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Original Article

Chemokine receptor-2 deficiency induced mild experimental periapical lesion in mice

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Abstract *Background/purpose:* Macrophages are considered to play an important role in the development of chronic apical periodontitis (CAP). However the function of tissue resident macrophages in CAP is unclear. This study aims to investigate the potential role of macrophages of different origins in CAP.

Materials and methods: Chemokine receptor-2 deficiency ($CCR2^{-/-}$) mice and C57BL/6N mice (control group, WT mice) were used to induce apical periodontitis. The pulp of mandibular first molars of both sides were exposed to the oral environment. After 0, 7, 21, 28 days of pulp explosion, animals were sacrificed, the mandibular bones were collected and scanned with micro-CT, further processed for HE & IHC Staining to analyze the development of CAP, as well as the expression of surface markers of macrophages.

Results: Both $CCR2^{-/-}$ and WT mice exhibited CCR2 negative macrophages in normal periapical area, which indicated the presence of tissue resident macrophages. CCR2 deficiency decreased the number of macrophages in periapical lesions, the M1 type macrophages' number as well as osteoclasts around the edge of the lesion decreased compared to wild type. Meanwhile CCR2 deficiency decreased the volume of periapical lesion significantly compared to wild type, but did not inhibite and disappeare the lesion thoroughly.

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Conclusion: Monocyte-macrophage system derived macrophages promote the progression of periapical lesions, while tissue resident macrophages in periodontal ligament might also be involved in the progression of periapical lesion.
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Introduction

Chronic Apical Periodontitis (CAP) is an infectious inflammatory disease affecting apical periodontal tissues, with a global incidence rate of 5% of teeth.[1] It often leads to pain and discomfort during biting, as well as increased systemic levels of inflammatory markers *in vivo*,[2,3] consequently diminishing patients' quality of life. The primary cause of this disease is a mixed bacterial infection, predominantly by anaerobic bacteria, which results in the necrosis of dental pulp tissue.[4] The hallmark symptoms are the infiltration of inflammatory cells around the apical foramen and the resorption of alveolar bone.[5,6] Acting as the first line of defense against bacterial infections, the innate immune system halts the spread of bacteria and their products to periapical tissues through the apical foramen.[7,8] Previous studies have demonstrated that the replacement of neutrophils by macrophages as the dominant immune cells signifies the transition of apical periodontitis to its chronic stage.[9,10] In this stage, monocyte-macrophage exhibit the CCR2 chemokine receptor on their surface, activated mainly by the MCP-1/CCR2 axis, to function within the local tissues.[11—13] The MCP-1/CCR2 axis has been shown to be associated with the progression of periodontitis.[14] Moreover, during the progression of apical periodontitis in rats and humans, a significant upregulation of MCP-1 and CCR2 expression has been observed.[11,15] Intriguingly, the application of intracanal metformin dressing in apical lesions can accelerate the healing of chronic apical periodontitis by inhibiting monocyte recruitment and inducible nitric oxide synthase, underscoring the significant impact of the monocyte-macrophage system in the evolution and treatment of chronic apical periodontitis.[16,17]

Research on macrophages reveals that the majority of macrophages with different origins can be classified as tissue-resident macrophages or the monocyte-macrophage system.[18—20] Monocyte-macrophage system derived macrophages might mainly appear during infection and inflammation processes, while tissue-resident macrophages are thought to contribute to organogenesis, promote tissue regeneration, and maintain local homeostasis and immune surveillance.[21—23] The dysfunction of resident macrophages might lead to auto-inflammatory diseases and cancer.[24] It is important to note that the role of tissue-resident macrophages varies across different organs and tissues.[25,26] Cardiac resident macrophages were found to promote clearance and degradation of apoptotic cardiomyocytes, while peritoneal tissue-resident macrophages were found to control the early stage of neutrophil recruitment during tissue inflammation. Some of them have self-renewal ability, and some rely on the supplementation of the circulatory system.[25—28] It has been proved that there are resident macrophages in normal periodontal ligament tissues.[29] A recent single-cell sequencing study uncovered the presence of tissue-resident macrophages in the mandibles of wild-type mice, although their specific distribution remains elusive.[30] Hence, further exploration is required to discern whether the function of tissue-resident macrophages in periapical tissues deviates from that of macrophages derived from the monocyte-macrophage system.

