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ORIGINAL ARTICLE

Progranulin Promotes Regeneration of Inflammatory Periodontal Bone Defect in Rats *via* Anti-inflammation, Osteoclastogenic Inhibition, and Osteogenic Promotion

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Abstract— Progranulin (PGRN) has been proved to play a crucial role in anti-inflammation and osteogenesis promotion; thus, it was hypothesized that PGRN could promote bone regeneration in periodontal disease. In this experiment, the periodontal bone defects were established in periodontitis rats; recombinant human progranulin (rhPGRN), tumor necrosis factor alpha inhibitor (anti-TNF-α), or phosphate buffer saline (PBS)-loaded collagen membrane scaffolds were implanted within defects and the rats were sacrificed at scheduled time points. Volume of new bone was assessed by radiological and histomorphometric analyses. Expression of osteogenesis-related markers and tumor necrosis factor-α (TNF-α) was evaluated using immunohistochemistry. Tartrate-resistant acid phosphatase (TRAP) staining was also performed to determine the number of osteoclasts. Immunofluorescence (IF) staining was performed to explore the interaction between rhPGRN and tumor necrosis factor receptors (TNFRs). The results showed that the rhPGRN group had significantly superior quantity and quality of newly formed bone, higher expression of alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), and TNFR2 compared with the PBS group and the anti-TNF-α group. Similarly to the anti-TNF-α group, the rhPGRN group also exhibited the significant inhibitory effect on the expression of TNF-α and the number of TRAP-positive cells compared with the PBS group. Hence, our experiment suggests that PGRN promotes regeneration of inflammatory periodontal bone defect in rats *via* anti-inflammation, osteoclastogenic inhibition, and osteogenic promotion. Local administration of PGRN may provide a new therapeutic strategy for periodontal bone regeneration.

KEY WORDS: Anti-inflammatory agents; Growth factors; Experimental periodontitis; Periodontal regeneration; Tumor necrosis factor-α.

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INTRODUCTION

Periodontitis is one of the most prevalent chronic infections in human which involves periodontal attachment apparatus and leads to alveolar bone loss and eventual teeth loss [1]. Besides, periodontitis may exert a definite negative influence on several systemic diseases such as diabetes, rheumatoid arthritis, and cardiovascular diseases [2–5]. Therefore, to restore the destructive periodontal attachment apparatus holds an urgent challenge for oral and systemic health.

Numerous regenerative approaches, for example, mesenchymal stem cells (MSCs), biological mediators, and scaffold-based techniques, had been come out and shown out the considerable success [6–9]. However, the current understanding on periodontitis pathogenesis mechanism holds that the major periodontal destruction associated with periodontitis progression results from host immune inflammatory response to the bacterial challenge [2, 10]. Among numerous pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) is a master regulator in the pathogenesis of periodontitis, participating in tissue destruction and osteoclastogenesis [11]. The activation of multinucleated osteoclasts was enhanced in alveolar bone resorption while TNF-α was involved in osteoclastogenesis and disturbing alveolar bone regeneration associated with progress of periodontitis [12]. The abundant evidences also show that TNF-α exerts some negative effects on osteogenic differentiation of MSCs and bone regeneration [12–16]. Moreover, pro-inflammatory cytokines could compromise the osteogenesis promotion ability of some biological mediators such as bone morphogenetic proteins (BMPs) [17]. Thus, the development of new techniques and strategies that play a dual effect of anti-inflammation and osteogenesis promotion is essential for periodontitis-associated bone regeneration.

Progranulin (PGRN), also known as GRN-epithelin precursor, is a 593-amino-acid pleiotropic growth factor which is involved in tumorigenesis, host defense, neurodegeneration, inflammation, endochondral ossification, and tissue healing [18–21]. Recent studies reported that PGRN exhibited the higher affinity to bind with tumor necrosis factor receptors (TNFRs) than TNF-α [22]. There were also studies indicating that PGRN could reverse TNF-α-induced osteoclastic differentiation promotion and osteogenic differentiation inhibition [23, 24]. Additionally, PGRN could bind with tumor necrosis factor receptor 2 (TNFR2) to directly induce bone regeneration [25]. Unfortunately, the effect of PGRN on regeneration of inflammatory periodontal bone defect remains unclear. In present study, the therapeutic effect of PGRN on regeneration of inflammatory periodontal bone defect was evaluated using an experimental periodontitis model in rats.

MATERIALS AND METHODS

Animals

Seven-to-eight-week-old male Wistar rats weighing 300 to 320 g were supplied by Institute of Shandong University Animal Experimental Center. The rats were housed in the specific pathogen-free (SPF) unit of the animal facility, under 12-h light/dark cycle and fed up with distilled water and food *ad libitum*. All animal experiment procedures were performed under guidelines approved by the Institutional Animal Care Committee (protocol GR201712).

