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Invisible appliance promotes bone reconstruction via modulating the periodontal immune microenvironment

HiuChing Wong[1,2], Yuching Huang[1,2] and Pu Yang[1,2*]

Abstract

Objectives Recently, invisible appliances have become popular due to their aesthetics and comfort, but their impact on periodontal health in periodontitis patients has not been fully elucidated. The purpose of this study was to investigate the effectiveness of fixed and invisible appliance treatment for periodontitis in experimental rats while monitoring the dynamics of Th17/Treg cells and osteoclast-related cytokines during treatment.

Methods SD rats were randomly divided into six groups: control, periodontitis model, scaling-0 (basic treatment), no appliance, fixed appliance, and invisible appliance. After successful establishment of ligature-induced periodontitis, rats in the treatment groups were first subjected to supra/subgingival scaling and daily brushing for 2 weeks. Next, except rats in the scaling-0 group, the periodontitis rats were treated with uncorrected, fixed, or invisible appliance, respectively. The CEJ-AC was examined using micro-CT. Osteoclast activation was detected with TRAP staining. The proportion of Th17 and Treg cells was measured by flow cytometry. Subsequently, potential mechanisms were explored by detection of RORγt, Foxp3, RANKL, OPG, and pathogenic bacteria (Pg, Aa, and Tf). Finally, the association between Th17/Treg cells and osteoclast-related indicators as well as pathogenic bacteria was evaluated by correlation analysis.

Results Among the three treatments, invisible appliance treatment provided optimal results in controlling periodontal infection while promoting periodontal tissue repair. Specifically, in the early phase of treatment (3–7 days), orthodontic force exerted by the appliance stimulates osteoclast and Th17 cell activation by increasing RANKL and RORγt expression, thereby inducing osteoclast generation and accelerating the amount of tooth movement. In the later stages of treatment (7–21 days), Foxp3 and OPG levels gradually increased to induce the dominant role of Treg cells, controlling osteoclast generation and bone resorption. Meanwhile, the invisible appliance can maintain a low number of pathogenic bacteria during treatment. Finally, Th17 cells showed significant positive correlation with RANKL, Pg, osteoclast percentage, and CEJ–AC; Treg cells were positively correlated with RANKL and OPG; Th17/Treg ratio displayed positive correlation with RANKL/OPG or osteoclast percentage.

Conclusions Invisible appliance has obvious advantages in periodontitis treatment, which can effectively improve periodontal condition and reduce inflammatory response.

Keywords Periodontitis, Appliance, Th17 cells, Treg cells, Osteoclasts

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Introduction

Periodontitis is the sixth most prevalent human chronic disease, affecting an estimated 60% of the adult population worldwide [1]. It is a disease caused by dysfunctional plaque biofilms and is characterized by progressive destruction of tooth support apparatus, which may lead to tooth loss as well as other serious complications, such as diabetes mellitus and cardiovascular system disorders [2–5]. For patients with severe periodontitis (stage IV), if not treated in time, it will cause irreversible damage to the alveolar bone and lead to tooth loss, negatively impacting the patients' masticatory function and overall quality of life [6]. In addition, pathological tooth migration caused by tooth loss poses a great challenge for clinicians. Currently, the treatment of severe periodontitis is usually multiscientific, and especially the benefits of orthodontic treatment in the overall rehabilitation of the occlusion are gradually being recognized [7]. Orthodontic treatment can help adjust pathologic tooth displacement, independent of deterioration of clinical periodontal parameters, and improves esthetics [8, 9]. Despite the increasing demand for orthodontic treatment in periodontally compromised patients, orthodontic treatment is rarely included in the overall treatment plan due to lack of knowledge in the dental community [10]. Therefore, it is urgent to study the interaction between periodontics and orthodontics.

Traditional orthodontic treatment, especially fixed appliance, provide a stable retention force, which can effectively control the direction of tooth movement and thus ensure the stability/precision of the correction effect [11]. However, fixed appliance hinders the maintenance of oral hygiene and can markedly increase the accumulation of plaque bacteria, which may cause the development of gingival inflammation [12]. Meanwhile, fixed appliance is inadequate in terms of aesthetics and comfort. In recent years, with increasing patient demands for aesthetics and advances in material science, invisible orthodontic technology has been developed [13]. Compared with fixed appliance, invisible appliance ensures adequate periodontal/gum health and better oral hygiene; and it also meet the patients' requirements for aesthetics and comfort [14–16]. Notably, in orthodontic patients with periodontitis, invisible appliance can shorten the correction time, maintain the alveolar bone height, and effectively improve the periodontal condition [17]. Although the results of the current evidences provide valuable information, research on the comparative analysis of the two appliances in periodontitis is still limited, particularly from the perspective of pathogenesis.

The pathogenesis of periodontitis is closely related to the dysregulation of host immune response [18, 19]. As key cells in the regulation of immune homeostasis, the role of $CD4^+$ T cells in the periodontitis progression has attracted special attention from researchers [20]. It has been reported that the imbalance between important $CD4^+$ T cell subsets including Th17 and Treg cells may be important in the pathogenesis of periodontitis [21]. An increased proportion of Th17 cells and decrease of Treg cells were detected in periodontitis [22]. In particular, aberrant activation of Th17 cells induces osteoclast generation through up-regulation of RANKL expression, followed by the production of cytokines contributing to periodontal destruction, such as IL-17 [23]. Conversely, Treg serves a protective role in periodontitis, and their elicitation of Foxp3 attenuates immune-mediated tissue damage by regulating effector T cell function to secrete anti-inflammatory cytokine [24]. Thus, maintaining the balance between Th17 and Treg cells is an important strategy to prevent and treat periodontitis [25]. Ge et al. found that orthodontic treatment was effective in avoiding alveolar bone over-resorption by affecting the Th17/Treg ratio and then altering osteoclast metabolism under periodontitis conditions [26]. However, their study only considered the regulation of immune factors by fixed appliance and did not include invisible appliance. Hence, we believe that it is necessary to supplement the regulation effect of invisible appliance on immune homeostasis, to provide theoretical reference for the selection of appliances in periodontitis treatment.

To the best of our knowledge, the purpose of periodontal treatment is to control infection and remove dental plaque as well as calculus, so scaling is also utilized in periodontal clinic [27]. Besides, adequate oral hygiene is a prerequisite for orthodontic appliance therapy and is important for periodontal care. In this study, we established a rat model of experimental periodontitis that first received basic treatment (supra/subgingival scaling), followed by either uncorrected or appliance treatment. Our objective was to compare the effects of no orthodontic treatment, fixed appliance, and invisible appliance on periodontal health status, Th17/Treg cell ration, and related regulator factors.

Materials and methods

Animals

Sixty male SD rats (age, 7 weeks; weight, 200–250 g) were acquired from Beijing HFK Bio-Technology co., LTD. All rats were placed under standard experimental facilities and acclimated for 7 days under the conditions of 45–65% humidity, 20–25 °C temperature, and 12 h of light–dark cycle. This study was authorized by the Animal Ethics Committee of West China School/

Hospital of Stomatology Sichuan University (No. WCHSIRB-D-2024-523) and all experimental procedures were conducted following the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

Animals were randomly divided into the following six experimental groups: (i) healthy control (control, $n=4$), (ii) periodontal disease (model, $n=4$), (iii) scaling-0 (basic treatment, $n=4$), (iv) no appliance ($n=16$), (v) fixed appliance ($n=16$), and (vi) invisible appliance ($n=16$). Meanwhile, to longitudinally observe the effects of different treatments on the periodontitis-related pathological states, biological samples from rat in groups iv, v, and vi at 3 day, 7 day, 14 day, and 21 day post-treatment were collected for subsequent analysis (four rats/group at a timepoint). These timepoints can cover the inflammatory acute phase, chronic phase, and tissue repair phase, which helps to systematically evaluate the process of orthodontic periodontal tissues remodeling [28, 29]. This study design involved changes in key measures between control, disease, and treatment groups, along with observations of differences in indicators at different timepoints within same group. Notably, scaling-0 day group served as the control for different treatment.

Establishment of ligation-induced periodontitis

Experimental periodontitis in rats were performed as described in previous study [30]. The preparation method of the experimental periodontitis model consisted of two stages. In the first stage, after SD rats were anesthetized and immobilized, a 0.25 mm stainless steel ligature wire (XIHUBIOM, Hangzhou, China) was placed between the first and second maxillary molar, followed by securing the ligature wire and 4-0 silk suture (Yangzhou Jinhuan Medical Appliance Factory, Yangzhou, China) around the neck area of the first molar. In the second stage, the original ligature wire was removed and new stainless steel wire ligatures continued to be placed around the same subgingival cervical area (pressing the wire under the gum as much as possible). Each phase lasted 15 days, and the *Porphyromonas gingivalis* (Pg, 1×10^9 CFU/mL, obtained from West China School/State Key Laboratory of Oral Diseases, Chengdu, China) was applied to the ligated gingival sulcus every other day. After ligation, rats were given 10% sugar water. Throughout the duration of the experiment (21 days), the ligatures were checked periodically and replaced if the ligatures were loose or detached. At the end of the modeling, one rat was randomly selected for observation. If there was obvious gingival redness, swelling, bleeding or erosion in the upper jaw, and alveolar bone resorption to 1/3–1/2 of the tooth root was clearly observed by Micro-CT, indicating that the periodontitis model was successfully constructed.