In this study, aiming to investigate the role of tissue-resident macrophages in the pathogenesis of chronic apical periodontitis, $CCR2^{-/-}$ mice were chosen to establish a chronic apical periodontitis model, and wild-type mice were set as controls. The histological alterations between the two groups during the chronic apical periodontitis progression were compared. We assumed that the absence of CCR2 would suppress the progression of chronic apical periodontitis in mice, leading to a reduction in lesion size and inflammation level.

Materials and methods

Induction of apical periodontitis and sample preparation

Six-week-old C57BL/6N mice, weighing around 20 g, as an unmodified group (WT) or with CCR2 knocked out ($CCR2^{-/-}$) were used, with 12 animals in each group. The mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and Ge's Lab (Peking University, Beijing, China). All animals were treated according to the ethical regulations for animal experimentation defined by the Animal Care Committee of Peking University Health Care Center, Beijing, China (Approval Number: LA2022401).

Apical periodontitis was induced as previously described.[31] Animals were anesthetized by intraperitoneal injection of 70 mg/kg Ketamine, and the pulp of both sides' first mandibular molar was exposed in the mesial pulp horns.[32] After awakening, the animals' weight was monitored, and they received soft food to reduce the pain while chewing. The animals were randomly divided into four groups ($n = 3$) and were euthanized after 0, 7, 21, and 28 days of pulp exposure. The mandibular bones were dissected and prepared for micro-CT, histologic, or molecular analysis.

The right part of the mandibular bone was fixed in 4% neutral paraformaldehyde for 24 h, preserved in 70% ethanol, and subjected to micro-CT observation. The left part of the mandibular bone was rinsed with PBS and then

demineralized in 17% ethylenediaminetetraacetic acid (EDTA) (pH 7, room temperature) for 2 weeks, and EDTA was replaced every 1—2 days. The decalcified samples were dehydrated in ascending concentrations of ethanol, cleared in dimethylbenzene, and finally embedded in paraffin. Longitudinal 6-μm-thick sections were cut in a mesiodistal orientation at the tooth level for hematoxylin-eosin staining, immunohistochemistry, or tartrate-resistant acid phosphatase (TRAP) detection.

Micro-computed tomography observation

Samples (n = 3 teeth per group) were placed in a test tube and scanned along the axis, then scanned with micro-CT (INVEON MM GANTRY-STD CT-3121, 2014, Germany) using the following parameters: energy of 220 kV, intensity of 60 mA, exposure time of 1500 ms, and slice increments of 9 μm in each plane. This gave sagittal slices with a central slice including the pulp chamber, mesial canals, and the apical foramens which were used for PA lesion analysis. Inveon Research Workplace software (Siemens, Munich, Germany) was used to reconstruct the periapical bone resorption region.

Hematoxylin-eosin staining

The slides were deparaffinized with dimethylbenzene and then rehydrated with descending concentrations of ethanol to double-distilled water. Thereafter, they were stained with hematoxylin and eosin for 3 min and 30 s separately, after being quickly rinsed with double-distilled water (ddH2O). Then the slides were placed in ddH_2O and dehydrated with ascending concentrations of ethanol, followed by dimethylbenzene, and covered with paraffin.

Immunohistochemical staining

The samples were immersed in 3 mM citrate buffer (pH 6.0) for 10 min at 95 °C for antigen retrieval. Subsequently, the sections were incubated in goat serum (Vector Laboratories, Burlingame, CA, USA) for 20 min. Then, IHC staining was incubated with primary antibody against mouse EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80) (1:300) (#70076; Affinity, Boston, MA, USA), Chemokine Receptor 2 (CCR2) (1:200) (Affinity) and Cluster of Differentiation 86 (CD86) (Affinity), and restored in 4 °C overnight. After incubation with the secondary antibodies (Aoqing Biotechnology, Beijing, China), the samples were stained with the 3,3′-Diaminobenzidine Staining Kit (Aoqing Biotechnology). Thereafter, they were stained with hematoxylin for 5 min. Then, the slides were placed in ddH2O and dehydrated with ascending concentrations of ethanol, followed by dimethylbenzene, and covered with Entellan. For quantification of immunostaining, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used.