Experimental Periodontitis Model

Rats were housed for acclimatization about 1 week and then randomly assigned into two groups with three animals in each group, which were named as the experimental periodontitis and the healthy group, respectively. In periodontitis group, rats were anesthetized by using intraperitoneal injection with pentobarbital (35 mg/kg) and placed on the operation table with horizontal supine position, which allowed an access to place 0.3-mm orthodontic ligature into the gingival sulci embracing the cervical margin of the left mandibular first molar. Rats were fed with standard rat feed and 10% sucrose solution drinking. In the healthy group, the rats were fed with standard rat feed and sterilized water without orthodontic ligature. After 2 weeks, the rats were sacrificed by cardiac perfusion, and the mandibular specimens were harvested. The distance from cemento-enamel junction (CEJ) to alveolar bone crest (ABC) of the distal root of the first molar was measured by using Mimics 15.0–3 matic to analyze two-dimensional images which were captured by micro-computed tomography (micro-CT SCANCO Medical AG, Switzerland). Then, the specimens were routinely fixed in 4% paraformaldehyde for 48 h, decalcified in 10% EDTA for 3 months, embedded in paraffin, and cut into 5-μm-thick serial mesial-distal sections for hematoxylin and eosin staining (HE Solarbio, Beijing, China).

Inflammatory Periodontal Bone Defect Model Establishment and rhPGRN Administration

To create clinically relevant periodontitis-associated bone defect model, the surgical periodontal defects were made by modifying the method reported by Nagata et al. [26] in molar teeth of rats with periodontitis induced.

Briefly, periodontitis was firstly induced as described above for 2 weeks. Then, an extraoral incision was made at the left of the mandible and the buccal plate was exposed. The buccal bone from the medial root of the first molar to the mesial root of the second molar was carefully removed until root surface was exposed and alveolar crest removed. The dimensions of the defect were 2 mm in height and 3 mm in width.

Rats were randomly allocated to the following groups with nine animals in each group: (1) PBS, (2) recombinant human PGRN (rhPGRN 8 μg; Sino Biological, Beijing, China), and (3) anti-TNF-α (lenalidomide, 3 μg; MCE, America). All of the reagents were diluted into 10 μl PBS, and dripped slowly and gently into the collagen membrane (ZhengHai Biotechnology Yantai, Shandong, China) to make them distribute well, and all of the process was performed on ice in the sterile environment. Then, the collagen membranes were implanted into bone defects, respectively. The internal overlying masseter and external skin were repositioned and closed with 4–0 gut sutures separately. One, 4 and 6 weeks after operation, rat was sacrificed by cardiac perfusion and the mandibles were isolated for further analysis.

Micro-Computed Tomography Scanning and Analysis

Each specimen was scanned at 15 × 15 × 15 voxels through μCT-100 (SCANCO Medical AG, Switzerland) and evaluated by V6.5–3 evaluation software. Through two-dimension (2D) radiography, the region of interest (ROI) was located and the distance from CEJ to ABC of the distal root of the first molar was measured. Through 3D reconstructed images, the volumetric parameters of alveolar bone were measured. The bone volume fraction (BV/TV, %) were calculated and analyzed to represent bone quantity, while the parameters of the trabecular thickness (Tb.Th; mm), total number of trabeculae (Tb.N; mm), and trabecular spacing (Tb.Sp; mm) were gathered to evaluate bone quality.

Histomorphometric Analysis

The mandibular blocks of the rats were fixed in 4% paraformaldehyde phosphate buffer for 1 day, decalcified in 10% ethylenediamine tetraacetate (EDTA) for about 3 months, and embedded in paraffin. Buccal-lingual sections of 5-μm thickness were obtained, and respective three sections from each mesial, middle, and distal level of the defects were stained with hematoxylin-eosin (H.E.) and Masson's trichrome stainings. Images were gathered with an Olympus microscope (Olympus, Tokyo, Japan), and the percentages of the newly formed bone area over original defect area were analyzed with the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemical Staining

To determine the anti-inflammatory effect of PGRN, rabbit polyclonal anti-TNF-α (1:100 dilution, Abcam, Cambridge, MA) was used as primary antibody for 1-week specimens. To explore the osteogenesis effect of PGRN, rabbit polyclonal anti-Runx2 (1:200 dilution; Abcam, Cambridge, MA) and anti-ALP (1:400 dilution; Abcam, Cambridge, MA) were used as primary antibodies for 1-, 4-, and 6-week specimens. SPlink Detection Kit (Biotin-Streptavidin HRP Detection Systems ZSGB-BIO, Beijing, China) was used and biotin-conjugated goat anti-mice/rabbit IgG were used for second antibody. Diaminobenzidine (DAB; ZSGB-BIO, Beijing, China) was used to immunoreaction staining, and cell nuclei were stained with hematoxylin. Images were captured by using an Olympus microscope. Three high-magnified microscopic fields in each slice were selected, and TNF-α and Runx2-positive cells, characterized respectively by brown cytoplasm staining and brown nuclei staining, were counted and the integral optical density (IOD) of ALP was calculated by Image-Pro Plus 6.0 software by two independents.

