Received: 12 January 2023 | Revised: 3 August 2023 | Accepted: 10 August 2023

DOI: 10.1111/jcpe.13872

ORIGINAL ARTICLE

Journal of Clinical Periodontology WILEY

CCL2 is a key regulator and therapeutic target for periodontitis

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Funding information
Peking University Clinical Scientist Program, Grant/Award Number: BMU2019LCKXJ010; National Natural Science Foundation of China, Grant/Award Number: 81970536; Research Foundation of Peking University School and Hospital of Stomatology, Grant/Award Number: PKUSS20220114

Abstract

Aim: Our previous study revealed that the C-C motif chemokine receptor 2 (CCR2) is a promising target for periodontitis prevention and treatment. However, CCR2 is a receptor with multiple C-C motif chemokine ligands (CCLs), including CCL2, CCL7, CCL8, CCL13 and CCL16, and which of these ligands plays a key role in periodontitis remains unclear. The aim of the present study was to explore the key functional ligand of CCR2 in periodontitis and to evaluate the potential of the functional ligand as a therapeutic target for periodontitis.

Materials and Methods: The expression levels and clinical relevance of CCR2, CCL2, CCL7, CCL8, CCL13 and CCL16 were studied using human samples. The role of CCL2 in periodontitis was evaluated by using CCL2 knockout mice and overexpressing CCL2 in the periodontium. The effect of local administration of bindarit in periodontitis was evaluated by preventive and therapeutic medication in a mouse periodontitis model. Microcomputed tomography, haematoxylin and eosin staining, tartrate-resistant acid phosphatase staining, real-time quantitative polymerase chain reaction, enzyme-linked immunosorbent assay, bead-based immunoassays and flow cytometry were used for histomorphology, molecular biology and cytology analysis.

Results: Among different ligands of CCR2, only CCL2 was significantly up-regulated in periodontitis gingival tissues and was positively correlated with the severity of periodontitis. Mice lacking CCL2 showed milder inflammation and less bone resorption than wild-type mice, which was accompanied by a reduction in monocyte/macrophage recruitment. Adeno-associated virus-2 vectors overexpressing CCL2 in *Ccl2*$^{-/-}$ mice gingiva reversed the attenuation of periodontitis in a CCR2-dependent manner. In ligation-induced experimental periodontitis, preventive or therapeutic administration of bindarit, a CCL2 synthesis inhibitor, significantly inhibited the production of CCL2, decreased the osteoclast number and bone loss and reduced the expression levels of proinflammatory cytokines TNF-α, IL-6 and IL-1β.

Conclusions: CCL2 is a pivotal chemokine that binds to CCR2 during the progression of periodontitis, and targeting CCL2 may be a feasible option for controlling periodontitis.

KEYWORDS
bindarit, CCL2, monocytes/macrophages, periodontitis, targeted therapy

Clinical Relevance

Scientific rationale for study: Our previous study revealed that CCR2 was a promising target for periodontitis. CCR2 is a receptor with multiple ligands, and which of these ligands plays a key role in periodontitis remains unclear. The effect of bindarit on periodontitis has not been reported.

Principal findings: The present study demonstrated for the first time that among the different ligands of CCR2, CCL2 is the key functional ligand in periodontitis. Local administration of bindarit provided good preventive and therapeutic effects on experimental periodontitis.

Practical implications: The present study provides a new therapeutic target for periodontitis, and bindarit may be a potentially effective drug for periodontitis prevention and treatment.

1 | INTRODUCTION

Periodontitis, one of the most prevalent oral diseases, is characterized by chronic inflammation and irreversible alveolar bone absorption (Kinane et al., 2017). Excessive activation of the host immune response sitimulated by microbial pathogens is the main pathogenesis of periodontitis (Hajishengallis et al., 2020). The current treatment for periodontitis mainly focuses on pathogenic plaque biofilm elimination, including scaling and root planing, antibiotic administration and surgical interventions (Mombelli, 2018). However, the direct damage of periodontitis is caused by the host immune response. Thus, it is essential to further uncover the mechanism of the host immune response in periodontitis and develop alternative host modulation therapies to manage this disease.

Chemokines and chemokine receptors participate in different biological processes, especially in immune cell infiltration and migration to specific inflammatory response sites (Bhusal et al., 2020; Hughes & Nibbs, 2018). The C-C motif chemokine receptor 2 (CCR2) is a key receptor that mediates immune cell mobilization. CCR2 ligands include CCL2, CCL7, CCL8, CCL13 and CCL16 (Nagarsheth et al., 2017). Our previous study revealed that CCR2 was critical for the development of periodontitis and that targeting CCR2 could effectively protect against experimental periodontitis in mice (Jiang et al., 2022). However, the specific ligands of CCR2 that play key roles in periodontitis have not been reported. Several studies have demonstrated that the expression of CCL2 is elevated in periodontitis (Boström et al., 2015; Kurtiş et al., 2005; Zhang et al., 2021). Moreover, the high level of CCL2 in the gingival crevicular fluid of patients with periodontitis was found to significantly decrease after periodontal treatment (Gupta et al., 2013; Pradeep et al., 2009). However, using knockout mice to prove that CCL2 plays a key role in periodontitis has not been reported.

Bindarit is a selective inhibitor of monocyte chemotactic proteins (Mirolo et al., 2008). Mora et al. found that bindarit exerted a specific inhibitory effect on p65- and p65/p50-mediated activation of the CCL2 promoter in vitro (Mora et al., 2012). In recent years, bindarit has been shown to be an anti-inflammatory factor in various CCL2-mediated disease models, including osteoarthritis, neuroinflammatory disease, cancer-related inflammation and diabetes-associated periodontitis (Ge et al., 2012; Liu et al., 2018; Raghu et al., 2017; Shen et al., 2021). Its safety and efficacy have been verified by phase II clinical trials in patients with lupus nephritis and coronary stent restenosis (Ble et al., 2011; Colombo et al., 2016).

In the present study, we found that CCL2 may be a key mediator in the progression of periodontitis through the detection and analysis of clinical gingival samples. In this study, CCL2 knockout mice were used for the first time to explore whether CCL2 plays a key role in the progression of periodontitis and to evaluate the potential of CCL2 as a therapeutic target for periodontitis. This study is also the first to investigate the effect of local administration of bindarit on periodontal inflammation and alveolar bone resorption in mice.

2 | MATERIALS AND METHODS

2.1 | Human subjects and ethics statements

In the present study, human gingival tissues were collected from 12 periodontally healthy donors and 12 patients with periodontitis in the Department of Periodontology, Peking University Hospital of Stomatology, China. The inclusion criteria for healthy individuals were: having no teeth with probing depth > 3 mm; no sites with attachment loss; and no history of periodontitis. Periodontitis patients were

included based on the presence of at least eight teeth with probing depth ≥ 4 mm and radiographic bone loss, based on the 2018 new classification of periodontal diseases (Shi et al., 2018). Healthy samples were taken from patients who underwent crown-lengthening surgery. Periodontitis-affected tissues were collected from periodontitis sites that had undergone tooth extraction and alveolar ridge preservation, which is a procedure consisting of an internal oblique incision 0.5–1 mm below the gingival margin to remove the inner wall of the periodontal pocket (Zhao et al., 2018); the removed tissues were collected. The harvested tissues were immediately placed in liquid nitrogen and stored at −80°C (under liquid nitrogen transportation).

The present study was approved by the Ethics Committee of the Peking University Health Science Center (PKUSSIRB-201310068a). All enrolled subjects signed informed consent forms. The clinical and demographic characteristics of the enrolled subjects are presented in Tables S1 and S2.