Periodontal treatment

Periodontitis rats were first treated with supragingival cleaning and subgingival curettage. Next, the rats were brushed daily for 2 weeks until the gums were no longer red and swollen. Expect rats in the scaling-0 group, the remaining rats after basic treatment were categorized into no appliance, fixed appliance, and invisible appliance groups. Specifically, rats in the no appliance group only received daily brushing; rats in the fixed appliance group wore the fixed appliance at the first molar and brushed their teeth daily; rats in the invisible appliance group wore the invisible appliance (Xian Henghui Technology Co., LTD.) in the upper jaw and brushed their teeth every day after removing the appliance. The invisible appliance was changed every 3.5 days during the treatment period, with the aligner moving 0.1 mm each time. The treatment lasted for 21 days. In addition, the design timeline and method of rats in each group are displayed in Fig. 1.

Detection of alveolar bone resorption by micro-CT

The maxillae of the rats were collected and fixed in 4% paraformaldehyde for 24 h. Then, we employed SkyScan 1176 machine (BRUKER, Beijing, China) to perfume micro-CT scanning of the specimen, with the following parameters: pixel size of 17.76 μm, X-ray voltage of 65 kV, and source current of 385 μA. The quadrilateral region from the alveolar ridge crest to root apex in the proximal–medial and distal–medial directions of the first molar root was measured in the proximal–medial and distal–medial directions of the roots of selected first molar teeth (CEJ–AC). The CEJ–AC distance can reflect the degree of alveolar bone resorption, and its increase usually indicates the intensification of alveolar bone resorption, which is an important sign of the periodontitis progression [31].

TRAP staining

TRAP staining was conducted as described in the previous article [32]. TRAP staining was performed on the maxilla of rats from each group to detect osteoclast activity. Briefly, the paraformaldehyde-fixed maxilla samples were decalcified with 10% EDTA solution for 3 weeks, followed by paraffin embedding and preparation into frozen sections (5 μm thick). The tissue sections were stained with TRAP solution (G1492, Solarbio, Beijing, China) for 60 min and then re-stained with methyl green agent for 5 min. Afterwards, stained sections were imaged using a light microscope, and the percentage of

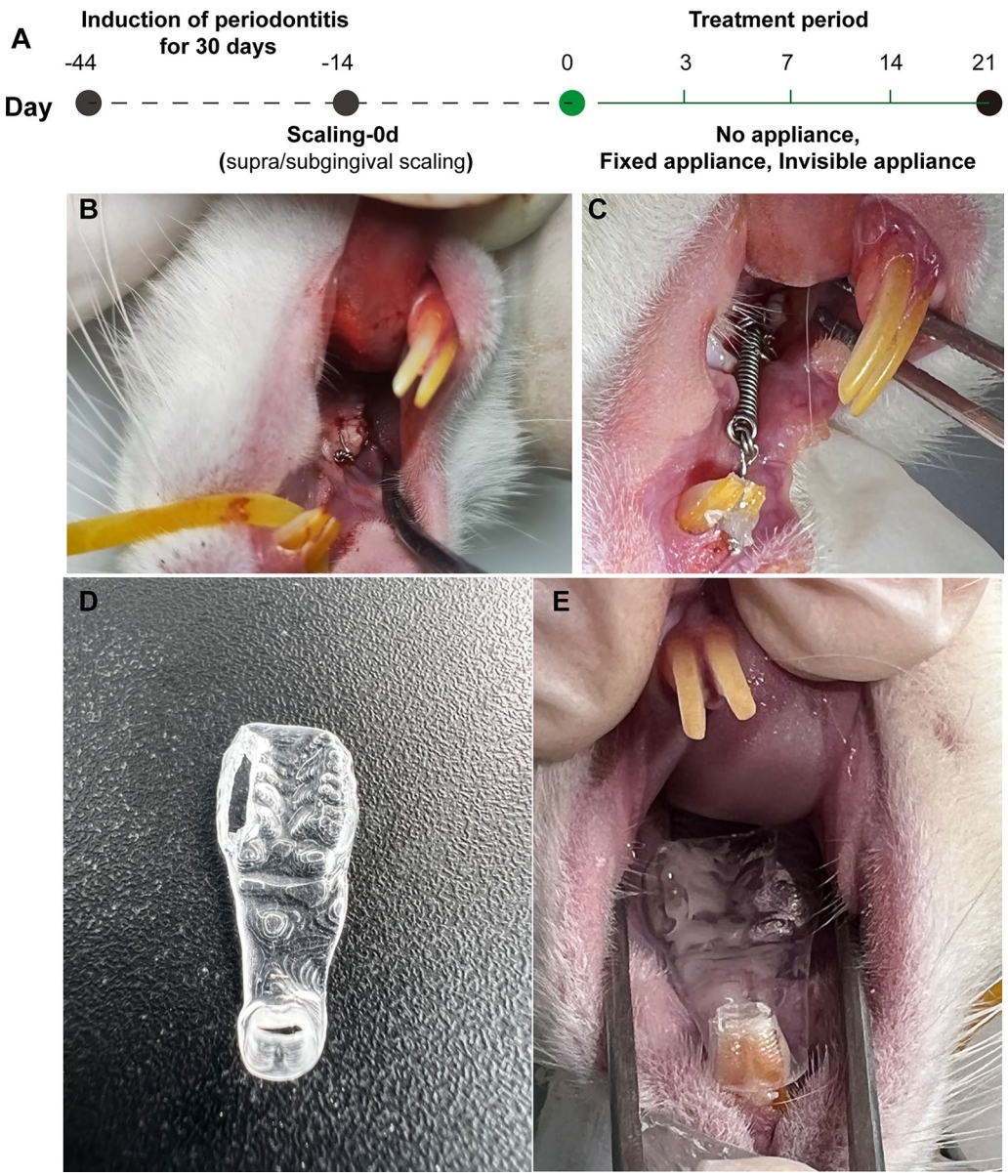

Fig. 1 Work flow chart of study design. **A** Timeline for constructing animal models. **B** Periodontitis model. **C** Rat model of fixed appliance treatment. **D** Appearance of invisible appliance. **E** Rat model of invisible appliance treatment

osteoclasts (1 field of view, × 400) as well as areal density (3 fields of view, × 200) were calculated. Specifically, the area percentage of osteoclasts is defined as osteoclast pixel area (purple)/field pixel area * 100; area density is defined as the positive cumulative optical density value (IOD)/pixel area (AREA) in each photograph [33].

Flow cytometry analysis of Th17 and Treg

The proportion of $CD8^{-}IL\text{-}17A^{+}$Th17 cells or $CD25^{+}FOXP3^{+}$Treg cells in each group was detected by flow cytometry [26]. Briefly, serum-free medium (125 μL), PMA/Ionomycin mixture (1 μL; HY-18739/HY-13434, MedChem Express, NJ, USA), and BFA/monensin mixture (1 μL; HY-16592/HY-N0150, MedChem Express) were added to rat abdominal aortic artery blood (125 μL), followed by incubation at 37 °C for 6 h to activate T cells. For the detection of Th17 cells, samples were stained with ER780-CD3 antibody (E-AB-F1228S, Elabscience, Hubei, China) and PerCP-eFluor™ 710-CD8a antibody (46-0084-82, ThermoFisher Scientific, MA, USA) for 60 min at 4 °C in the dark. Cells were further fixed and permeabilized according

to the manufacturer's procedure. Subsequently, the samples continued to be incubated with PE-labeled IL-17A antibody for 60 min in the dark. As for Treg cells, APC-coupled CD25 antibody (70-F3102503-100, Multi Sciences, Zhejiang, China) and FITC-coupled CD4 antibody (E-AB-F1105C, Elabscience) stained the cell surface for 60 min at 4 °C. After fixing and permeabilizing the cells based on the manufacturer's protocols, intracellular staining was performed using PE-coupled Foxp3 (12-5773-82, eBioscience) for 30 min. Finally, the percentage of Th17 or Treg cells in T cells after 4% paraformaldehyde fixation was determined by flow cytometry (CytoFLEX, Beckman coulter, California, USA). We also analyzed the Th17/Treg cell ratio in the samples.

Immunohistochemical (IHC) assay

To further assess the inflammatory response in periodontitis, the expression levels of Foxp3, RANKL, RORγt, and OPG in maxillary samples were examined using IHC assay, according to the methods reported in previous article [34]. The sample sections were heated to restore antigen reactivity, and then 3% H_2O_2 solution was added to block endogenous peroxidase activity. Afterwards, the slices were incubated with goat serum for 15 min and treated with the following primary antibodies at 4 °C overnight: anti-FOXP3 (No. bs-10211R, Bioss, Woburn, MA, USA), anti-RANKL (No. bs-0747R, Bioss), anti-RORγt (No.bs23110R, Bioss), and anti-OPG (No.A2100, ABclonal, Woburn, MA, USA). After washing three times with PBS, the sections were incubated with secondary antibody (Goat anti-Rabbit IgG, No.GRA0072, Multi Sciences, Hangzhou, China) solution for 15 min. The samples were washed with PBS three times again, and then stained with DAB reagent as well as hematoxylin. The stained sections were observed under a microscope (CX40, SOPTOP, Zhejiang, China), with counting of positive cells.

qRT-PCR detection

We also examined key genes through qRT-PCR, including RORγt, Foxp3, *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), and *Tannerella forsythia* (Tf), to observe periodontitis severity or immune mechanisms at the transcriptomic level. Total RNA (genes) or DNA (bacteria) was extracted from periodontal tissues of rats in each group using TriQuick Reagent (R1100, Solarbio), and cDNA (only for RNA) was subsequently obtained by reverse transcription using the All-in-One First-Stand cDNA Synthesis kit (AT341, TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Finally, qRT-PCR was conducted using the Green qPCR SuperMix kit (AQ602, TransGen Biotech) on a fluorescent quantitative PCR instrument (ABI QuantStudio 1, ThermoFisher Science). The relative expression levels of target mRNA were calculated using $2^{-\Delta\Delta CT}$ method, with GAPDH or Universal primer as the normalized gene. Primers used for qRT-PCR are listed in Table 1.