Tartrate-Resistant Acid Phosphatase Staining

Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify and quantify osteoclast-like cells using The Acid Phosphatase Leukocyte Kit (TRAP; Sigma-Aldrich, Saint Louis, MO). Images of coded specimens were captured by using an Olympus microscope. Three high-magnified microscopic fields in each slice were selected, and TRAP-positive cells which were stained in red with three or more nuclei were counted by Image-Pro Plus 6.0 software.

Immunofluorescence Staining

To identify the mechanism of PGRN-mediated bone formation in periodontitis, TNFR1 mouse monoclonal antibody (1:200 dilution, 60192–1-Ig; Proteintech, USA) and TNFR2 rabbit polyclonal antibody (1:100 dilution, 19272–1-AP; Proteintech, USA) were used to stain the 4-week specimens. Alexa Fluor 594-conjugated goat anti-rabbit (1:100 dilution, SA00006–4; Proteintech, USA) and Alexa Fluor 488-conjugated affinipure goat anti-mouse (1:100 dilution, SA00006–1; Proteintech, USA) were used as fluorescent second antibodies. The

cell nuclei were stained with 4,6-diamino-2-phenylindole (DAPI). The stained specimens were visualized by using a fluorescent microscope (Olympus, BX51, Japan). Images were captured by a CCD camera (CoolSNAP-Pro *cf.*, Media Cybernetics, USA). The TNFR1- and TNFR2-positive cells were counted in selected merged microscopic images by Image-Pro Plus 6.0 software.

The *In Vitro* Coimmunoprecipitation Assay

The human periodontal ligament stem cells (PDLSCs) were cultivated and identified by the method reported by Shulan Chen et al. [27]. The rhPGRN (0.1 μg/ml) was used to stimulate PDLSCs. After 36 h, the medium was discarded, and the PDLSCs were washed with PBS for twice and total protein from PDLSCs was extracted in coimmunoprecipitation (Co-IP) buffer (50 mM Tris-HCl (pH 7.5), 1 mM NaF, 2 mM EDTA, 100 mM NaCl, 1 mM $NaVO_3$, 1 mM PMSF, 10 mM β-glycero phosphate, 0.1% (*v*/*v*) Triton X-100, 0.5% (*v*/*v*) Nonidet P-40, and 1× protease inhibitor cocktail), then incubated with anti-PGRN antibody (ab208777; Abcam, RabMAb Technology, USA) or normal rabbit IgG antibody (2729S, Cell Signaling Technology, USA) for 1 h at 4 °C. The mixture was incubated with protein A/G PLUS-Agrose (sc-2003, Santa Cruz Biotechnology Inc., USA) with gentle rotation overnight at 4 °C. Protein A beads were washed five times with Co-IP buffer and treated with SDS-PAGE sample buffer. The binding proteins were detected *via* western blot. In brief, the samples were separated by SDS-PAGE. The proteins were transferred onto a PVDF membrane. Membranes were then incubated with anti-TNFR1 (1:1000 dilution, C25C1; Cell Signaling Technology, USA), anti-TNFR2 (1:1000 dilution, ab109322; Abcam, RabMAb Technology, USA) and anti-PGRN (1:1000 dilution) antibody overnight at 4 °C. VeriBlot for IP detection (HRP; 1:2000 dilution, ab131366; Abcam, RabMAb Technology, USA) was used as secondary antibody. Images were collected with Amersham Imager 600 (GE Healthcare, Stockholm, Sweden).

Statistical Analysis

Statistical analyses were performed by GraphPad Prism 5.0 software (version 6, by Mackiev software; Boston, MA, USA). The differences among groups were assessed by one-way analysis of variation (ANOVA) and Student's *t* test. The values of statistical significance were confirmed by $P < 0.05$. Each data were presented as the mean ± standard deviation. All assays were performed at least thrice and representative data are presented.

RESULTS

Establishment of Experimental Periodontitis

To investigate the effect of rhPGRN on bone defect healing under inflammatory condition, the experimental periodontitis model was established at first. Micro-computed tomography (micro-CT) analysis showed that 2-week ligation with high sugar diet led to significant bone absorption in the interdental septum between the first and the second molar teeth (Fig. 1a, b, e). Meanwhile, histological observation demonstrated that there was abundant inflammatory cell infiltration in the connective tissues and obvious bone resorption in the experimental periodontitis (Fig. 1c), as compared to the healthy group (Fig. 1d).

PGRN Enhanced Inflammatory Periodontal Bone Defect Regeneration

HE staining showed that at 1 week after operation, less pink osteoid was formed around the defect edges and under the collagen membrane dispersedly, but there was no significant difference in the amount of newly formed bone among three groups. At 4 weeks, the percentage of new bone area in rhPGRN group was significantly higher than in the PBS group, whereas there was no significant difference between the PBS group and the anti-TNF-α group. At 6 weeks, the percentage of new bone area in the rhPGRN group and the anti-TNF-α group was significantly higher than in the PBS group, and the rhPGRN group also presented significantly higher percentage of new bone area than the anti-TNF-α group (Fig. 2).