2.2 | Mice and animal experiments

To exclude the interference of gender-related factors, all mice used in this study were males. C57BL/6 mice were purchased from SPF Biotechnology (Beijing, China). CCL2 knockout (*Ccl2*$^{-/-}$) mice on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbour, USA). CCR2 knockout (*Ccr2*$^{-/-}$) mice were gifts from Professor Yu Zhang. CCL2 and CCR2 double-knockout *Ccl2*$^{-/-}$*Ccr2*$^{-/-}$ mice were generated by crossing *Ccl2*$^{-/-}$ mice and *Ccr2*$^{-/-}$ mice. We generated all data from age-matched male littermates. All animals were maintained in specific pathogen-free conditions with 12-h light/dark cycles. The sample size of each experiment was determined based on the effect size, assumed significance level (0.05) and assumed power (90%), and the effect size was obtained according to the standard deviation and mean difference observed in the pilot studies (Festing & Altman, 2002). All animal experiments were approved by the Peking University Health Science Center's Ethics Committee (Ethics number: LA2021494) and conformed to the updated ARRIVE 2.0 guidelines (Supporting Information).

To determine the role of CCL2 in the development of periodontitis, 7-day ligation-induced experimental periodontitis was established in *Ccl2*$^{-/-}$ and wild-type (WT) littermate mice. To examine the preventive and therapeutic effects of bindarit on periodontitis, 8-week-old C57BL/6 male mice were used. The bindarit suspension was injected daily into the ligation around the maxillary second molar. Mice in the control group were administered an equal volume of the vehicle. In the preventive group, the animals received medical intervention from day 0 until they were sacrificed on day 7. In the therapeutic group, the animals received the drug from day 3 to day 7 or day 9 after ligature placement. In all experiments involving drug intervention, the ligatures were not removed until the animals were sacrificed at the end of the experiment. The gingival tissues were collected for total RNA extraction, and the maxillae were post-processed for microcomputed tomography (micro-CT) scanning and histological analyses.

2.3 | Mouse adeno-associated virus 2 construction and overexpression

The adeno-associated virus 2 (AAV2) delivery system to overexpress the CCL2 gene in mouse gingiva was constructed by Vigene Bioscience (Shandong, China). The empty adeno-associated virus vector (AAV2-null) served as a control. To overexpress CCL2, 6-week-old male *Ccl2*$^{-/-}$ and *Ccl2*$^{-/-}$*Ccr2*$^{-/-}$ mice were used. Into the palatal mucosa of the maxillary molar area, 2.5 μL of virus solution (3.28×10^{13} μg/mL) was injected into with a graded Hamilton syringe (33-G needle). Injections were given once every other day, and a total of three injections were given; experimental periodontitis was induced 2 weeks later.

2.4 | Statistical analysis

Comparisons between two groups were performed using Student's *t* test, and analysis of covariance (ANCOVA) was used to adjust for age and gender. Comparisons between multiple groups were performed using one-way ANOVA or two-way ANOVA with Brown–Forsythe and Welch tests. Pearson correlation analysis was used to assess the correlation between two variables. The correlations were defined as very strong ($r = 0.8–1.0$), strong ($r = 0.6–0.79$), moderate ($r = 0.4–0.59$) and weak ($r = 0.2–0.39$) (Gulbrandsen et al., 2021; Hyde et al., 2020). All experiments were performed at least twice independently. All data were expressed as mean ± standard deviation, and a value of $p < .05$ was considered statistically significant. All analyses were conducted using GraphPad Prism (GraphPad Software, Version 8.2.1, San Diego, CA, USA) and SPSS (SPSS, Version 27; SPSS Inc., Chicago IL, USA).

Complete details of the Materials and Methods are given in Supporting Information.

3 | RESULTS

3.1 | CCL2 is highly expressed in periodontitis and is positively associated with the severity of periodontitis

To determine the functional ligand of CCR2 in periodontitis, gene expression profiles of gingival tissues collected from healthy donors and patients with periodontitis were performed. Compared with other ligands of CCR2, including CCL7, CCL8, CCL13 and CCL16, CCL2 was highly expressed in human gingival tissues and significantly up-regulated in periodontitis (Figure 1a). Furthermore, significantly positive correlations were found between CCL2 and probing depth (PD) of the sampling sites (Figure 1b). In accordance with the real-time quantitative polymerase chain reaction (real-time qPCR) results, an enzyme-linked immunosorbent assay showed that the protein level of CCL2 was significantly elevated in the periodontitis group (Figure 1c), and significantly positive correlations were found