Statistical analysis

Data from experiments repeated at least three times were expressed as mean ± SD. GraphPad Prism (version 8.0, GraphPad, CA, USA) was utilized for statistical analysis and plotting of histograms. One-way ANOVA was used to determine the effect of different time periods in the same treatment regimen on disease outcomes, while two-way ANOVA was used to measure the effect of different treatments and times on disease outcomes. Tukey test was further performed to complement the ANOVA. Besides, Spearman analysis was employed to confirm correlations between variables, mainly including associations between immune cells (Th17, Treg, or Th17/Treg) and indicators of disease severity (key factor level, TRAP activity, and CEJ–AC). The significance level for the results was set at $P < 0.05$.

Table 1 Primer sequences used for qRT-PCR

Gene name	Primer sequence (Forward, 5′-3′)	Primer sequence (Reverse, 5′-3′)
GAPDH	ACTCTACCCACGGCAAGTTC	TGGGTTTCCCGTTGATGACC
RORγt	CACAGAGACACCACCGAACA	GTGCAGGAGTAGGCCACATT
Foxp3	CCAGTACCCCCAAATTCCTG	AGGACATACGCAGAAAGCCAG
Universal primer	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT
Pg	AGGCAGCTTGCCATACTGCG	ACTGTTAGCAACTACCGATGT
Aa	AGAGTTTGATCCTGGCTCAG	CACTTAAAGGTCCGCCTACGTGCC
Tf	ATCCTGGCTCAGGATGAACG	TACGCATRCCCATCCGCAA

Results

Micro-CT assay and TRAP staining reveals effect of different treatments on bone resorption

We first observed the effects of different treatment methods on periodontitis in rats, and collected periodontal tissues for micro-CT and histological analysis. The main manifestation of periodontitis is alveolar bone destruction, so we mainly calculated the CEJ–AC score of each group (Fig. 2). Micro-CT analysis showed that compared with fixed appliance, CEJ–AC was reduced in the no appliance and invisible appliance groups, without significant difference. Notably, at the late stage of treatment (21 days), the CEJ–AC was slightly lower in the invisible aligner group than in the no appliance group, suggesting that it could reduce periodontitis-induced alveolar bone loss in rats.

Following, we assessed osteoclasts on the interradicular alveolar bone surface by TRAP staining (Fig. 3A). Almost no TRAP-positive cells were detected in the control group. TRAP-positive osteoclasts (purple staining) were observed around alveolar bone of rats in the model group, and statistical analysis also confirmed that the number of positive cells was significantly higher than that in the control group (Fig. 3B, C). Compared to the model group, the periodontal basic therapy (scaling-0 day group) markedly reduced the number of positive cells, indicating that it inhibited osteoclastogenesis. We also observed the effect of different treatments on the differentiation of osteoclasts. For no appliance group, the number of osteoclasts showed a decreasing trend with extension of treatment time. For fixed appliance group, the osteoclast counts displayed a trend of increasing and then decreasing during period of treatment, with a peak at 14 days. Similarly, during treatment with invisible appliance, the number of osteoclasts was observed to increase and then decrease, reaching a peak at 7 days. Notably, the inhibition of osteoclast activation by no appliance was superior to the other two aligners. In addition, the number of osteoclasts in the fixed appliance group was higher than that in the invisible appliance throughout the treatment period.

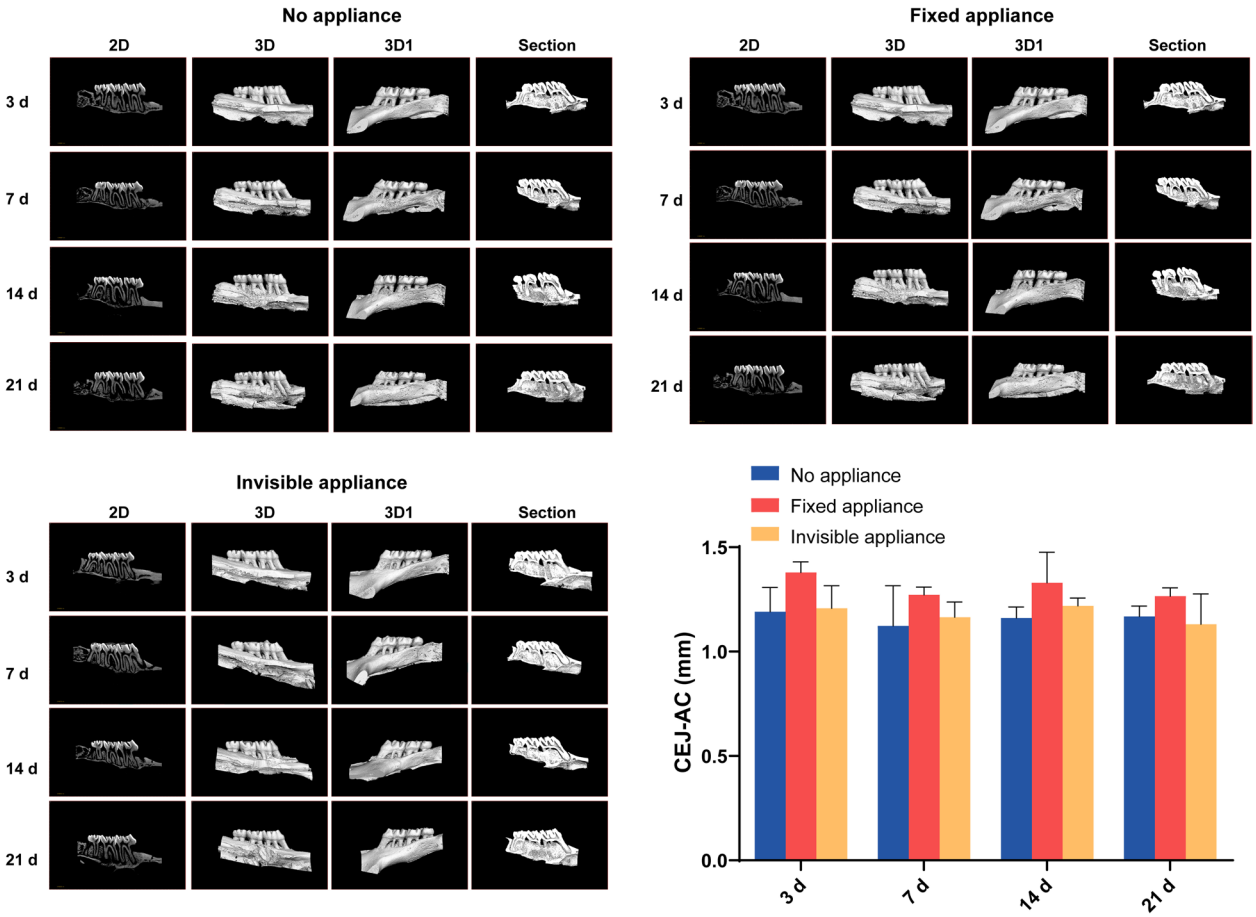

Fig. 2 Micro-CT images showing bone loss measurements for each group at day 3, day 7, day 14, and day 21. *N* = 4 per group