Masson's trichrome staining was performed to distinguish the newly formed bone and original mineralized bone tissue. Briefly, the newly formed bone which was made up of collagen type I was stained in green. The results observed by Masson's trichrome staining were similar to HE staining (Fig. 3).

Reconstructed micro-CT 3D images showed that the quantity and quality of newly formed bone in the rhPGRN group were superior to the PBS group and in some aspects, the anti-TNF-α group. At 4 and 6 weeks, there was significantly higher BV/TV in the anti-TNF-α group than in the PBS group, and the rhPGRN group had significantly higher BV/TV than the anti-TNF-α group (Fig. 4a, b). Both the rhPGRN and the anti-TNF-α group exhibited significantly more Tb.Th (at 6 weeks) and Tb.N (at 4 weeks) than the PBS group, and the former also had significantly more Tb.N (at 6 weeks) than the later. There was significantly lower Tb.Sp in the rhPGRN group (at 4 and 6 weeks) and in anti-TNF-α group (at 4 weeks) than in the PBS group (Fig. 4b).

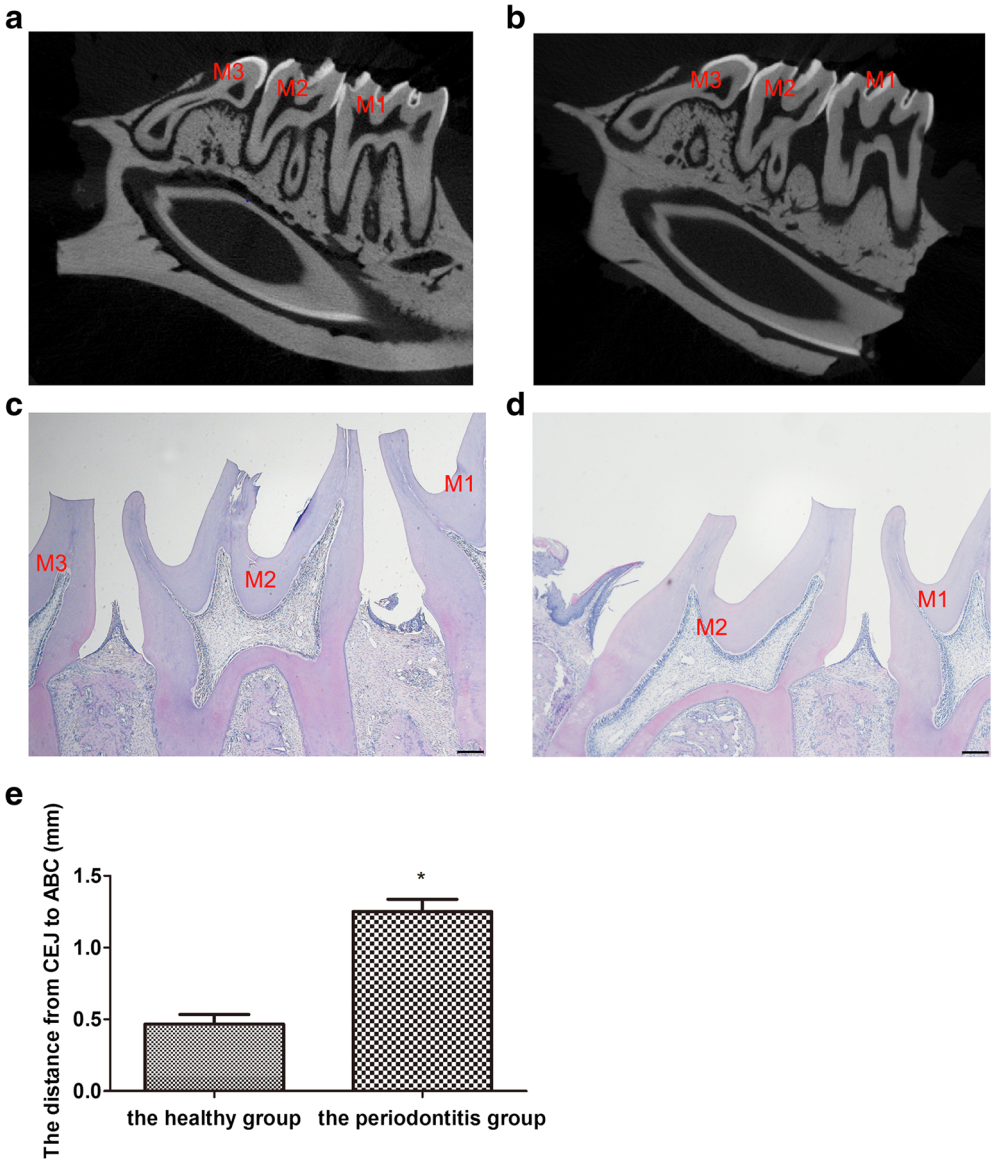

Fig. 1. Histological analysis and micro-CT analysis of alveolar bone resorption in rats with experimental periodontitis for 2 weeks. **a** The alveolar bone level in healthy group. **b** The alveolar bone resorption in periodontitis group. **c** HE staining for obvious inflammatory cell infiltration and bone absorption in the interdental septum between the first and the second molar. **d** HE staining for the healthy group. There were no obvious bone absorption in the interdental septum between the first and the second molar. **e** Statistical analysis of the distance from CEJ to ABC of the distal root of the first molar (mm). *M1* the first mandibular molar, *M2* the second mandibular molar, *M3* the third mandibular molar. $^{*}P < 0.05$ *vs* the healthy group; scale bars, 200 μm.

PGRN Increased Expression of Osteogenesis-Related Markers During Healing Process of Inflammatory Periodontal Bone Defect

Immunohistochemical staining was performed to analyze the expressions of ALP and Runx2. As compared to the PBS group, the rhPGRN group and the anti-TNF-α group revealed a significantly higher expression of ALP around the defect edges at 1 week. At 4 and 6 weeks, the IOD of ALP in the rhPGRN group was significantly higher than in the other two groups, but there was little difference between the anti-TNF-α group and the PBS group. The number of Runx2-positive cells was not significantly different among three groups at 1 week. However, there were