Tartrate resistant acid phosphatase staining

Tartrate-resistant acid phosphatase (TRAP) staining was performed using a commercially available TRAP staining kit according to the manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA). Osteoclasts were identified as TRAP-positive multinucleated (three or more nuclei) cells.

Statistical analysis

All statistical data were expressed as mean ± standard deviation. An independent-sample t-test was used to compare differences between the experimental group and the control group. The statistical significance was set at $P < 0.05$.

Results

Chemokine receptor-2 deficiency resulted in smaller periapical lesion and reduced inflammation level in mice

To investigate the influence of CCR2 deficiency on lesion size of apical periodontitis, the radiolucency zones of the $CCR2^{-/-}$ group and WT group were observed by micro-CT (Fig. 1A). A typical image of periapical radiolucency zone was shown in Fig. 1. After 7 days of pulp exposure, there was no significant difference between $CCR2^{-/-}$ mice and WT mice. However, after 21 days ($P = 0.017$) and 28 days ($P = 0.003$) of pulp exposure, a larger radiolucency zone could be seen in WT mice compared to the $CCR2^{-/-}$ group (Fig. 1C), indicating that CCR2 deficiency could decrease the volume of the periapical lesion size at the same time points. HE staining was used to observe the difference in the inflammation process between the $CCR2^{-/-}$ group and the WT group (Fig. 1B). The HE staining of periapical area showed less inflammatory cell infiltration around $CCR2^{-/-}$ group's lesion area compared to WT group, revealed that without monocyte-macrophage system-derived macrophages, $CCR2^{-/-}$ group exhibited less inflammation compared with the WT group (Fig. 1A).

Chemokine receptor-2 deficiency decreased macrophages' infiltration and M1 macrophages' number

IHC staining was applied to detect the expression of the macrophage biomarker F4/80 (Fig. 2A), CCR2 (Fig. 2B), and the M1 macrophage biomarker CD86 (Fig. 3). In both unexposed groups, positive staining of F4/80 could be found in normal periapical tissues, with no significant difference in the quantity of those markers, but less than in exposed groups. CD86 and $CCR2^{+}$ cells could not be found in normal periodontal ligament, indicating that those macrophages are not inflammatory monocyte-derived subsets and may be tissue-resident macrophages.

In the WT group, F4/80 and CD86 expression gradually increased as the exposure time of the pulp prolonged. The statistical results are presented in a bar chart (Fig. 2C and D). CCR2 expression increased at the first stage of apical periodontitis, then slightly decreased as the inflammation progressed into the chronic phase. At day 28 there were still some $CCR2^{+}$ cells in the periapical lesion, with a significant difference compared to the day 7 group