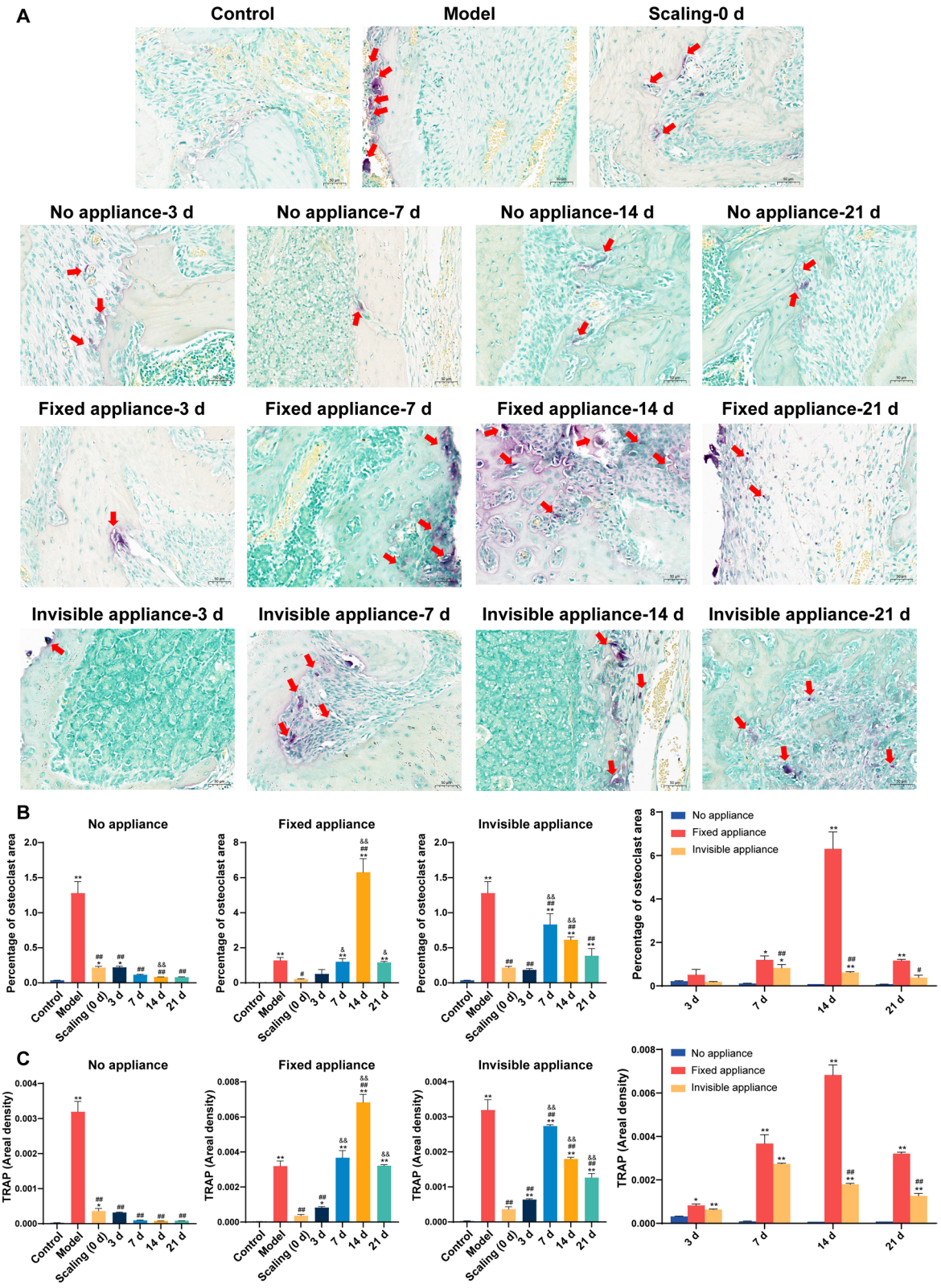

Fig. 3 Detection of osteoclast activity in periodontal tissue by Trap staining. **A** Representative stained sections (arrows indicate osteoclasts that are dyed red). **B**, **C** Quantitative analysis of osteoclast number. In the same treatment, *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. model; &$P < 0.05$, &&$P < 0.01$ vs. scaling-0 day. In the comparison of different treatment, *$P < 0.05$, **$P < 0.01$ vs. no appliance; #$P < 0.05$, ##$P < 0.01$ vs. fixed appliance. $n = 4$ per group

Effect of different treatment modalities on Th17, Treg, or Th17/Treg cells analyzed by flow cytometry

Considering that the disturbed balance between Th17 and Treg cells can aggravate periodontitis. Thus, we focused on the ability of different treatments to affect Th17 and Treg populations using flow cytometry (Fig. 4A). Results indicated that the percentages of Th17 and Treg cells were significantly elevated in the model group compared to the control group, and basic treatment (scaling-0) can dramatically reduce Th17 while increasing the Treg cell count. We also observed a severe imbalance of Th17/Treg ratio in rats from the model group compared to the control group, whereas basic treatment (scaling-0) notably restored Th17/Treg homeostasis (Fig. 4B). In the no appliance group, the number of Th17 cells tended to increase with treatment duration; Treg cells tended to decrease at the beginning of the treatment period (no appliance-3 days), then began to increase and peaked at 14 days, followed by a sharp decrease at the end of treatment (no appliance-21 days). Consistent with this, the rats in the no appliance-14 day group had the lowest Th17/Treg ratio throughout the treatment period (Fig. 4B). In the fixed appliance treatment group, the proportion of Th17 cells displayed an increasing and then decreasing trend with the prolonged treatment time, reaching a maximum on day 14; the percentage of Treg cells reached a maximum at 3 days, then decreased sharply to reach a minimum on day 7, and finally showed an increasing trend again. In addition, the least and most abundant Th17/Treg cell ratio were detected at 3 and 7 days after fixed aligner treatment, respectively (Fig. 4C). For the invisible appliance group, the number of Th17 cells increased first and then decreased during the treatment period, achieving a peak at 7 days. Notably, the changing trends of Treg cells and Th17/Treg ratio in this group were consistent with those observed in the fixed appliance group (Fig. 4D). Afterwards, we compared the differences in the regulation of immune homeostasis among three treatment modalities (Fig. 4E). Compared to two appliance treatments, the no appliance group maintained the lowest percentage of Th17 cells throughout the treatment period. At the beginning (3 days) and end (21 days) of treatment, appliances treatment significantly remained higher Treg cell counts compared with the no appliance group, especially the invisible group. Besides, at the beginning of treatment, the appliances markedly suppressed the Th17/Treg ratio compared with the no appliance group. However, with increasing treatment time, the two appliances significantly promoted an elevated Th17/Treg ratio, while then decreased it in the later stage of treatment, with the invisible appliance having the most obvious effect.

Effect of different treatment modalities on Th17/Treg balance-associated regulatory factors

Evidence suggests that periodontal pathogen-mediated Th17/Treg cell imbalance may regulate tooth bone resorption through the receptors, so we examined immunomodulatory factors (RORγt and Foxp3) and key signals of osteoclast differentiation (RANKL and OPG) by IHC to explore the specific mechanisms by which different treatments regulate immune homeostasis. RORγt and Foxp3 are key transcription factors of Th17 and Treg cells, respectively. As shown in Figs. 5A and 6A, the protein expression of RORγt and Foxp3 in the model group was significantly up-regulated in the model group compared with the control group; the basic treatment (scaling-0) induced a down-regulation of RORγt and an up-regulation of Foxp3. For the no appliance group, the expression level of RORγt decreased with increasing time. Meanwhile, for the fixed and invisible appliance groups, the protein level of RORγt increased and then decreased within 3–21 days, reaching the highest value at 14 (fixed appliance-14 day group) and 7 (invisible appliance-7 day group) days, respectively. Undoubtedly, the changes in the expression level of Foxp3 during the treatment period was consistent with the change pattern in Treg cells. Briefly, the Foxp3 level in the no appliance group first declined within 3 days, then increased during 3–14 days, and finally decreased in 21 days. The Foxp3 level in the two appliance groups was up-regulated on day 3 of treatment (highest value), then rapidly down-regulated on day 7, and finally displayed a gradual upward trend within 7–21 days. As for the difference among three treatment methods, RORγt level in the no appliance group was significantly lower than in the other two groups through the treatment period. Meanwhile, the suppression effect of invisible appliance on RORγt level was better than the fixed appliance group. We also observed that in the middle and later stages of treatment (7–21 days), the no appliance group could slightly inhibit Foxp3 expression, while the appliance administrations exerted the opposite effect, and again, the invisible appliance was found to promote the gene level better than the fixed group. As expected, similar changes in transcriptional levels of RORγt and Foxp3 were also seen in each group (Figs. 5B, 6B).

Effect of different treatment modalities on osteoblast-related cytokines

In the periodontal tissues of periodontitis model rats, a significant increase in RANKL expression and decrease in OPG expression was detected (Figs. 7, 8). Notably,

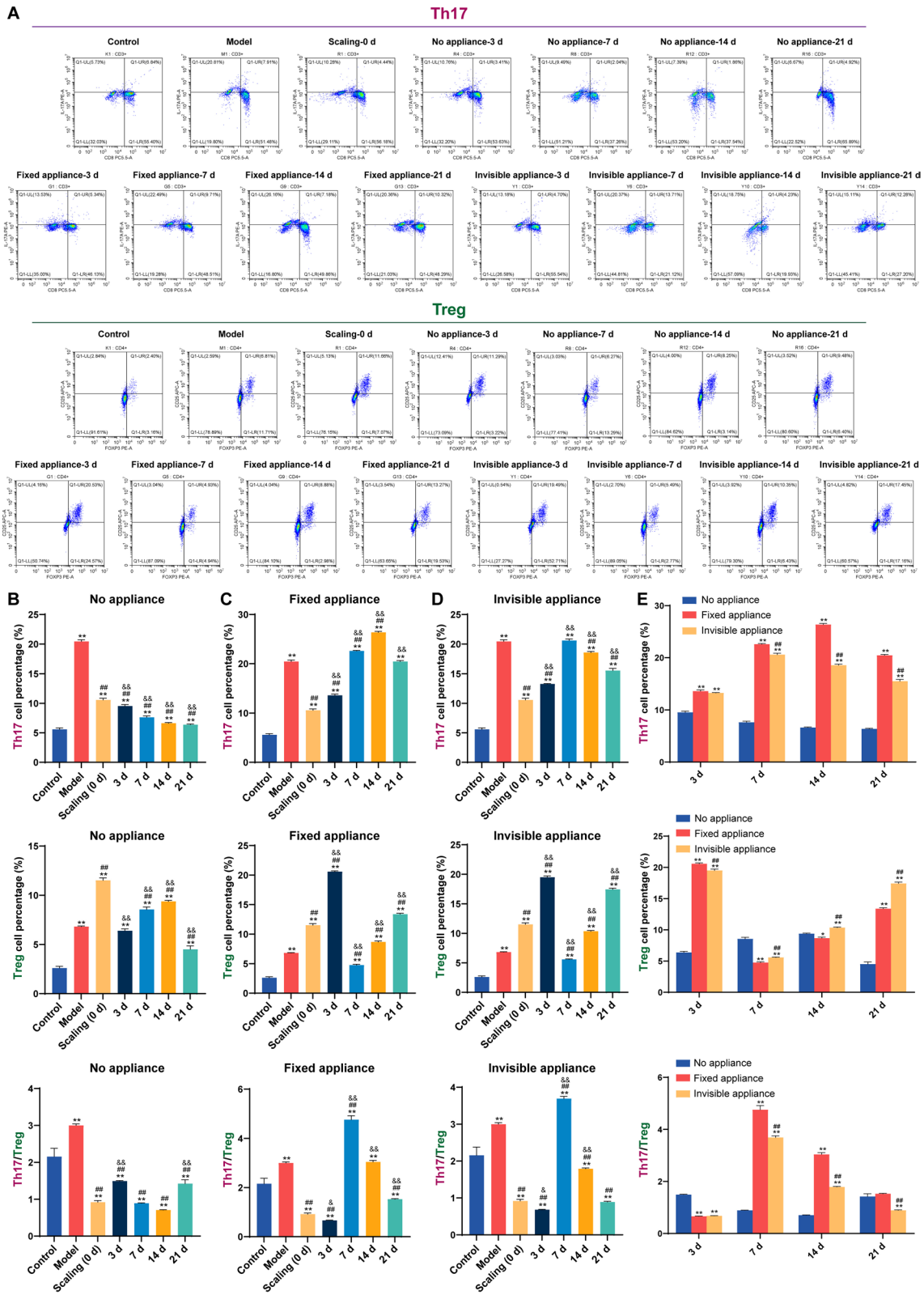

Fig. 4 Detection of Th17, Treg, and Th17/Treg ratio in the blood from rats. **A** Proportion of Th17 and Treg cells analyzed by flow cytometry. Dynamic changes of Th17, Treg, and Th17/Treg ratio during treatment with the no appliance (**B**), fixed appliance (**C**), and invisible appliance (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $\#P < 0.05$, $\#\#P < 0.01$ vs. model; $\&P < 0.05$, $\&\&P < 0.01$ vs. scaling-0 day. **E** Comparison of Th17, Treg, and Th17/Treg in three treatment modalities. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. no appliance; $\#P < 0.05$, $\#\#P < 0.01$ vs. fixed appliance. $n = 4$ per group