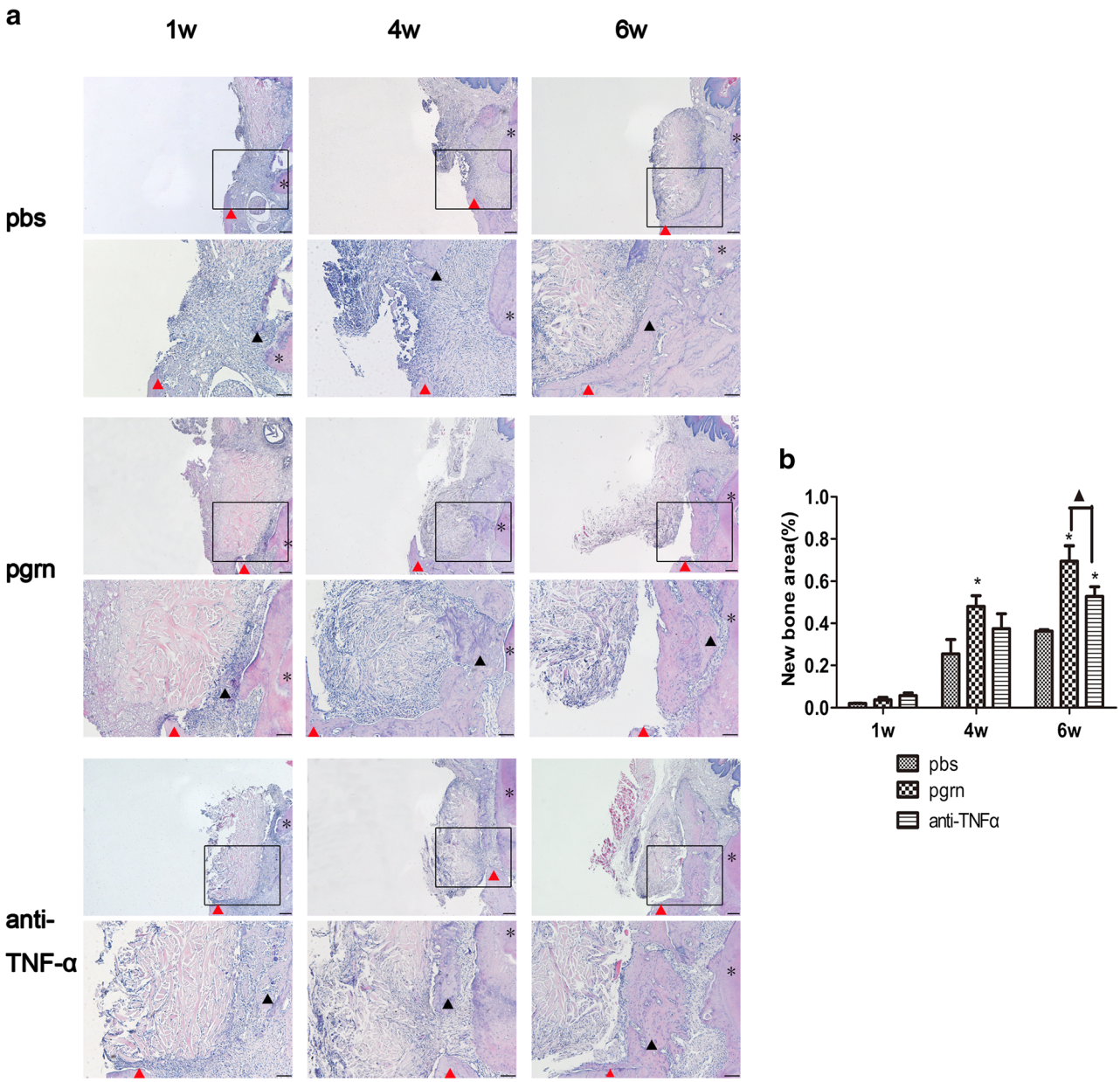

Fig. 2. Histological analysis of bone defects at 1, 4, and 6 weeks. **a** HE staining of bone defects. The visual fields framed by the black line in low-magnification images (scale bars, 200 μm) were magnified in the inferior (scale bars, 100 μm) of the each images. *Asterisks* dentin, *black arrows* new bone, *red arrows* the boundary of bone defects. **b** Statistical analysis for new bone area within bone defects. *$P < 0.05$ *vs* the control group, ▲$P < 0.05$ *vs* the anti-TNF-α.

significantly more Runx2-positive cells in the rhPGRN group at 4 weeks than in the other two groups. Furthermore, the rhPGRN group and the anti-TNF-α group had significantly more Runx2-positive cells than the PBS group at 6 weeks (Fig. 5).

PGRN Reduced Periodontal Inflammatory Response and Inhibited Osteoclastogenesis During Healing Process of Inflammatory Periodontal Bone Defect

To evaluate the anti-inflammation effect of rhPGRN, immunohistochemical staining for TNF-α was conducted