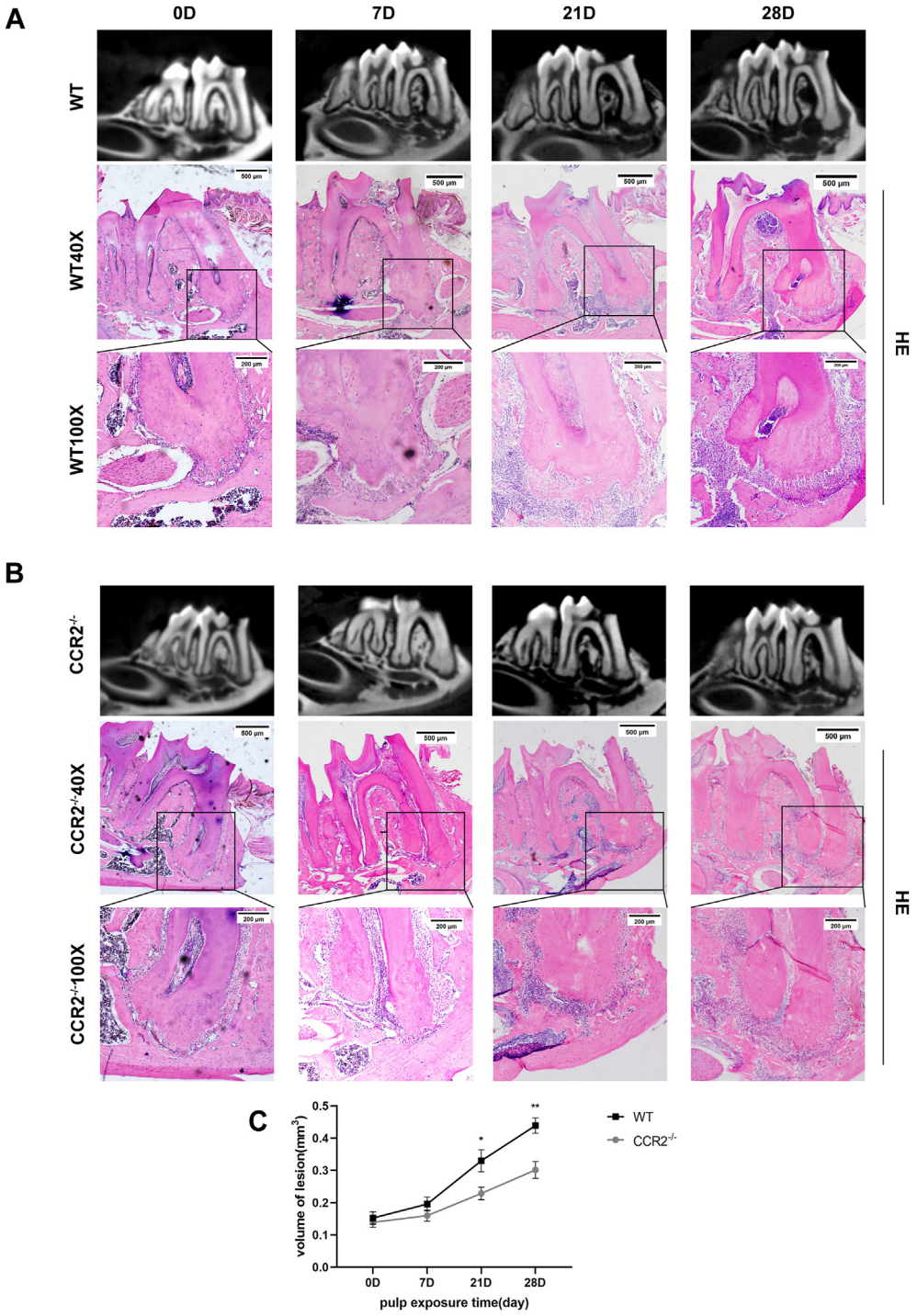

Figure 1 The difference in radiolucency zone and inflammatory infiltration between wild type (WT) mice and chemokine receptor-2 deficiency ($CCR2^{-/-}$) mice. (A) Micro-CT & Hematoxylin-eosin (HE) staining of WT mice after 0, 7, 21, and 28 days of pulp exposure. (B) micro-CT & HE staining of $CCR2^{-/-}$ mice after pulp exposure. (C) The volume of radiolucency zone in each group, the asterisks indicate statistically significant differences. (*: $P < 0.05$, **: $P < 0.01$).

($P = 0.016$). At the same time point, although the number of F4/80 and CD86 positive-staining cells in the $CCR2^{-/-}$ group increased, was significantly lower than that in the WT group ($P = 0.018$ and $P = 0.003$). We observed that M1 macrophages were also presented in the periapical lesion of the $CCR2^{-/-}$ group, but less than in the WT group. CCR2 was not expressed in all CCR2-deficiency mice group. Taken together, the expression of CCR2 in the WT group changed