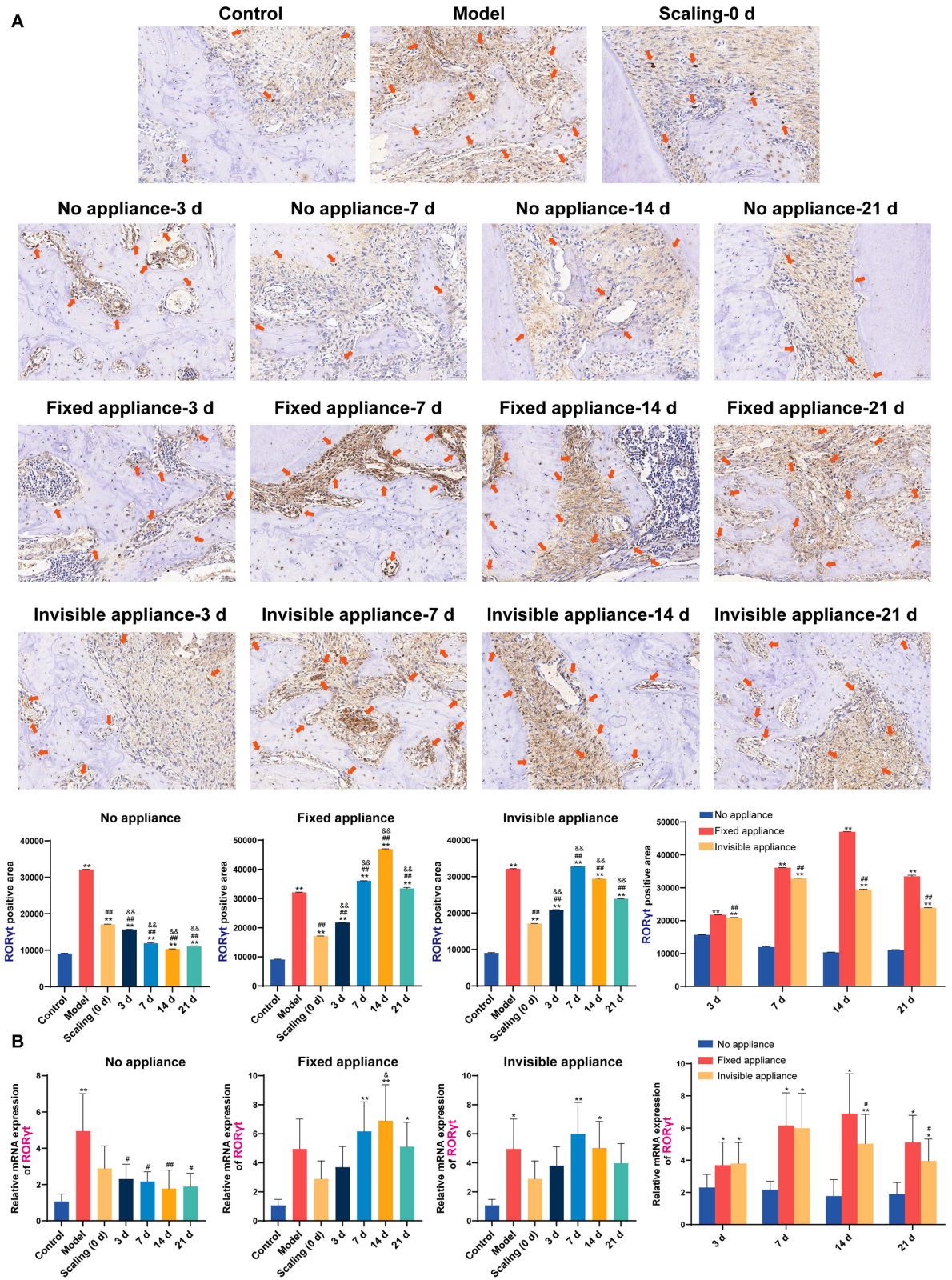

Fig. 5 Effect of different treatment modalities on Th17-related transcription factor-RORγt. **A** Representative immunohistochemical staining images (positive staining for biomarkers (indicated by arrows) red to brown in color) and statistical analysis of RORγt. **B** mRNA expression level of RORγt detected by qRT-PCR. In the same treatment, $^{*}P<0.05$, $^{**}P<0.01$ vs. control; $\#P<0.05$, $\#\#P<0.01$ vs. model; $\&P<0.05$, $\&\&P<0.01$ vs. scaling-0 day. In the comparison of different treatment, $^{*}P<0.05$, $^{**}P<0.01$ vs. no appliance; $\#P<0.05$, $\#\#P<0.01$ vs. fixed appliance. $n=4$ per group

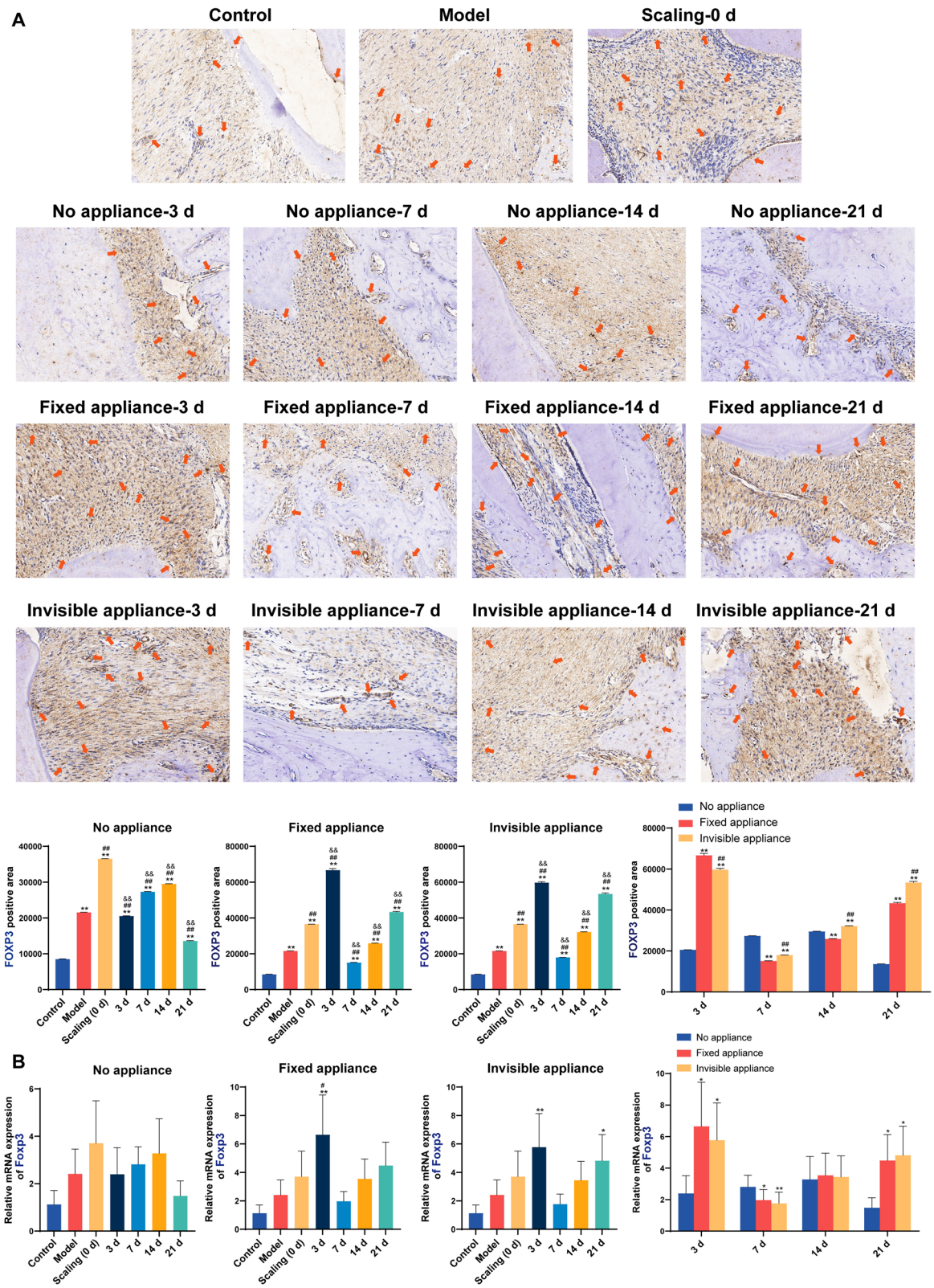

Fig. 6 Effect of different treatment modalities on Treg-related transcription factor—Foxp3. **A** Representative immunohistochemical staining images (positive staining for biomarkers (indicated by arrows) red to brown in color) and statistical analysis of Foxp3. **B** mRNA expression level of Foxp3 detected by qRT-PCR. In the same treatment, *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. model; &$P < 0.05$, &&$P < 0.01$ vs. scaling-0 day. In the comparison of different treatment, *$P < 0.05$, **$P < 0.01$ vs. no appliance; #$P < 0.05$, ##$P < 0.01$ vs. fixed appliance. $n = 4$ per group