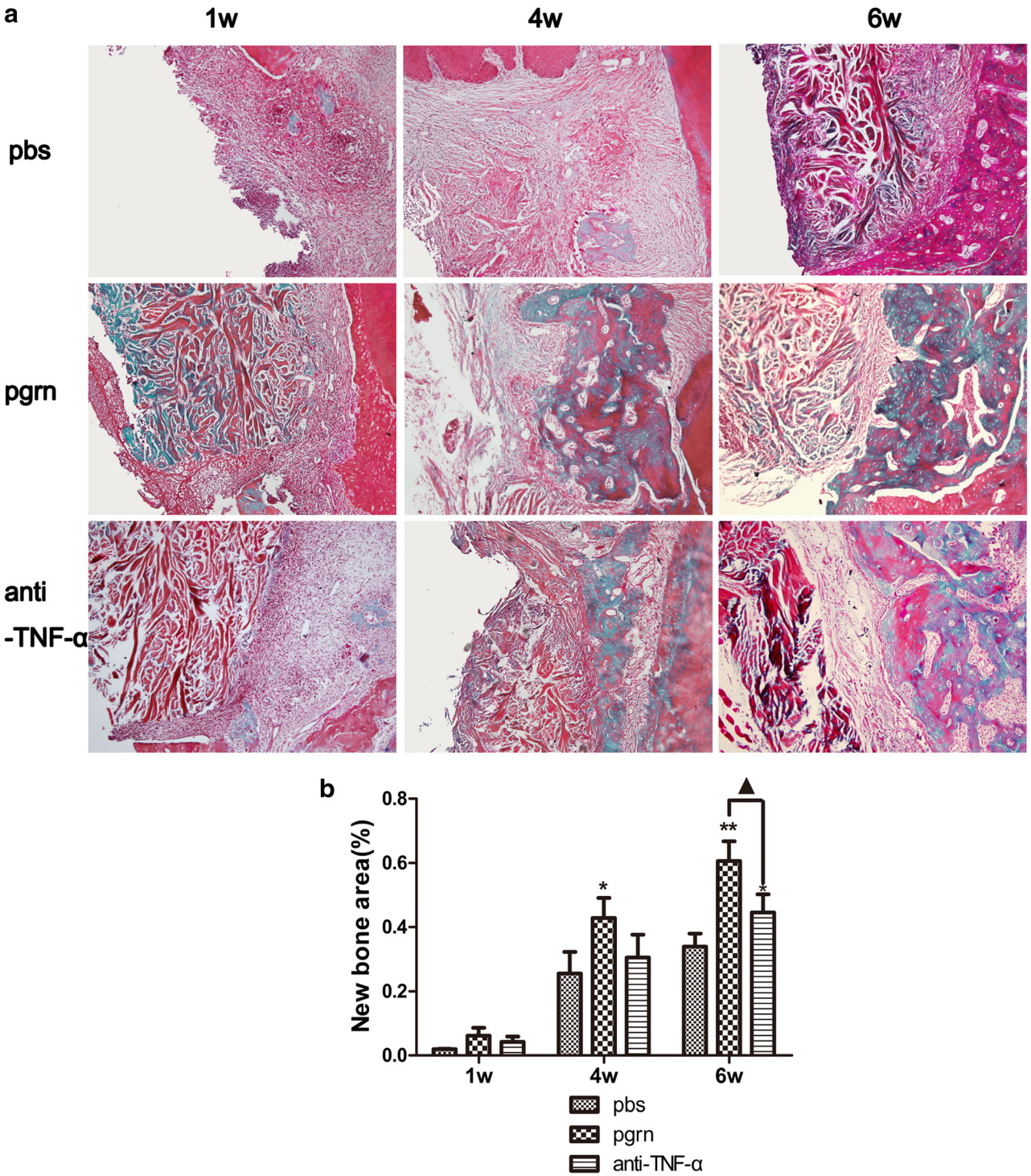

Fig. 3. Masson's trichrome staining of bone defects at 1, 4, and 6 weeks. **a** Masson staining of bone defects among three groups. The newly formed bone was stained with green and the mineralized bone tissue was stained with red. At 1 week, there was little newly formed bone among three groups. At 4 weeks, the amount of new bone was much higher in the rhPGRN groups. At 6 weeks, much of the newly formed bone (green) in the defect site was visible among three groups, but this amount was much higher in