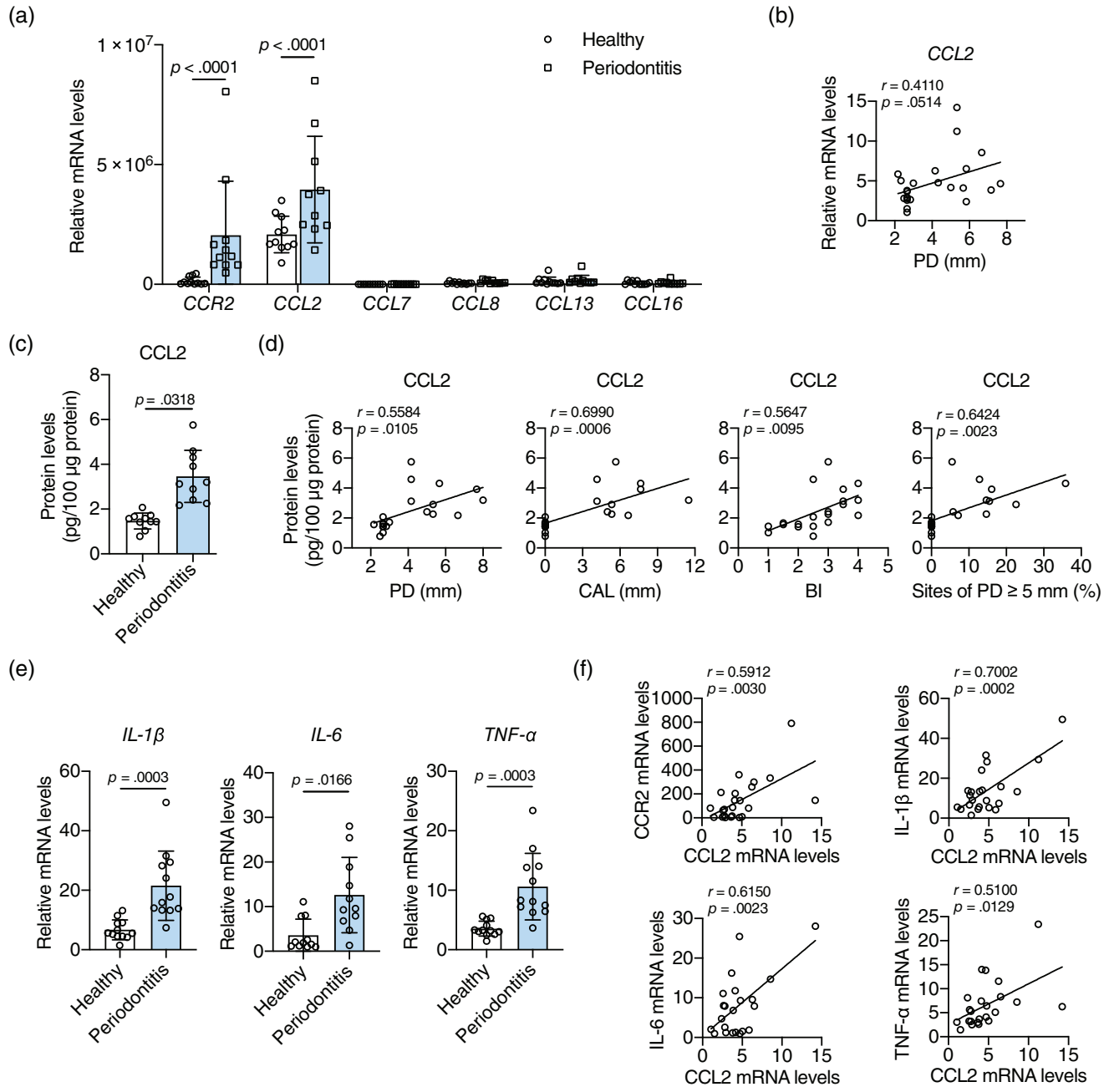

FIGURE 1 CCL2 is highly expressed in periodontitis and is positively associated with the severity of periodontitis. (a) Expression profile of CCR2 and its ligands in human gingival tissues (healthy, $n = 12$; periodontitis, $n = 12$). (b) Correlation analysis of the mRNA level of CCL2 and PD. (c) Protein level of CCL2 in human gingival tissues and the correlation with clinical probing depth (healthy, $n = 10$; periodontitis, $n = 10$). (d) Correlation analysis of the protein level of CCL2 and PD, CAL, BI and the percent of sites with PD ≥ 5 mm of the full mouth. (e) The mRNA levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in human gingival tissues. (f) Correlation analysis of the expression levels of CCR2, IL-1β, IL-6 and TNF-α with CCL2. The data are presented as the mean ± SD. Statistical significance was determined by two-way ANOVA, Pearson correlation analysis (the correlations were defined as very strong [$r = 0.8$–1.0], strong [$r = 0.6$–0.79], moderate [$r = 0.4$–0.59] and weak [$r = 0.2$–0.39]) and unpaired, two-tailed Student's *t* tests and analysis of covariance. BI, bleeding index; CAL, clinical attachment loss; PD, probing depth. Sites of PD ≥ 5 mm (%), percentage of sites with PD ≥ 5 mm of the full mouth.

between the protein level of CCL2 and PD, the clinical attachment loss (CAL) and the bleeding index (BI) of the sampling sites and the percentage of sites with PD ≥ 5 mm for the full mouth (Figure 1d). To further examine the relationship between CCL2 and periodontitis-associated proinflammatory cytokines, we detected the mRNA levels of IL-1β, IL-6 and TNF-α and found that their expressions were up-regulated in the gingival tissues of patients with periodontitis (Figure 1e). Moreover, significantly positive correlations were also found between CCL2 and the expression levels of CCR2, IL-1β, IL-6 and TNF-α (Figure 1f).

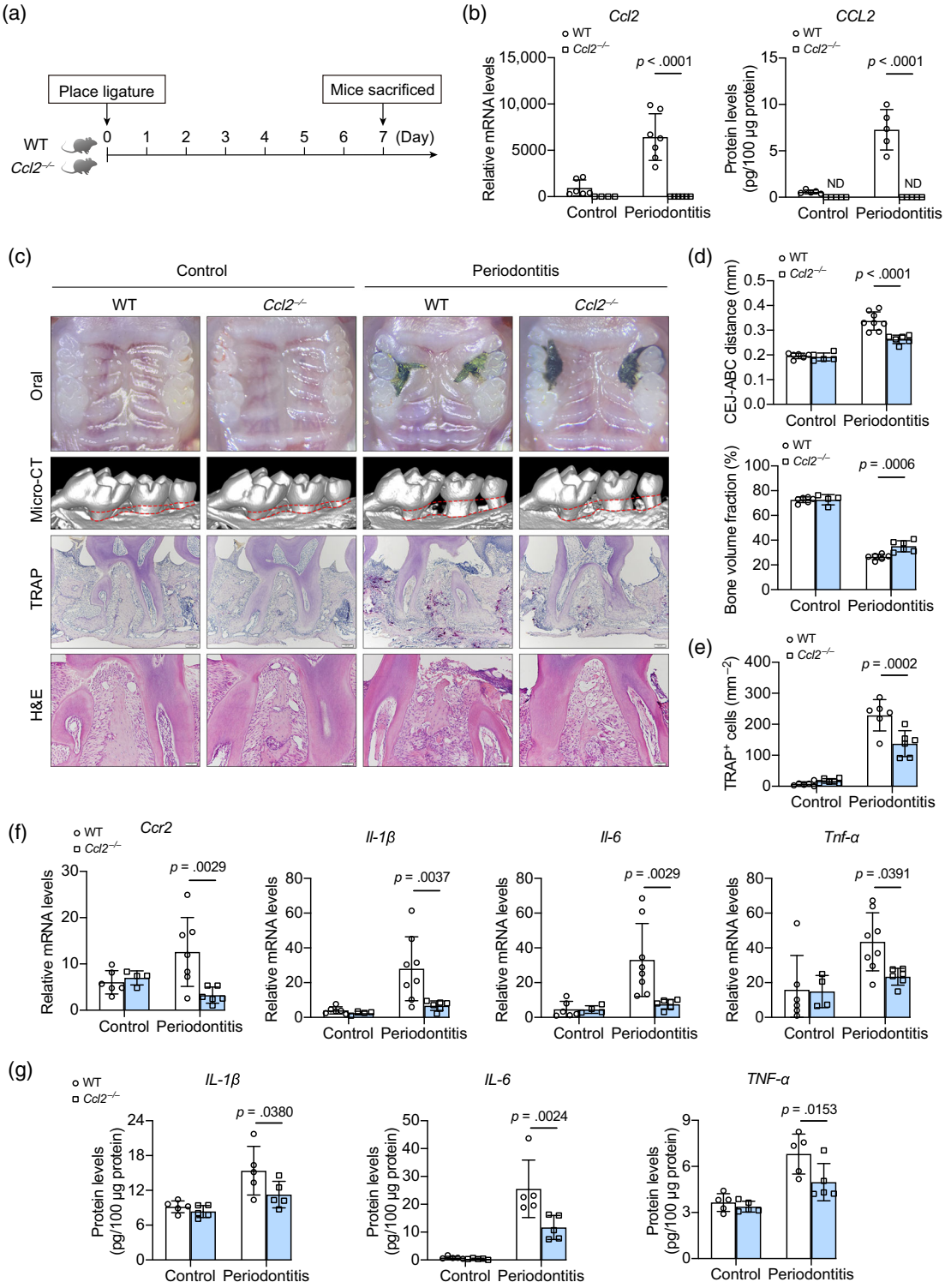

FIGURE 2 Genetic knockout of CCL2 reduces alveolar bone resorption, periodontal inflammation and monocyte/macrophage infiltration in mice. (a) Schematic diagram of the experimental design. (b) The mRNA level and protein level of CCL2 in murine gingival samples. (c) First line: intra-oral images were taken with a stereomicroscope. Second line: three-dimensional reconstruction images of microcomputed tomography (micro-CT) scanning. Third line: tartrate-resistant acid phosphatase (TRAP) staining images of maxillae sections (scale bar = 100 μm). Fourth line: haematoxylin and eosin (H&E) staining images of the furcation region of the second molar (scale bar = 50 μm). (d) Bone loss and bone volume fraction were analysed by micro-CT. (e) The number of osteoclasts per square millimetre was analysed by TRAP staining (scale bar = 100 μm). (f) The mRNA levels of CCR2, IL-1β, IL-6 and TNF-α in murine gingival tissues. (g) The protein levels of IL-1β, IL-6 and TNF-α in murine gingival tissues. $n = 6, 4, 8, 6$ for each group in a–f. The data are presented as the mean ± SD. Statistical significance was determined by two-way ANOVA. ABC, alveolar bone crest; CEJ, cemento-enamel junction; H&E, haematoxylin and eosin; Micro-CT, microcomputed tomography; ND, not detected; TRAP, tartrate-resistant acid phosphatase.