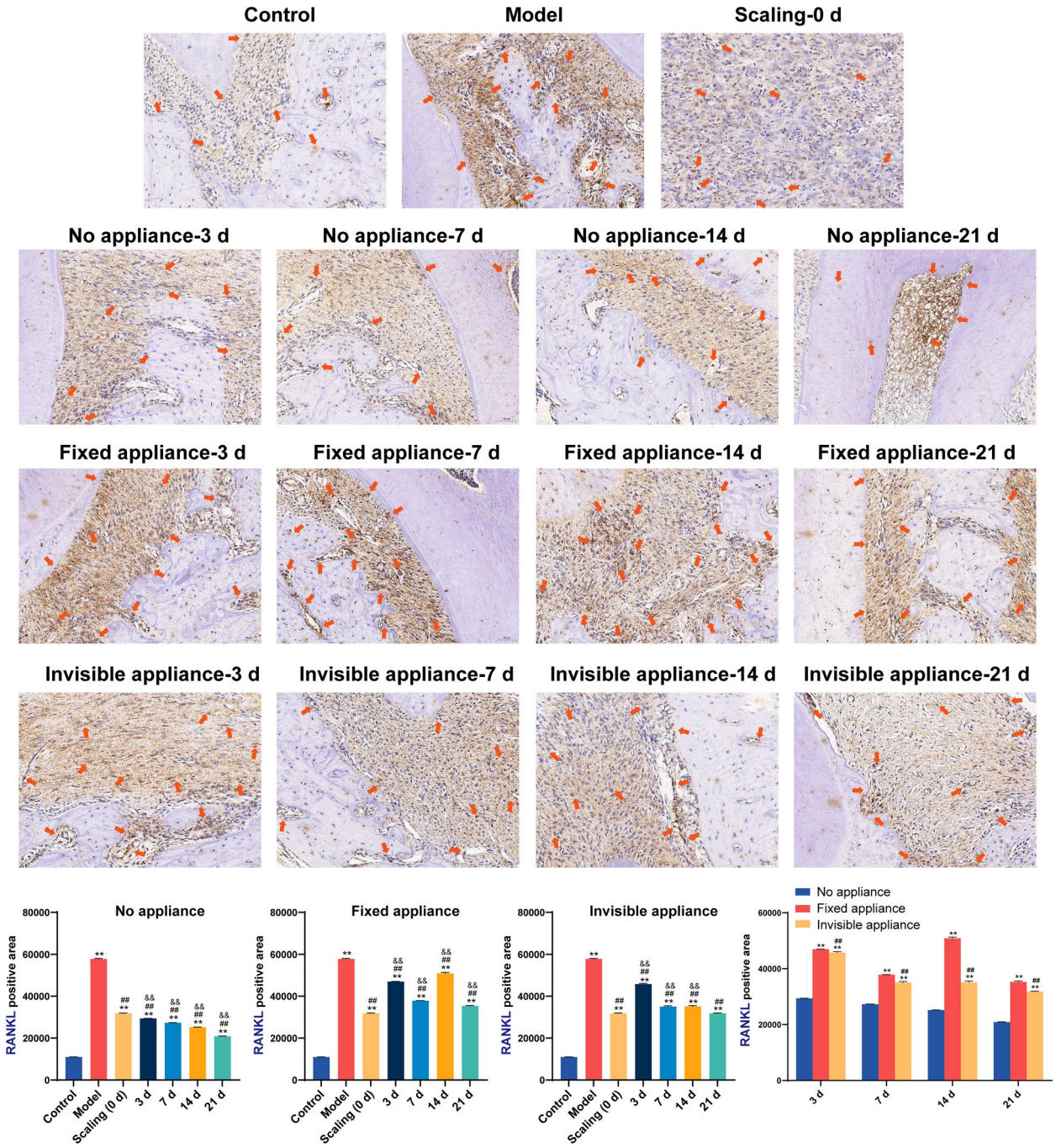

Fig. 7 Representative immunohistochemical staining images (positive staining for biomarkers (indicated by arrows) red to brown in color) and statistical analysis of osteoblast-related key signal-RANKL. In the same treatment, *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. model; &$P < 0.05$, &&$P < 0.01$ vs. scaling-0 day. In the comparison of different treatment, *$P < 0.05$, **$P < 0.01$ vs. no appliance; #$P < 0.05$, ##$P < 0.01$ vs. fixed appliance. $n = 4$ per group

basic treatment (scaling-0) clearly inhibited RANKL production while stimulating enhanced OPG expression. During no appliance period, we found that the synthesis of RANKL gradually decreased over time, accompanied by an increase in OPG production. After fixed appliance stimulation, RANKL expression was significantly higher during the whole treatment period than at day 0 of scaling, reaching a peak at 14 days and then decreasing. Similarly, OPG levels were highest at 14 days. Moreover, compared to the scaling-0 day group, invisible appliance

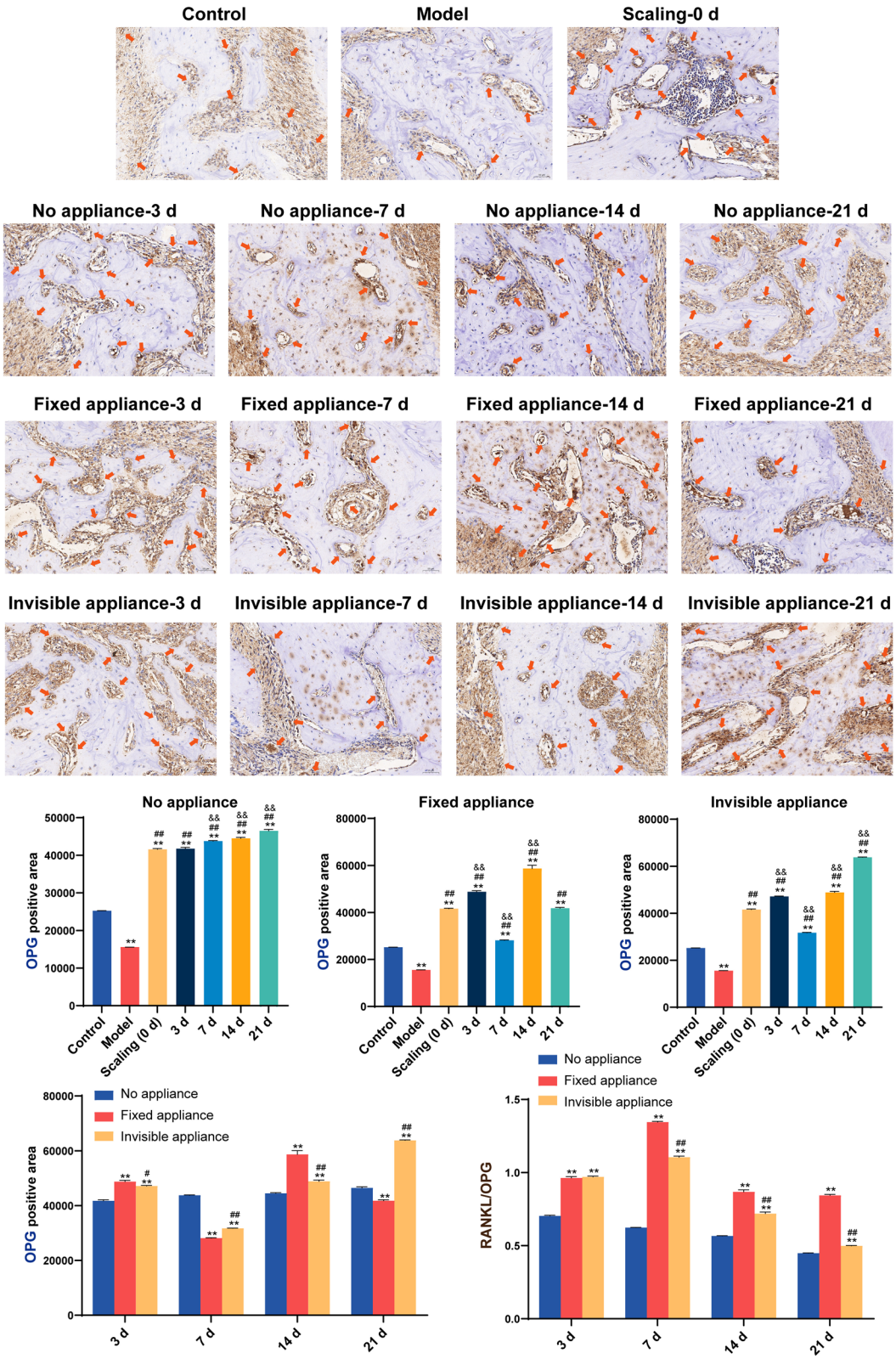

Fig. 8 Effect of different treatment modalities on OPG expression (positive staining for biomarkers (indicated by arrows) red to brown in color) and RANKL/OPG ratio. In the same treatment, *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. model; &$P < 0.05$, &&$P < 0.01$ vs. scaling-0 day. In the comparison of different treatment, *$P < 0.05$, **$P < 0.01$ vs. no appliance; #$P < 0.05$, ##$P < 0.01$ vs. fixed appliance. $n = 4$ per group

treatment could promote RANKL expression to a certain extent. In detail, it reached the highest value at 3 days and then showed a decreasing trend. On the contrary, the OPG level displayed a gradual increase in the later stage of invisible appliance treatment (7–21 days). The three treatments presented different modulation patterns on RANKL and OPG expression levels. Compared with the two appliance groups, the no appliance group maintained dramatically lower levels of RANKL during the treatment period. Furthermore, the invisible appliance treatment significantly suppressed RANKL levels in periodontitis rats compared to the fixed appliance. Following, two appliances notably increased the OPG levels at the beginning of treatment (3 days), while invisible devices still showed a promoting effect at the end of treatment. We also evaluated the changes in the RANKL/OPG ratio among different groups. Both appliance treatments exhibited higher RANKL/OPG ratios at different timepoints compared to the no appliance. Intriguingly, the ability of invisible appliance to suppress the RANKL/OPG ratio was clearly superior to that of fixed appliance. After basic scaling treatment (scaling-0 day), there was a clear decrease in Pg and Tf while an increase in Aa level.