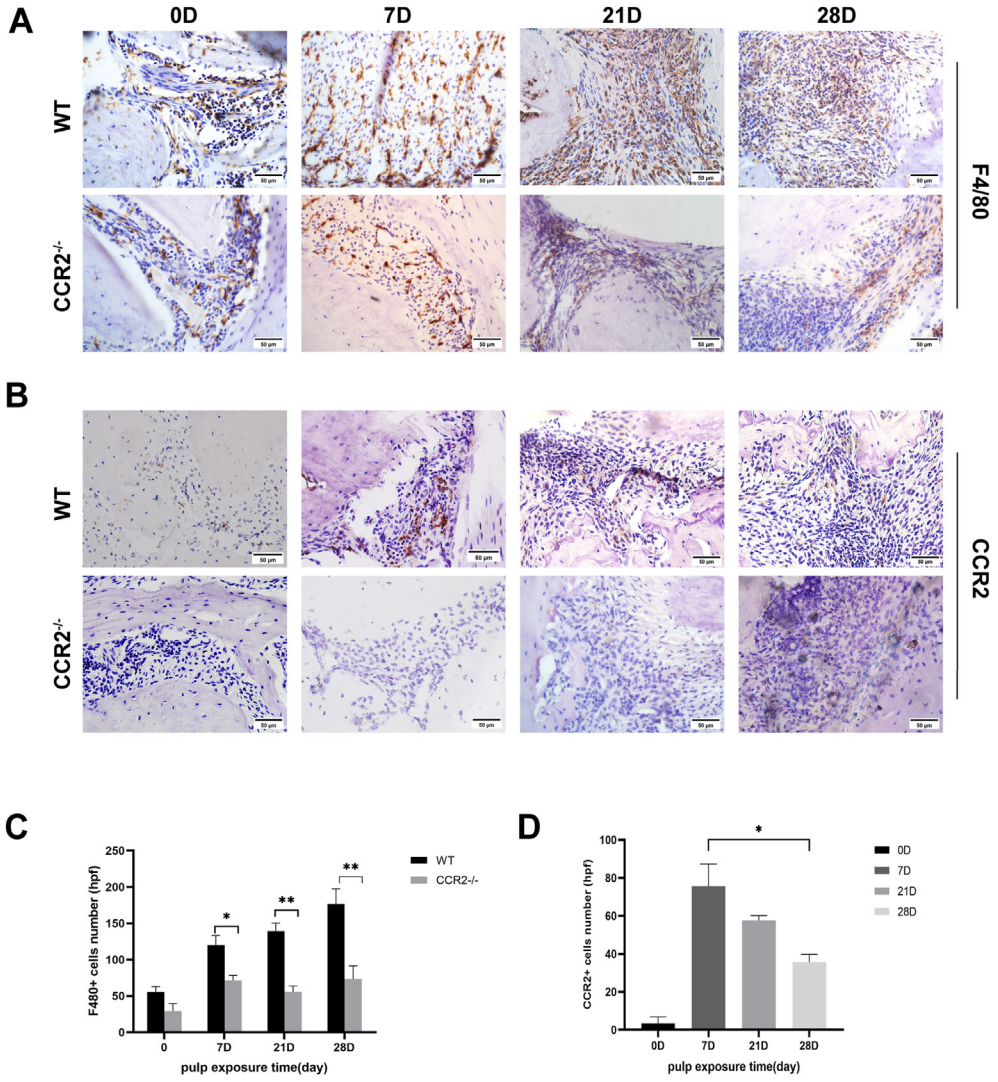

Figure 2 Representative images from immunohistochemical staining to detect the expression of F4/80 and chemokine receptor-2 (CCR2). (A) IHC staining of F4/80 in different groups. (B) IHC staining of CCR2 in different groups, (C) The number of $F4/80^{+}$ cells in different groups, (D) The number of $CCR2^{+}$ cells in WT groups, the asterisks indicate statistically significant differences. (*: $P < 0.05$, **: $P < 0.01$) WT: wild type.

dynamically, and CCR2 expression in apical periodontitis could increase macrophages infiltration, as well as the number of M1 macrophages.

Chemokine receptor-2 deficiency decreased the formation of osteoclasts

TRAP staining was applied to detect the number of osteoclasts at the frontline of the periapical lesion (Fig. 3B). The results revealed that the number of osteoclasts in the $CCR2^{-/-}$ group was significantly lower than that in the WT group at each time point ($P = 0.024$ at day 21 and $P = 0.017$ at day 28) (Fig. 3D).

Discussion

In this research, a reduction in the total number of inflammatory cells infiltrating the apical lesions in $CCR2^{-/-}$ mice compared to wild-type mice was observed, accompanied by reduced lesion volume, reduced inflammation level, and decreased number of local osteoclasts. The findings revealed that when the chemotaxis of the mononuclear macrophage system was obstructed in mice, the volume of chronic apical periodontitis lesions significantly diminished compared to that of the wild-type mice within the same group at the same juncture, showing statistically significant distinctions.