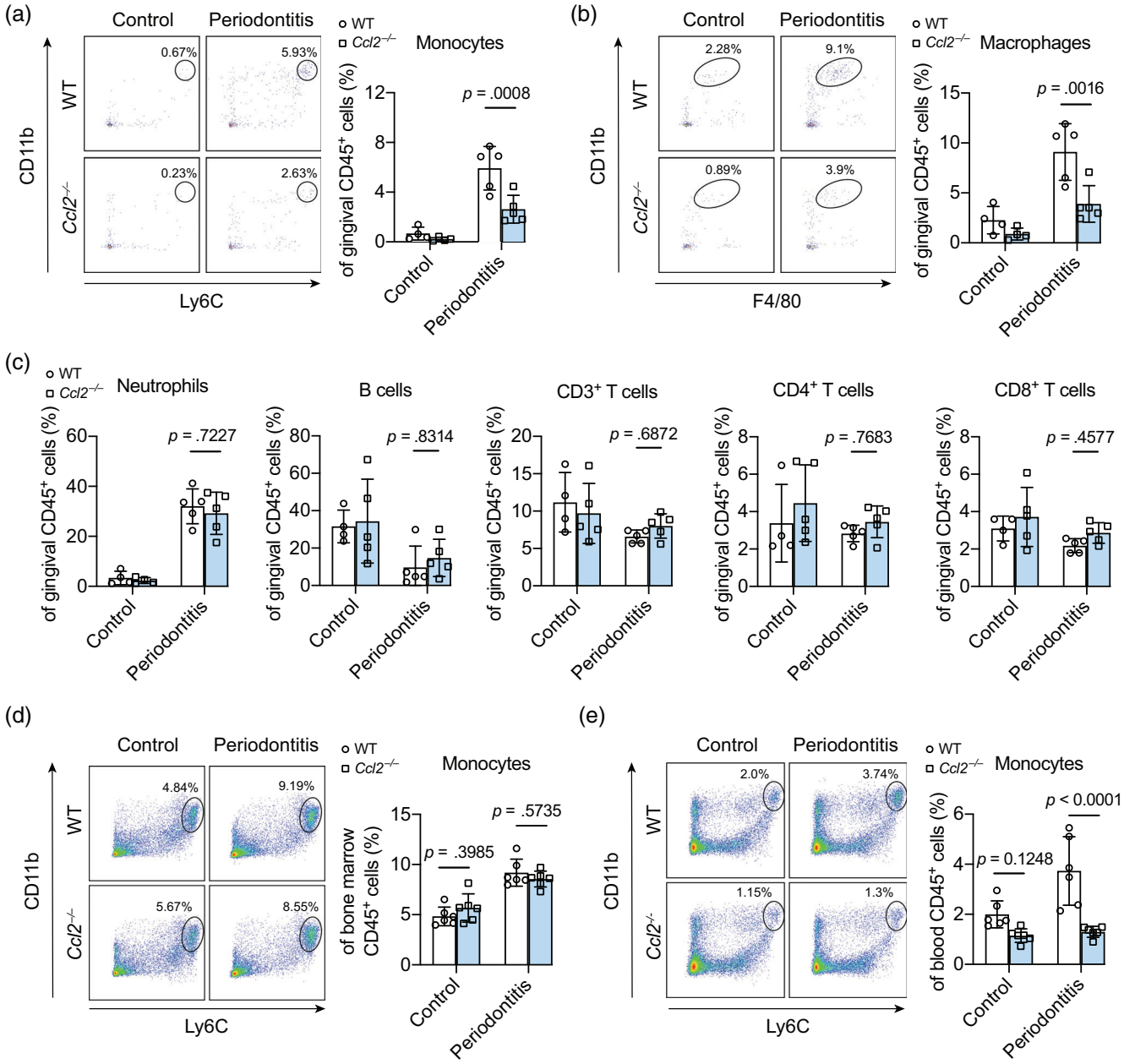

FIGURE 3 CCL2 deficiency hinders monocyte/macrophage infiltration in mice. (a) Representative flow cytometric images and statistical analysis of monocytes ($CD45^+CD11b^+Ly6G^-Ly6C^{hi}$) in murine gingival tissues. (b) Representative flow cytometric images and statistical analysis of macrophages ($CD45^+CD11b^+Ly6G^-F4/80^+$) in murine gingival tissues. The data are representative of three independent experiments. (c) Gingival neutrophils and lymphocytes analysis. (d) Representative flow cytometric analysis images and statistical analysis of monocytes in bone marrow. (e) Representative flow cytometric analysis images and statistical analysis of monocytes in peripheral blood. $n = 4, 4, 5, 5$ for each group. The data are presented as the mean ± SD. Statistical significance was determined by two-way ANOVA.

3.2 | Genetic knockout of CCL2 reduces alveolar bone resorption and periodontal inflammation in experimental periodontitis

To investigate the role of CCL2 in periodontitis in vivo, we established ligature-induced periodontitis in WT and *Ccl2*$^{-/-}$ littermate mice (Figure 2a). CCL2 was undetectable in the gingival tissues of *Ccl2*$^{-/-}$ mice, indicating successful knockout (Figure 2b). During maxillary sample collection, we found that in the periodontitis groups, gingival swelling in *Ccl2*$^{-/-}$ mice was significantly milder than that in WT mice (Figure 2c, first line). Micro-CT analysis showed that there was no significant difference in alveolar bone height and volume between WT and *Ccl2*$^{-/-}$ mice in the control groups, while in the experimental groups the alveolar bone destruction caused by periodontitis in *Ccl2*$^{-/-}$ mice was significantly reduced compared with that in WT mice (Figure 2c, second line, Figure 2d). To clarify the effect of CCL2 deficiency on bone resorption, we examined osteoclasts by tartrate-resistant acid phosphatase (TRAP) staining (Figure 2c, third line,