Effect of different treatment modalities on pathogenic bacteria of periodontitis

We further observed the effects of different treatments on periodontal pathogens, including Pg, Tf, and Aa, through qRT-PCR. Compared with the control group, higher levels of Pg, Tf, and Aa mRNA levels were detected in the periodontal group of model rats, and only Tf results were statistically significant. In the rats received the no appliance, we found that there was no significant change in the Pg and Tf levels throughout the treatment; the Aa level showed a tendency to increase and then decrease, reaching a peak at 14 days (Fig. 9A). For fixed appliances, Pg and Aa levels exhibited a gradual increase in the early–mid treatment period (3–14 days) and a

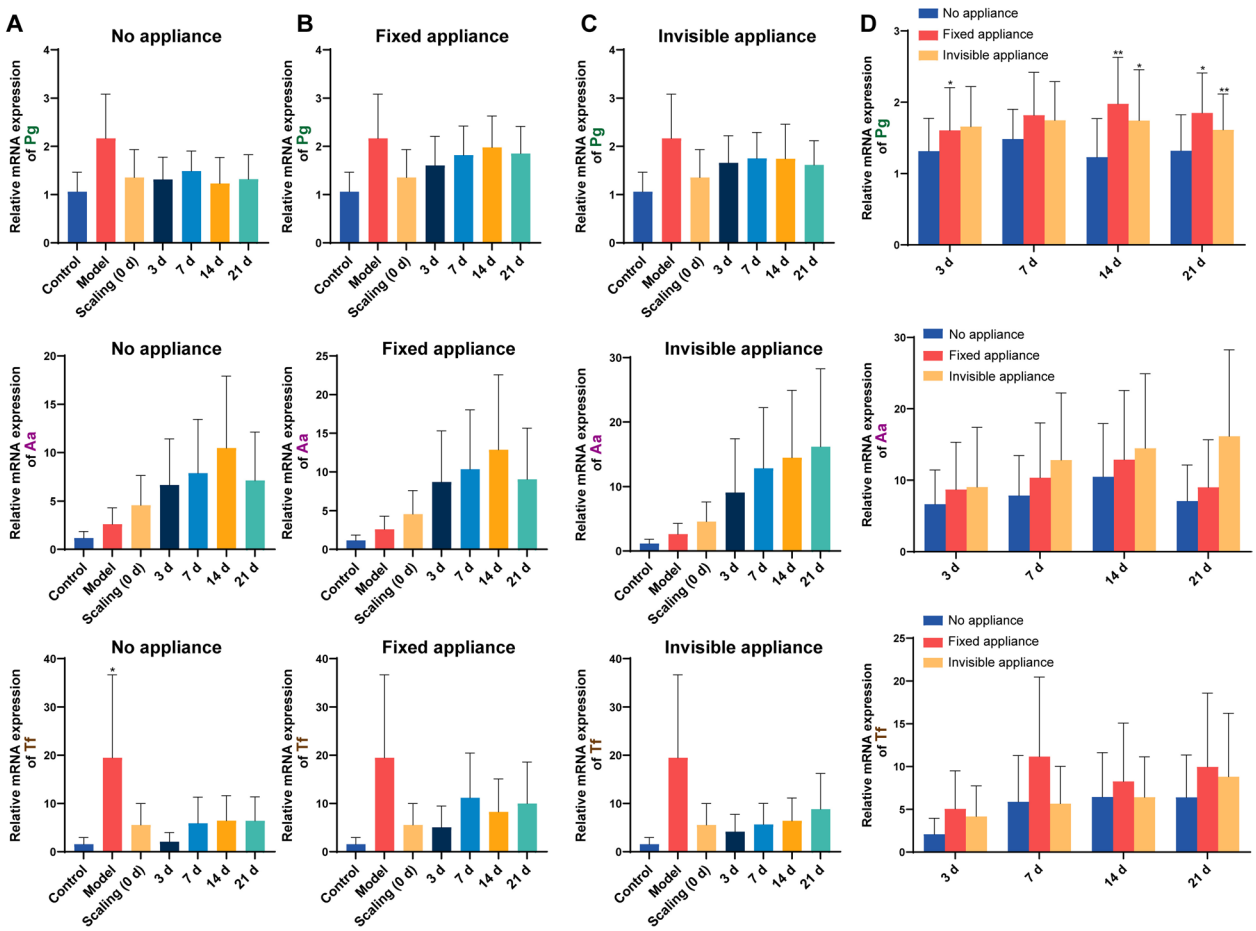

Fig. 9 Effect of different treatment modalities on pathogenic bacteria (Pg, Aa, Tf)-related mRNA level. **A** No appliance, **B** fixed appliance, **C** invisible appliance. *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. model; &$P < 0.05$, &&$P < 0.01$ vs. scaling-0 day. **D** Comparison of three treatment modalities. *$P < 0.05$, **$P < 0.01$ vs. no appliance; #$P < 0.05$, ##$P < 0.01$ vs. fixed appliance. $n = 4$ per group

decrease in late period; Tf content slowly elevated at the beginning of the treatment period, reached a peak on day 7, and then decreased. However, none of the changes were statistically significant (Fig. 9B). In the invisible appliance treatment group, Pg and Tf mRNA levels did not change significantly, while the Aa mRNA level gradually increased with the extension of treatment time, without statistical significance (Fig. 9C). In addition, a comparative analysis of the three groups revealed that Pg levels were significantly lower in the no appliance group than in the two appliances; the invisible appliance maintained Pg mRNA at relatively low level, but not statistically significant. All three methods promoted an increase in Aa levels during treatment, especially the invisible appliance. Besides, Tf mRNA level in fixed appliance were higher than in no appliance and invisible appliance. However, there was no statistical significance in the comparison of Aa or Tf levels among the three treatment methods (Fig. 9D).

Correlation analysis of Th17/Treg cells and osteoblasts-related factors or periodontal health

Pearson correlation analyses were performed to analyze the association of Th17 cells, Treg cells, and Th17/Treg ratio in the blood of periodontitis rats with osteoblast-associated factors and disease severity-related indicators. Th17 cell proportion was positively correlated with RANKL protein expression and Pg mRNA expression levels, but negatively connected with OPG protein expression (without statistical significance). There was a significant positive correlation between Treg cell proportion and RANKL or OPG levels. Meanwhile, the Th17/Treg ratio showed a significantly positive correlation with RANKL/OPG (Fig. 10A). For disease severity, Th17 cells and Th17/Treg ratio displayed a positive relationship with osteoclast count, and Th17 also showed a positive correlation with CEJ–AC, with statistically significant results (Fig. 10B).

Discussion

Periodontitis mainly affects bone homeostasis and host immune system to cause periodontal tissue destruction, which ultimately leads to gingival retraction and alveolar bone resorption [35]. Currently, it is a pressing challenge in the periodontitis treatment to promote the recovery of periodontal tissues while controlling the infection [36]. According to the available evidence, orthodontic treatment can effectively reduce periodontal trauma and promote periodontal recovery, especially invisible appliances are becoming increasingly popular due to their aesthetic and comfort features [37]. However, the current reports on the outcome of orthodontic treatment are limited, and there is a lack of objective evaluation of its impact on periodontal tissue. Besides, disease progression in patients with periodontitis depends on the severity of the microbial biofilm, so periodontal disease should be controlled prior to the formal initiation of orthodontic treatment, with the aim of avoiding further tissue loss caused by periodontal inflammation combined with orthodontic-induced inflammation [38]. Periodontal basic therapy is the main clinical treatment of periodontitis, which can effectively control periodontal inflammation [39]. In this work, based on a rat model of periodontitis, we comprehensively compared the therapeutic efficacy of without orthodontic treatment, fixed appliance, and invisible appliance. We

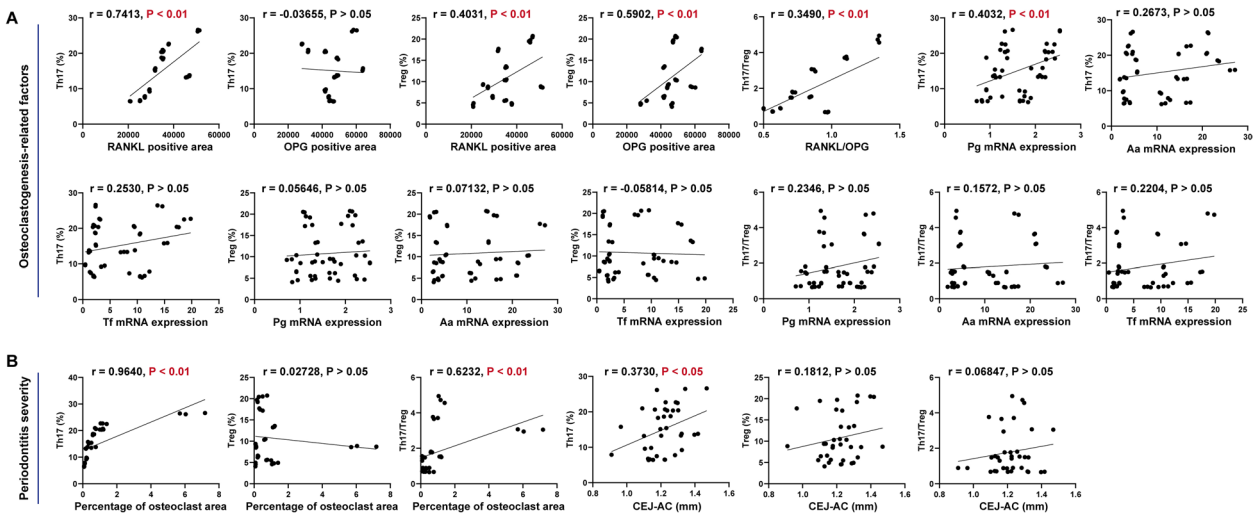

Fig. 10 Correlation analysis of the association between Th17/Treg cells with osteoblasts-related factors (**A**) and periodontal health (**B**)

also monitored the changes of immune factors and bone resorption modifiers during the treatment period, aiming to provide more data for the clinical treatment of periodontitis.