the rhPGRN group than the other two groups. **b** Statistical analysis for new bone area within bone defects. $^{*}P < 0.05$ *vs* the control group, $^{**}P < 0.01$ *vs* the control group, $^{\blacktriangle}P < 0.05$ *vs* the anti-TNF-α.

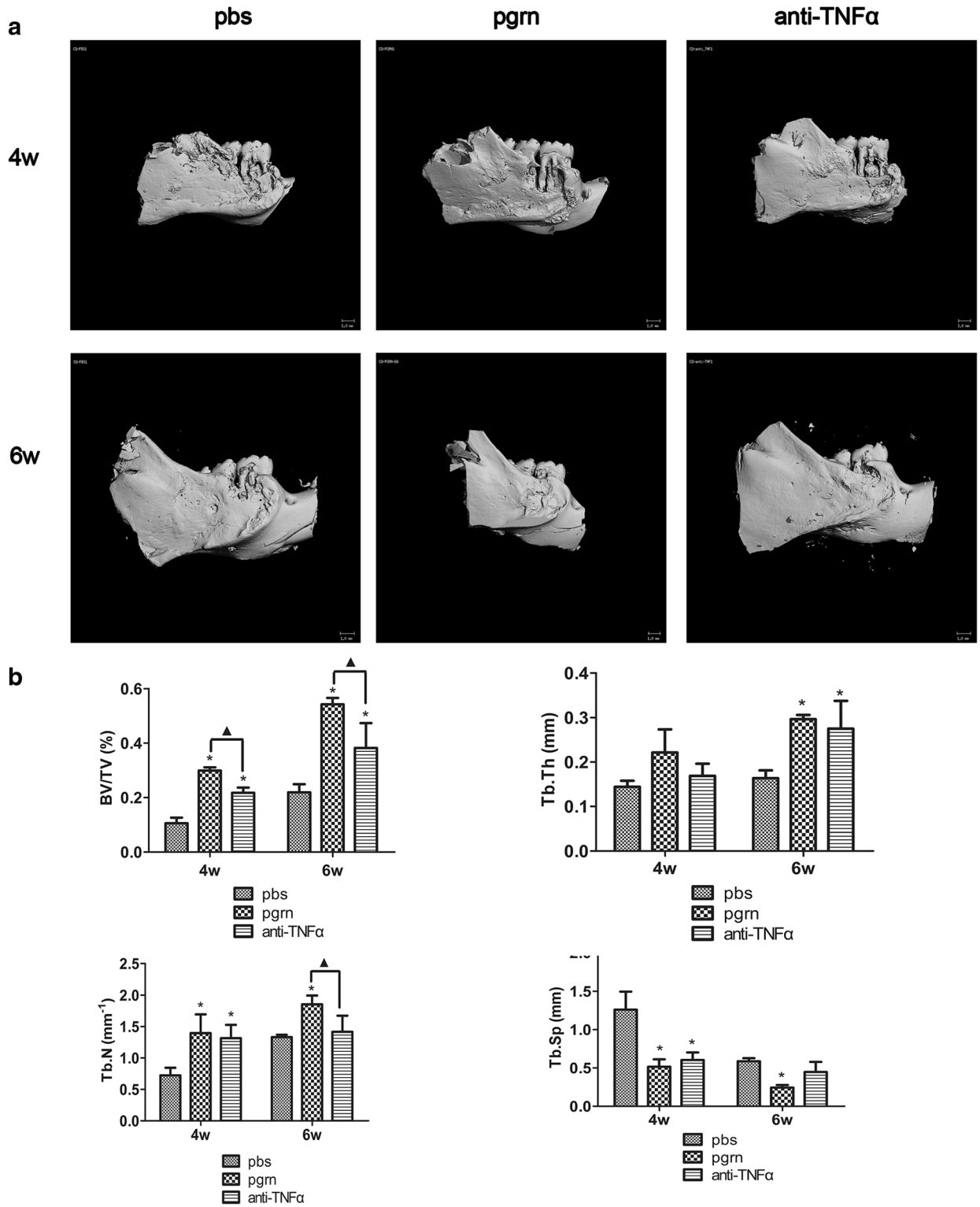

Fig. 4. Micro-CT analysis of the bone defects among three groups at 4 and 6 weeks. **a** 3D reconstructive images of bone defects. **b** Statistical analysis of BV/TV, Tb.Th, Tb.N, and Tb.Sp. *$P < 0.05$ *vs* the control group, ▲$P < 0.05$ *vs* the anti-TNF-α group.