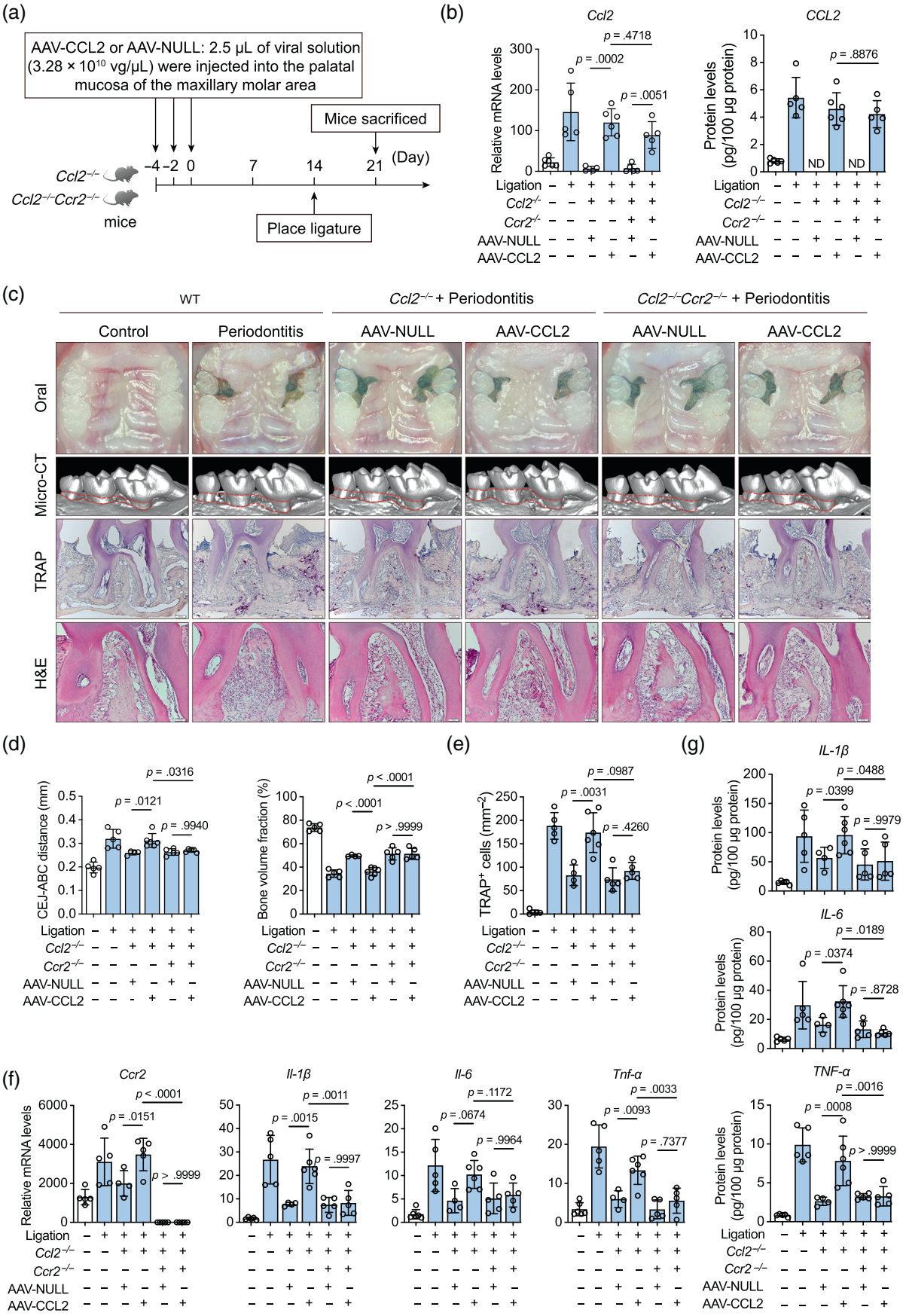

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Figure 2e). No significant difference in TRAP+ cells was observed between healthy WT and *Ccl2*$^{-/-}$ mice, while the number of TRAP+ cells in the *Ccl2*$^{-/-}$ + periodontitis group was significantly reduced compared with that in the WT+ periodontitis group. Furthermore, we used haematoxylin and eosin (H&E) staining and real-time qPCR to evaluate periodontal inflammation. Compared with that in WT mice, the number of infiltrating inflammatory cells in the furcation region (Figure 2c, fourth line) as well as the expression levels of CCR2, IL-1β, IL-6 and TNF-α (Figure 2f) and the protein levels of IL-1β, IL-6 and TNF-α (Figure 2g) in gingival tissues were significantly reduced in knockout mice. In addition, we examined the effect of CCL2 deficiency on the antibacterial activity of mice and found that it had no negative effect on periodontal microbial load or systemic responses to infection (Figure S1).

3.3 | CCL2 deficiency hinders monocyte/macrophage infiltration in ligature-induced periodontitis

To explore the underlying mechanism, we analysed periodontal immune cell composition by flow cytometry. The proportions of infiltrating monocytes and macrophages in the gingival tissues were significantly reduced in *Ccl2*$^{-/-}$ mice (Figure 3a,b), while the proportions of other immune cells, such as neutrophils, B cells and T cells (CD3+ T cells, CD4+ T cells, CD8+ T cells), were not significantly affected (Figure 3c). To understand whether CCL2 deficiency affects the formation or recruitment of monocytes/macrophages, we further analysed monocytes in the bone marrow and peripheral blood. No significant difference was found in bone marrow monocytes (Figure 3d), but the proportion of monocytes in the peripheral blood of *Ccl2*$^{-/-}$ mice was markedly lower than that of WT mice (Figure 3e).

3.4 | AAV2-CCL2 reverses gingival CCL2 expression and promotes periodontitis in a CCR2-dependent manner

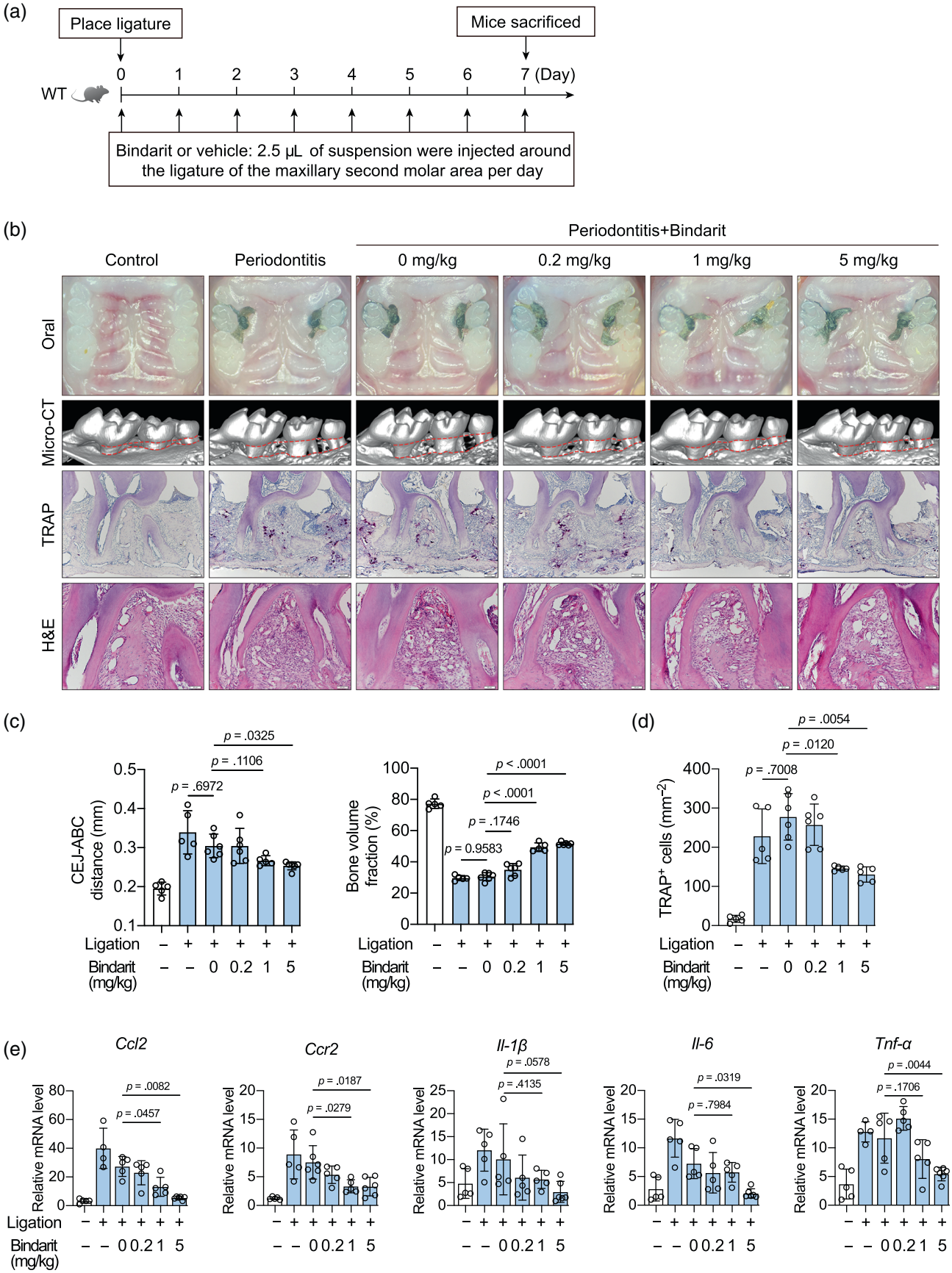
To confirm the role of CCL2 in the development of periodontitis, we overexpressed CCL2 in the periodontal tissue of *Ccl2*$^{-/-}$ mice with adeno-associated virus 2 (AAV2) (Figure 4a). Compared with the mice that received the empty vector (AAV2-null), *Ccl2*$^{-/-}$ mice that received AAV2-CCL2 showed markedly increased mRNA level and protein level of CCL2, indicating successful overexpression (Figure 4b). To examine the consequences of CCL2 overexpression in the context of periodontitis, 14 days after AAV2 injection, the second molars were ligatured for another 7 days to induce periodontitis (Figure 4a). We found that CCL2 overexpression significantly aggravated periodontitis-induced gingival swelling, alveolar bone destruction and inflammation compared with those of the mice injected with AAV2-NULL (Figure 4c–g). Furthermore, to evaluate whether the receptor CCR2 was required for CCL2-mediated periodontitis, we generated *Ccl2*$^{-/-}$ *Ccr2*$^{-/-}$ mice. The results showed that CCL2 and CCR2 double knockout did not restore the attenuation of periodontal inflammation and destruction observed in *Ccl2*$^{-/-}$ mice (Figure 4c–g).