In this study, micro-CT revealed that compared with no appliance treatment, two kinds of appliance treatment could improve the alveolar bone height of periodontitis-infected teeth. For the three treatment modalities, only a small number of Trap-positive cells (osteoclasts) were observed at different timepoints in the no appliance group, whereas a significant increase in the number/area of Trap-positive cells was detected in two appliance treatments, with a tendency to first increase and then decrease during the treatment period. The core of orthodontic treatment is the reconstruction of periodontal tissue induced by orthodontic forces, thereby moving the misaligned teeth into the appropriate position [40]. It has been reported that orthodontic tooth movement mainly involves two steps, that is, the pressure exerted by the appliance induces the activation of osteoclasts on the "compression side" and promotes bone resorption; moreover, the recruitment of osteoblasts on the "tension side" causes bone formation, which accelerates tooth movement [41]. This mechanism of bone cell response to mechanical loading explains the behavior of osteoclasts during orthodontic tooth movement, leading to bone remodeling.

Of concern, orthodontic tooth movement is a sterile inflammatory response induced by mechanical force, especially within few hours of mechanical load that triggers an acute inflammatory response, stimulates the release of inflammatory mediators from immune cells, and exacerbates osteoclastogenesis [42]. Recent evidence has found that adaptive immune response is involved in the regulation of this inflammation [43]. Hence, we analyzed the proportion of Th17 and Treg cells in the rat blood. In the model rats, we first observed significant increase in Treg, Treg, and Th17/Treg ratio caused by periodontal inflammation. Then, basic treatment (scaling-0) induced an increase in Th17 cells and a decrease in Treg or Th17/Treg ratio, thereby informing an anti-inflammatory environment to reduce periodontal inflammation [44]. However, the alleviating effect of no appliance on periodontitis was short-lived, with a tendency for a significant increase in the Th17/Treg ratio on 21 days. During two appliance treatment, Th17 cells and Th17/Treg ratio peaked at 7–14 days, followed by a downward trend with orthodontic tooth movement. This is supported by Wald et al., who found that most immune cells remain highly active during the initial phase of orthodontic treatment, occurring between 7 and 14 days after orthodontic loading in rats [45]. Moreover, this phenomenon may be related to the orthodontic tooth movement and the stress response triggered by the existing inflammatory environment. Proliferation of Th17 cells sustains the release of a large amounts of cytokines, such as IL-17A, which favors osteoclastogenesis in response to orthodontic forces [46]. We also observed that orthodontic appliances can cause an increase in Treg cell activity from 7 to 21 days after treatment. One study reported that Treg cells can increase during the lag phase of orthodontic tooth movement, inducing bone formation and subsiding inflammation through suppressing the Th17 cell activity [47]. Notably, compared with fixed appliance, invisible appliance can maintain a dominant position of Treg cell proliferation and inhibit Th17 cells during treatment, thus exerting a maximum protective effect on periodontal tissue. In further explorations, we found that the appliance similarly elicited significant changes in RORγt (Th17 cell transcription factor) and Foxp3 (Treg cell transcription factor) during treatment, consistent with changes in cell ratio. The invisible appliance significantly inhibited RORγt expression and increased Foxp3 levels in the later stages of treatment, reconfirming its role in regulating Th17/Treg cell balance and possibly protecting tissue from progressive damage during periodontitis recovery.

Orthodontic forces are also involved in orthodontic bone remodeling in periodontitis by affecting osteoclasts and their related regulatory factors [48]. Compared to the model group, the basic treatment (scaling-0) can significantly up-regulated RANKL and down-regulated OPG to exert an inhibitory effect on inflammation. However, no appliance had a negligible effect on RANKL and OPG during the treatment period. Under the effect of appliances, RANKL as well as RANKL/OPG levels increased at the beginning of the treatment and then showed a decreasing trend, whereas OPG levels tended to increase at the later stage of treatment (7–21 days), especially with invisible appliance. The bone remodeling process is a balanced regulation between the RANKL–RANK system and OPG [49]. Evidence suggests that during experimental tooth movement in rats, mechanical stress stimulates the up-regulation of RANKL expression (stimulated by Th17 cells) and reduces OPG secretion in periodontal tissues, induces osteoclastogenesis, and thus accelerating the amount of tooth movement [50, 51]. Up-regulation of OPG and decrease of RANKL/OPG ratio after 2 weeks of orthodontic treatment in rats were also found in the study of Ge et al. [26]. This may be caused by the activation of Treg, which secretes immunomodulatory factors (such as IL-10, TGF-β, and CTLA-4) that decrease the RANKL/OPG ratio, thereby inhibiting osteoclastogenesis and bone resorption. Further correlation analysis also confirmed this result. Besides, the basic treatment (scaling-0) can effectively

inhibit periodontal plaque, including Pg and Tf. During the treatment period, only a slight increase in periodontal bacteria was observed in the appliance groups, but it was not statistically significant, suggesting that orthodontic treatment after periodontal stabilization had no harmful effects on periodontal health [52]. Notably, we still observed that inviable appliance was more advantageous in maintaining oral health. This finding is confirmed by various studies [14, 53, 54].

Although the clinical application and therapeutic benefits of invisible appliance have been extensively studied over the past decade, these reports have been mainly oriented to orthodontic outcomes, and few studies have explored their mechanisms involved in remodeling periodontal tissues from the etiological perspective [55, 56]. There is evidence that mechanical force serves an important role in the regulation of oral microenvironment, inflammatory infiltration, and bone metabolic balance during appliance treatment [57, 58]. Inspired by this, we designed the present study to compare the therapeutic efficacy of no appliance, fixed appliance, and invisible appliance for periodontitis, and then reveals the complex regulatory role of Th17/Treg and osteoclast-related factors during treatment. Despite the topic of this study is novel, it has to admitted that there are some limitations. First, the results of this study are obtained based on an experimental periodontitis model. Although the pathologic manifestations of the rat periodontitis model are similar to those of human periodontitis, the disease progresses more rapidly in rats and their dental anatomy and immune system different from those of human. Thus, the clinical applicability of our results should be interpreted with caution. Second, this study focused on the efficacy and cleaning characteristics of these appliances, and other factors that may affect oral health status and treatment outcomes have not been considered. Statistically, 5–10% of orthodontic patients fail to complete treatment due to poor oral hygiene [59]. Previous study found a higher incidence of minor and severe breakage of thermoplastic clear retainer, which may lead to less cleaning of the appliance by the patients, followed by possible concomitant plaque/stone accumulation and gingival problems over time [60]. Thus, these removable orthodontic appliances require a high degree of patient compliance for optimal results. Besides, the assessment of cost-effectiveness in clinical practice is meaningful for the selection of appliances. Evidence suggests that the cost of treatment with fixed appliance (Hawley appliance) is about 20% higher than invisible appliance (vacuum-formed retainer) over a 6-month retention period, revealing invisible appliance has superior cost-effective [61]. However, the present work was conducted in experimental periodontitis rats and did not emphasize the differences in compliance and treatment costs between the two appliances. Third, previous study revealed that in periodontist-affected inflammatory tissues, excessive orthodontic forces may prevent the restoration of homeostasis to the tissues, leading to "maladaptive homeostasis" as well as tissue loss due to uncoupled bone resorption and formation [62]. Unfortunately, this study did not consider the effect of the orthodontic force on the immune response or treatment outcomes. Thus, a subsequent comprehensive evaluation of the two treatment modalities in a clinical trial with long-term follow-up is needed to make conclusive recommendations.

Conclusion

In summary, orthodontic treatment is superior to periodontal basic treatment (scaling) for the improvement of periodontitis, especially invisible appliance. Invisible appliance can accelerate orthodontic tooth movement by inducing osteoclasts, Th17/Treg cells, and altering the expression osteoclast-associated factors (RANKL and OPG) in the early stage; and then in the late stage of treatment, it can suppress osteoclastogenesis and bone resorption, exerting a controlling effect on periodontal inflammation. Meanwhile, the invisible appliance facilitates the maintenance of a favorable oral environment during treatment. Overall, invisible appliance is effective in the treatment of patients with periodontitis, and play a positive synergistic role in controlling periodontal infection and repairing periodontal tissues.

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Author contributions

HiuChing Wong: Conception and design of the research, Acquisition of data, Analysis and interpretation of data, Drafting the manuscript. Yuching, Huang: Acquisition of data, Analysis and interpretation of data. Pu Yang: Conception and design of the research.

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Availability of data and materials

The raw data of experiments are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was authorized by the Animal Ethics Committee of West China School/Hospital of Stomatology Sichuan University (No.WCHSIRB-D-2024-523) and all experimental procedures were conducted following the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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