study should be undertaken to clarify the association between the disease process and root resorption.

In our results, we discovered statistically significant differences in the numbers of both M1 and M2 macrophages in root resorption patients. According to other studies, the imbalance of M1 and M2 monocytes contributed to the formation of osteoclasts and was related to bone destruction diseases such as rheumatoid arthritis.²⁴ Furthermore, M1 activation increased the expression of proinflammatory cytokines and the production of reactive oxygen species, which are directly associated with age-related chronic inflammatory diseases.²⁵ Macrophages not only promote inflammation and tissue dysfunction but also are essential for the resolution and healing of inflammation as well as the maintenance of tissue homeostasis.²⁵ Iglesias determined that M1 macrophages promote inflammation through the secretion of proinflammatory cytokines such as TNF- α and NO, which leads to the activation of osteoclasts, thus destroying the bone and root.¹⁸ By contrast, M2 macrophages have an inhibitory effect on inflammation mediated by interleukin-10.¹⁸ This might explain why the total number of macrophages

increased in the root resorption area and could explain the results of our study.

Our result also determined that Axl expression was high in patients with root resorption. Documented reported have demonstrated that Axl/Gas6 expression in multiple types of cells included fibroblast and other stromal cells that are important component cells of periodontium.²⁶ According to other relevant studies, Axl can promote NF- κ B expression through the PI3K-AKT pathway and affected the development of macrophages in the bone marrow area.²⁷ Moreover, NF- κ B was related to cytokines with respect to bone destruction and cyst formation.²⁸ Notably, Gas6 also can bind to other receptor like MerTK and TYRO3, which may cause the difference between these two groups not so significantly.²⁹ Investigating the mechanism of Axl in apical periodontitis should be of interest for further researching.

In conclusion, we determined that the numbers of M1 and M2 macrophages were higher in cysts than in granuloma. The expressions of M1 and M2 macrophages and Axl were also stronger in root resorption teeth and may be associated with severe inflammation. Establishing the relationship between pathology and clinical presentation is considerable interest, and the additional mechanisms is still waiting for exploration.

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