3.5 | Local administration of bindarit alleviates periodontitis-induced bone loss in mice

Based on the results above, we confirmed that CCL2 was closely related to the development of periodontitis and predicted that targeting this molecule may be a promising strategy for the prevention and treatment of periodontitis. Here, we employed bindarit as an inhibitor of CCL2. We first evaluated the protective effect of bindarit on ligation-induced periodontitis: mice were treated with different doses of bindarit (0.2 mg/kg, 1 mg/kg and 5 mg/kg) from the day of ligature placement (Figure 5a). Encouragingly, continuous medication with bindarit markedly reduced the severity of periodontitis in a dose-dependent manner. Local administration of 1 mg/kg and 5 mg/kg bindarit significantly mitigated gingival swelling and alveolar bone resorption, reduced the number of osteoclasts and the infiltration of inflammatory cells in periodontitis mice, and down-regulated the expression levels of CCL2 and CCR2 (Figure 5b–e). The 5 mg/kg dose showed a more effective anti-inflammatory effect than the other doses (Figure 5e).

Next, to investigate the therapeutic potential of bindarit, we administered drugs starting from day 3 after ligature placement (Figure 6a). Intra-oral photographs showed milder gingival swelling after bindarit treatment compared with that in the vehicle-treated group (Figure 6b, first line). Micro-CT analysis indicated less bone loss and higher bone volume fraction in the treatment group (Figure 6b, second line, Figure 6c). Accordingly, bindarit significantly reduced the number of TRAP+ osteoclasts and infiltrated inflammatory cells (Figure 6b, third and fourth lines, Figure 6d). The mRNA expression of CCL2, CCR2, IL-1β, IL-6 and TNA-α was also markedly reduced after bindarit treatment (Figure 6e). To further confirm the therapeutic potential of bindarit, we established a 9-day ligature-induced

FIGURE 4 AAV2-CCL2 reverses gingival CCL2 expression and promotes periodontitis in a CCR2-dependent manner. (a) Schematic diagram of the experimental design. (b) The mRNA level and protein level of CCL2 in murine gingival samples. (c) First line: intra-oral images were taken with a stereomicroscope. Second line: three-dimensional reconstruction images of micro-CT scanning. Third line: TRAP staining images of maxillae sections (scale bar = 100 μm). Fourth line: H&E staining images of the furcation region of the second molars (scale bar = 50 μm). (d) Bone loss and bone volume fraction were analysed by micro-CT. (e) The number of osteoclasts per square millimetre was analysed by TRAP staining. (f) The mRNA levels of CCR2, IL-1β, IL-6 and TNF-α in murine gingival tissues. (g) The protein levels of IL-1β, IL-6 and TNF-α in murine gingival tissues. $n = 6, 5, 4, 6, 5, 5$ for each group in (b) and (f), and $n = 5, 5, 4, 6, 5, 5$ for each group in (c–e). The data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. ABC, alveolar bone crest; CEJ, cemento-enamel junction; H&E, haematoxylin and eosin; Micro-CT, microcomputed tomography; ND, not detected; TRAP, tartrate-resistant acid phosphatase.



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periodontitis model in order to extend the treatment time. The results showed that compared with the placebo treatment group, the bindarit treatment group had significantly lesser periodontal destruction and inflammation (Figure S2).

To explore whether bindarit affects the progression of periodontitis by inhibiting CCL2, a rescue experiment was performed (Figure S3a). We found that bindarit reduced the expression of CCL2 and periodontal destruction and that CCL2 overexpression with AAV2-CCL2 in murine gingival samples could counteract the inhibitory effect of bindarit (Figure S3b–g).

4 | DISCUSSION

Periodontitis is a consequence of the host immune inflammatory response to periodontal pathogens (Hajishengallis et al., 2020; Kinane et al., 2017). CCL2 is an important mediator of the cascade amplification of the host immune response (Xu et al., 2021). In several diseases, CCL2 mediates destructive inflammation by promoting the recruitment of monocytes and macrophages (Ge et al., 2012; Liu et al., 2018; Ma et al., 2020; Raghu et al., 2017; Shen et al., 2021). In the present study, we provided evidence for the first time that CCL2 is closely related to the inflammation and bone resorption associated with periodontitis and showed that this process depended on its receptor CCR2. In addition, we demonstrated that bindarit, a potent inhibitor of CCL2, ameliorated periodontitis-induced destruction by reducing the release of proinflammatory cytokines and the number of osteoclasts (Figure 7).

In our previous work, we showed that CCR2, which is a key G protein-coupled receptor of multiple chemokines such as CCL2, CCL7, CCL8, CCL13 and CCL16, plays an important role in the occurrence and development of periodontitis (Jiang et al., 2022; Nagarsheth et al., 2017). However, which ligands of CCR2 are involved in the development of periodontitis remains unclear. Thus, we first measured the expression levels of CCR2 and its ligands in human gingival tissues in homeostasis and disease. The expression levels of CCL7, CCL8, CCL13 and CCL16 in gingival tissues were extremely low, and they were not significantly up-regulated under inflammatory conditions. In contrast, CCL2 was significantly higher in periodontitis gingival tissues than in healthy gingival tissues, and the expression of CCL2 was positively correlated with the expression of CCR2 and the probing depth, indicating that CCL2 was a key functional ligand of CCR2 involved in the progression of periodontitis.

To confirm the effect of CCL2 up-regulation on periodontitis, we first used *Ccl2*−/− mice to verify that genetic inactivation of CCL2 attenuated periodontal destruction. Additionally, overexpressing CCL2 in $Ccl2^{-/-}$ mice with AAV2 vectors reversed the amelioration of periodontal inflammation and alveolar bone resorption, indicating that CCL2 promotes periodontitis. However, this effect was not observed in $Ccl2^{-/-}$ $Ccr2^{-/-}$ mice, indicating that CCL2 promotes periodontitis in a CCR2-dependent manner. Thus, we concluded that the specific binding of CCL2 and CCR2 promoted the progression of periodontitis.

Monocytes originate from bone marrow progenitors, and can be recruited to inflamed peripheral tissues via the blood circulation and can differentiate into macrophages (Jakubzick et al., 2017). Our findings demonstrated that compared with those in healthy mice, monocytes in peripheral blood and bone marrow, as well as monocytes and macrophages in gingival tissues, were significantly increased in mice with periodontitis. $Ccl2^{-/-}$ mice exhibited no changes in monocytes in the bone marrow but displayed reduced monocytes in peripheral blood and reduced monocytes and macrophages in gingival tissues, which was consistent with a previous study showing that the CCL2–CCR2 axis is the main signal for inflammatory monocyte/macrophage trafficking (Griffith et al., 2014; White et al., 2013).

The migration and infiltration of monocytes/macrophages induced by CCL2–CCR2 signalling have been implicated in many chronic inflammatory diseases and skeletal diseases, such as intestinal infection, osteoarthritis and osteoporosis (Binder et al., 2009; Raghu et al., 2017; Seo et al., 2015). Macrophages derived from monocytes function after activation. First, macrophages amplify the inflammatory response by secreting the proinflammatory cytokines CCL2, IL-1β, IL-6 and TNF-α (Lira-Junior et al., 2020). Second, macrophage-secreted proinflammatory cytokines (IL-1β, IL-6 and TNF-α) further promote bone resorption, and IL-1β, IL-6 and TNF-α up-regulate the expression of RANKL, an osteoclast differentiation factor, to promote osteoclastogenesis and osteoclast activation (Takeuchi et al., 2021; Tsukasaki & Takayanagi, 2019; Yao et al., 2021). Third, macrophages fuse to form multinucleated osteoclasts, which in turn promote bone resorption (Boyle et al., 2003). It was reported that monocytes and macrophages are key immune cells that promote the development of periodontitis (Almubarak et al., 2020; Lira-Junior et al., 2020). In the present study, data from human samples showed that CCL2, IL-1β, IL-6 and TNF-α levels increased when periodontitis occurred. In mice, periodontitis induced increases in IL-1β, IL-6 and TNF-α levels and osteoclast numbers were significantly reduced by CCL2 knockout. These findings suggest that CCL2–CCR2 signalling promotes the production of proinflammatory cytokines and the formation of osteoclasts by recruiting monocytes and activating macrophages, leading to alveolar bone resorption.