It was discerned that both the $CCR2^{-/-}$ group and the WT group possess macrophages in normal mandibular tissue.[26] These macrophages, lacking CCR2 expression on their surface, can be classified as tissue-resident macrophages.[33,34] Although the number of local macrophages augmented with the prolongation of pulp exposure time post pulp exposure in $CCR2^{-/-}$ mice, owing to the MCP-1/CCR2 axis-mediated inhibition of inflammatory mononuclear macrophage system migration and differentiation, it was hypothesized that the increased macrophages in

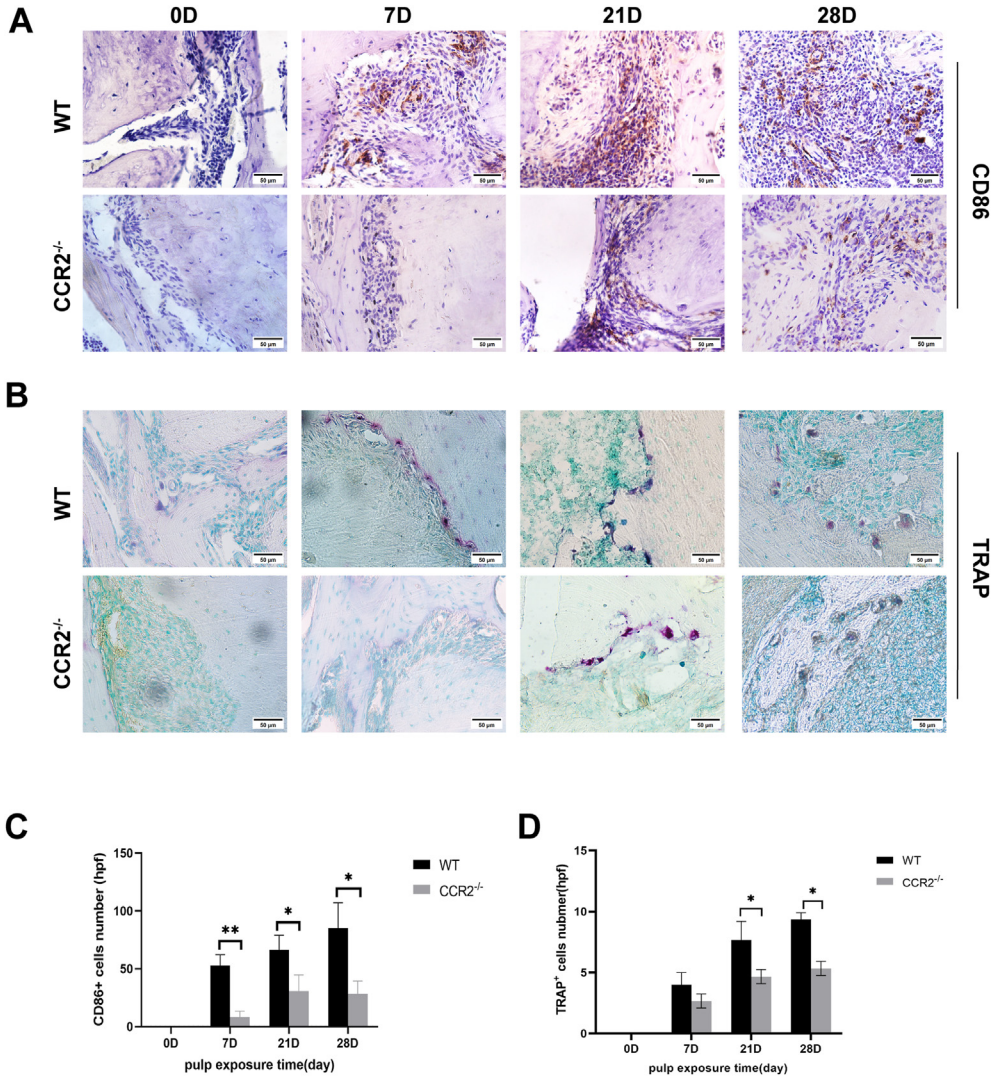

Figure 3 Representative images from immunohistochemical (IHC) staining and tartrate-resistant acid phosphatase staining (TRAP) staining to detect the expression of CD86 and the number of osteoclasts in different groups. (A) IHC staining of Cluster of Differentiation (CD86) in different groups, (B) TRAP staining in different groups, (C) The number of $CD86^{+}$ cells in different groups, (D) The number of osteoclasts in different groups, the asterisks indicate statistically significant differences. (*: $P < 0.05$, **: $P < 0.01$) WT: wild type.

$CCR2^{-/-}$ mice with extended pulp exposure time could stem from the local proliferation of tissue-resident macrophages, this hypothesis is consistent with the conclusions obtained in previous experiments of tissue resident macrophages.[20,30] These macrophages also possess pro-inflammatory attributes.

In $CCR2^{-/-}$ mice, a difference in lesion volume alongside a decrease in macrophage numbers was observed, and the count of M1-type macrophages also decreased. Additionally, *in vitro* experimental investigations demonstrated that CCR2 deficiency would facilitate the differentiation of macrophages into M1-type.[35—39] In this study, an increase in the expression of CD86 in the lesions of $CCR2^{-/-}$ mice was also observed, indicating that under inflammatory conditions, tissue-resident macrophages in the periapical area can differentiate into a pro-inflammatory phenotype, propelling lesion formation and development. This variance could be ascribed to either the absence of macrophages from the MCP-1/CCR2-mediated mononuclear macrophage system, resulting in a reduction in total macrophage numbers and a decrease in M1-type macrophage numbers, or to the disparity between local tissue-resident macrophages transitioning into M1-type macrophages under inflammatory processes and macrophages emanating from the bone marrow, necessitating further experimental examination for verification.

TRAP staining showed a significant reduction of osteoclasts in periapical lesions after inhibition of the mononuclear macrophage system compared to the wild-type group. During the formation process of periapical lesions, osteoclasts mediate bone resorption.[16,38] The phenomenon noted in this experiment aligns with *in vitro* experiment results from the literature, confirming that after inhibition of mononuclear macrophage system migration, the

osteoclast formation process is inhibited, leading to a decrease in osteoclast numbers and subsequently, a reduction of the lesion volume.[39] Since some osteoclast precursor cells originate from the mononuclear macrophage system, it is speculated that the reduction in the number of macrophages will also lead to a decrease in osteoclast numbers.[40,41] In the chronic apical periodontitis tissue of $CCR2^{-/-}$ mice, an increase in the number of osteoclasts over time was also observed, suggesting that these osteoclasts might be generated by the differentiation and fusion of tissue-resident macrophages, implying that tissue-resident macrophages can also engage in the osteoclast formation process, leading to periapical alveolar bone destruction.

This study shows that $CCR2^{-/-}$ macrophages are present in normal periapical tissue. In chronic apical periodontitis, the lack of the mononuclear macrophage system's infiltration results in a reduction in the number of macrophages within lesions and a decrease in the number of osteoclasts at the lesion front, which could be correlated with the contraction in lesion volume. Nonetheless, after the elimination of the MCP-1/CCR2-mediated mononuclear macrophage system, the count of local macrophages still increased. These $CCR2^{-/-}$ macrophages, suggesting that the tissue-resident macrophages within periapical tissues possess self-proliferation and renewal capacities, contribute a pro-inflammatory role in the progression of chronic apical periodontitis. As various CCR2 inhibitors have now been developed for treatment purposes, these research results will contribute to further study on the treatment of chronic apical periodontitis targeting the MCP-1/CCR2 axis.

The limitation of this study was that we could not extract tissue-resident macrophages from the periapical membrane of mouse chronic apical periodontitis lesions for phenotype determination. Moreover, as this study introduced infection via natural infection, the species of pathogens in the environment remain unknown, contrasting with previous studies employing four common mixed species for infection. This hints that the interplay between CCR2 and different species of pathogens may also influence the disease process, necessitating further research for confirmation. In this experiment, only staining for macrophages- and osteoclasts-related markers was conducted, and the relationship between CCR2 and other types of immune cells within chronic apical periodontitis lesions was not investigated, requiring further research for elucidation.

Declaration of competing interest

The authors declare no conflicts of interest.

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