FIGURE 5 Local and preventive administration of bindarit alleviates periodontitis-induced bone loss in mice. (a) Schematic diagram of the experimental design. (b) First line: intra-oral images were taken with a stereomicroscope. Second line: three-dimensional reconstruction images of micro-CT scanning. Third line: TRAP staining images of maxillae sections (scale bar = 100 μm). Fourth line: H&E staining images of the furcation region of the second molars (scale bar = 50 μm). (c) Bone loss and bone volume fraction were analysed by micro-CT. (d) The number of osteoclasts per square millimetre was analysed by TRAP staining. (e) The mRNA levels of CCL2, CCR2, IL-1β, IL-6 and TNF-α in murine gingival tissues. $n = 5, 5, 6, 6, 5, 6$ for each group. The data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. ABC, alveolar bone crest; CEJ, cemento-enamel junction; H&E, haematoxylin and eosin; Micro-CT, microcomputed tomography; TRAP, tartrate-resistant acid phosphatase.

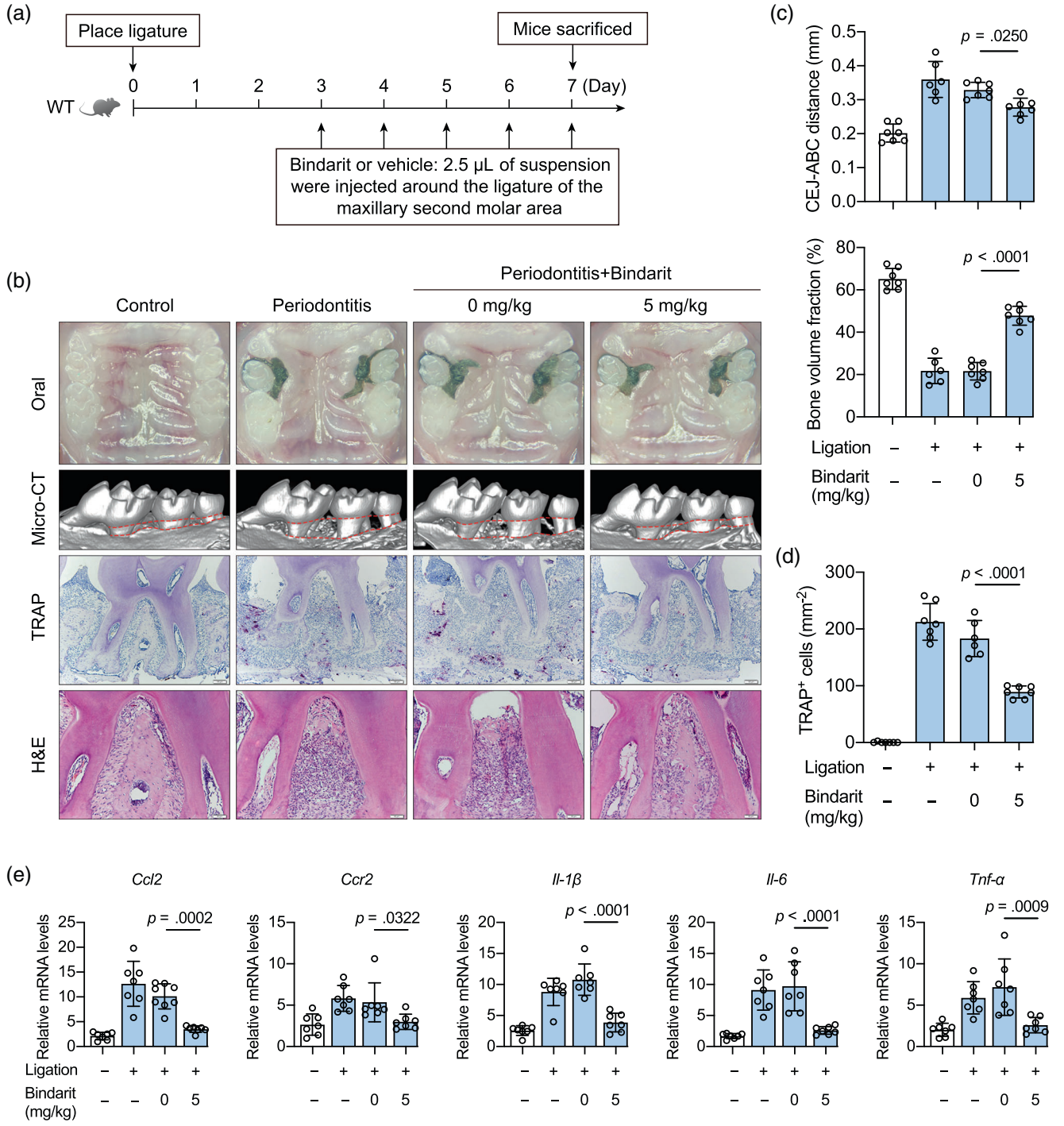

FIGURE 6 Local and therapeutic administration of bindarit alleviates periodontitis-induced bone loss in mice. (a) Schematic diagram of the experimental design. (b) First line: intra-oral images were taken with a stereomicroscope. Second line: three-dimensional reconstruction images of micro-CT scanning. Third line: TRAP staining images of maxillae sections (scale bar = 100 μm). Fourth line: H&E staining images of the furcation region of the second molars (scale bar = 50 μm). (c) Bone loss and bone volume fraction were analysed by micro-CT. (d) The number of osteoclasts per square millimetre was analysed by TRAP staining. (e) The mRNA levels of CCL2, CCR2, IL-1β, IL-6 and TNF-α in murine gingival tissues. $n = 7$ for each group in (b)–(e). The data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. ABC, alveolar bone crest; CEJ, cemento-enamel junction; H&E, haematoxylin and eosin; Micro-CT, microcomputed tomography; TRAP, tartrate-resistant acid phosphatase.

In recent years, therapeutic methods targeting the CCL2–CCR2 axis have attracted extensive attention and shown promising prospects. Bindarit is an anti-inflammatory compound that can inhibit the synthesis of CCL2 and has been proven to have beneficial effects on various inflammatory diseases (Ge et al., 2012; Liu et al., 2018; Raghu et al., 2017; Shen et al., 2021). Phase II clinical trials have

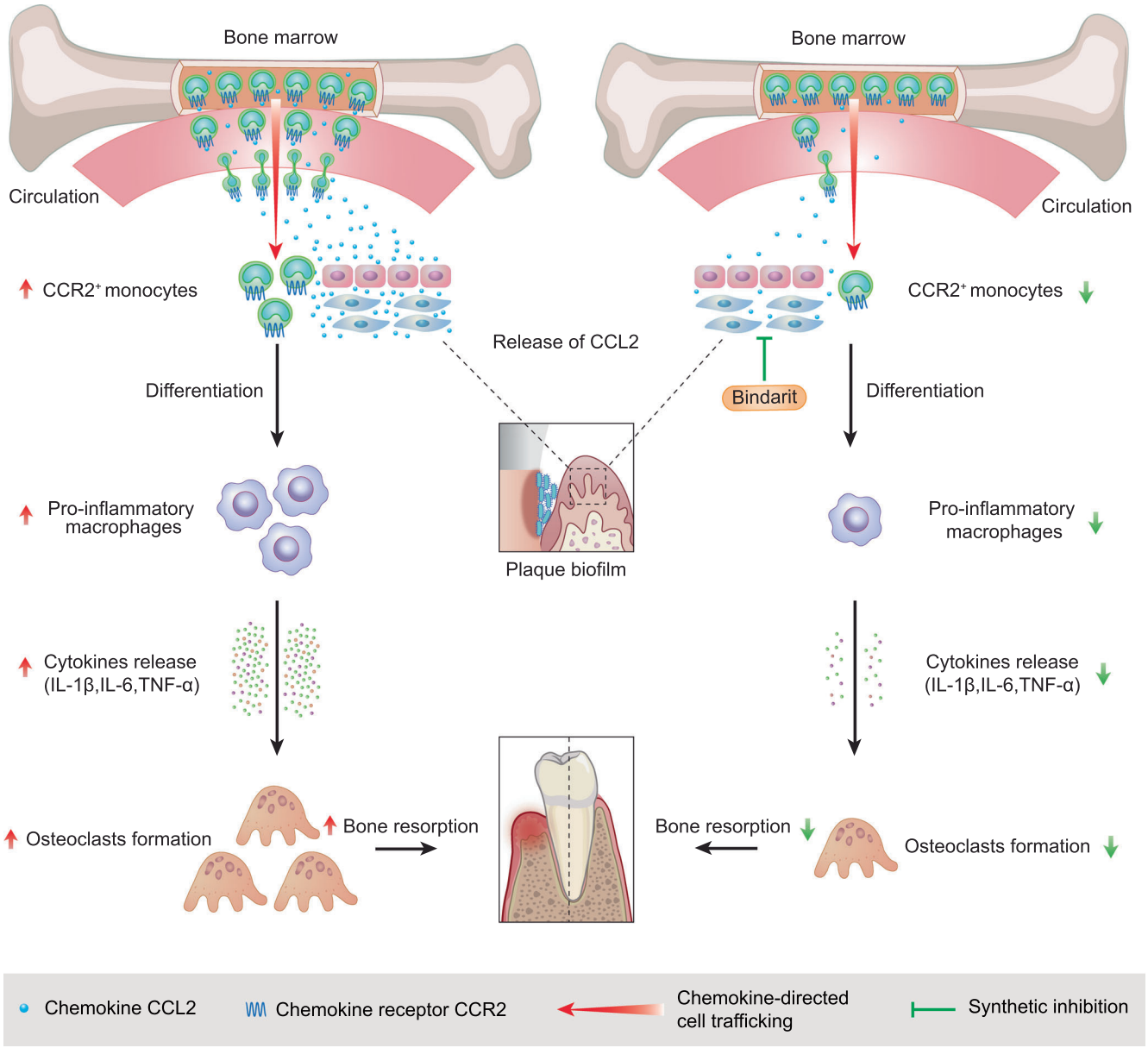

FIGURE 7 Proposed summary diagram of CCL2–CCR2 signalling involved in the development of periodontitis. Bindarit inhibits the development of periodontitis by inhibiting the synthesis of CCL2.

demonstrated the efficacy of bindarit in patients with lupus nephritis and coronary stent restenosis and also the safety and tolerability of bindarit (Ble et al., 2011; Colombo et al., 2016). In the present study, we selected this agent as a pharmacological tool to control periodontitis. Bindarit effectively inhibited the production of CCL2 and reduced the expression levels of proinflammatory cytokines and osteoclast numbers, resulting in the delayed progression of periodontitis-induced alveolar bone loss. Moreover, both prophylactic and therapeutic administration achieved favourable results, showing that bindarit not only has a protective effect against periodontitis but can also effectively inhibit further progression of existing disease. Our study is the first attempt to administer bindarit locally, since it had been administered orally in all previous studies. The effective dose of systemic administration of bindarit was at least 50 mg/kg (Raghu et al., 2017). Our results showed that local administration of 5 mg/kg bindarit could achieve excellent effect on periodontitis control, which reduced the impact of systemic administration on other organs in the body. Therefore, targeting the receptor CCR2 effectively delayed the progression of periodontitis (Jiang et al., 2022), and targeting CCL2 also achieved good results. In the past decade, soluble protein ligands have become an increasingly important class of drug targets. A recent report showed that targeting ligands might be preferable to their receptors in some disease contexts because it is easier to target ligands than receptors in general (Attwood et al., 2020). In addition, CCR2 is a receptor for several ligands, and our findings showed that only CCL2 was closely related to the progression of periodontitis. Accordingly, we believe that targeting CCL2 with bindarit could be a potential treatment strategy for periodontitis. In the last few decades, the

pharmaceutical concept of local administration of sustained-release medications in the periodontal pocket has been encouraged because of its multifaceted advantages, such as maintaining effective therapeutic drug concentrations at the target site, reducing unnecessary systemic loading of patients, improving the bioavailability of drugs and minimizing the frequency of drug intake (Steinberg & Friedman, 2020; Zidar et al., 2021). It may be a feasible choice to convert bindarit into a sustained-release delivery system for the treatment of periodontitis in the future.

In addition, as the main factor responsible for periodontitis is the pathogenic bacteria in the plaque biofilm (Hajishengallis et al., 2020), we evaluated the bacterial load of the ligature from the WT and *Ccl2*$^{-/-}$ littermate mice. There were no significant differences in the total oral bacterial load between the two groups. Furthermore, no adverse effect was found on the systemic defence against infection in mice, and CCL2 knockout tended to promote the elimination of bacteria. Our findings were consistent with a study by Souto et al., which showed that elimination or inhibition of CCR2 had no significant effect on bacterial load of peritoneal cavity with a cecal ligation and puncture model (Souto et al., 2011). However, in the transition from health to periodontitis, the total load of periodontal microbiome changes, and the alterations in the microbial population structure and functional properties of the microbial community are also extremely critical (Abusleme et al., 2021). In the future, more in-depth studies should be performed to analyse the effect of bindarit or other inhibitors targeting CCL2–CCR2 on the periodontal microbial community.

5 | CONCLUSION

Collectively, the present study for the first time proved that CCL2 plays a key role in the progression of periodontitis and that CCL2 could be a therapeutic target for periodontitis by detecting clinical samples, using CCL2 knockout mice and overexpressing CCL2 in the periodontium. This study is also the first to demonstrate that local administration of bindarit has excellent preventive and therapeutic effects on periodontal inflammation and alveolar bone resorption in mice, which provides a new potential strategy for the clinical management of periodontitis.

AUTHOR CONTRIBUTIONS

Wenting Jiang contributed to conception, design, sample collection, data acquisition, analysis and interpretation and drafted and critically revised the manuscript. Ying Wang and Wenjie Hu contributed to project approval, conception, design, data acquisition, analysis and interpretation and critically revised the manuscript. Tao Xu, Cui Wang, Gang Yang and Jie Cao provided human samples. Zhanming Song, Xuekang Wang, Shasha Yuan, Qingqing Li, Yiping Wei, Yaqian Mo, Zhongtian Liu, Ning Li, Siqi Li and Ping Lv contributed to specific experiments. Yu Zhang contributed to conception and provided CCR2 knockout mice. All authors gave their final approval and agree to be accountable for all aspects of the work.

ACKNOWLEDGEMENTS

This work was supported by the Peking University Clinical Scientist Program (BMU2019LCKXJ010), the National Natural Science Foundation of China (81970536) and the Research Foundation of Peking University School and Hospital of Stomatology (PKUSS20220114).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available in the main text or the Supplementary Materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Jiang, W., Xu, T., Song, Z., Wang, X., Yuan, S., Li, Q., Wei, Y., Wang, C., Yang, G., Cao, J., Mo, Y., Liu, Z., Li, N., Li, S., Lv, P., Zhang, Y., Wang, Y., & Hu, W. (2023). CCL2 is a key regulator and therapeutic target for periodontitis. *Journal of Clinical Periodontology*, 1–14. https://doi.org/10.1111/jcpe.13872