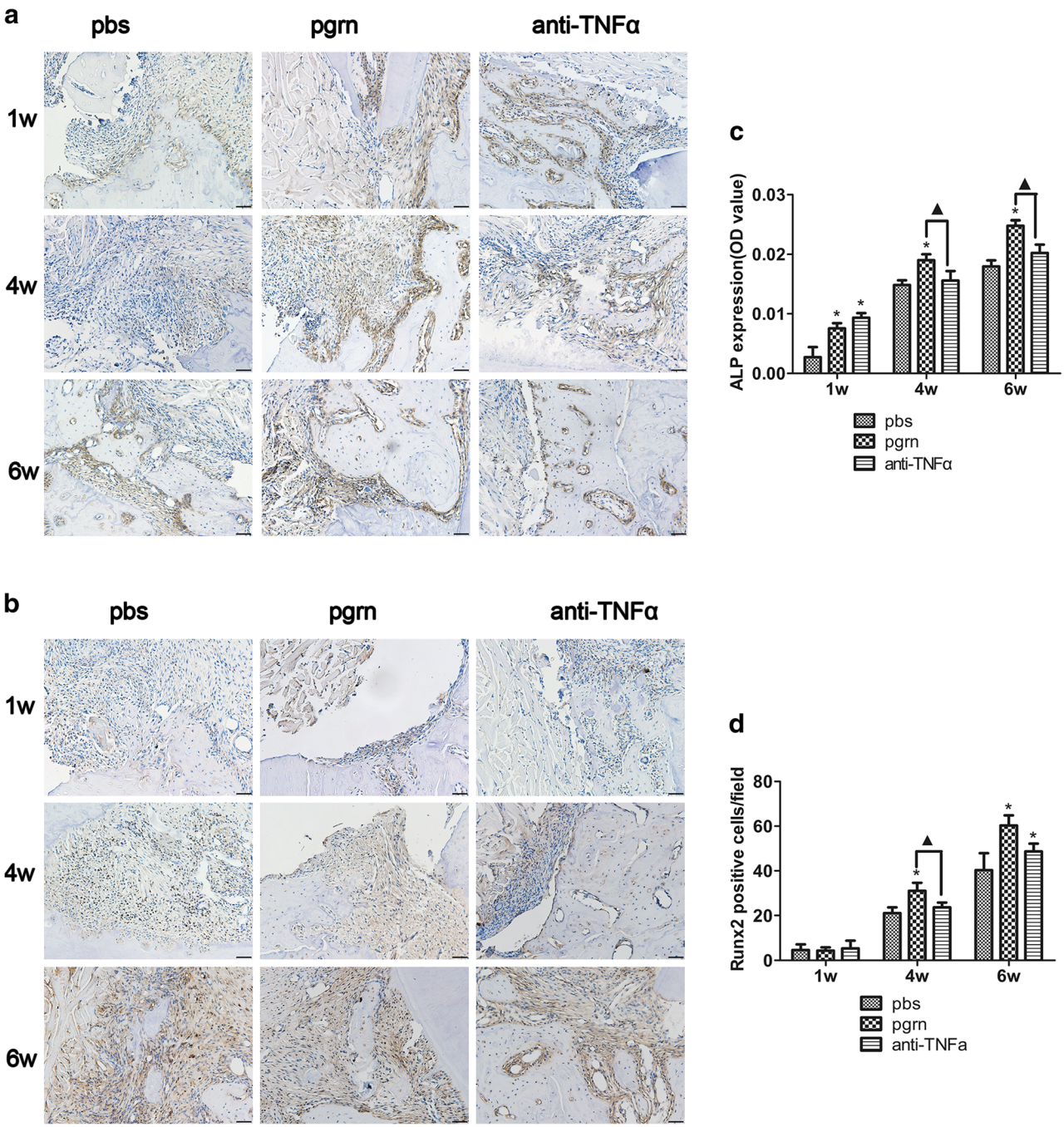

Fig. 5. Immunohistochemical analysis of osteogenesis-related marker ALP and Runx2 at 1, 4, and 6 weeks among three groups. **a** The expression of ALP (brown) among three groups. **b**. Immunohistochemical staining of Runx2-positive cells around the defect edges. **c** Quantitative evaluation of the integral optical density (IOD) of ALP staining. **d** Statistical analysis of the number of Runx2-positive cells among three groups. *$P < 0.05$ *vs* the control group, ▲$P < 0.05$ *vs* the anti-TNF-α group; scale bars, 50 μm.

for 1-week specimens and TNF-α-positive cells were counted. The results showed that the number of TNF-α-positive cells was significantly decreased in the rhPGRN group and anti-TNF-α group, as compared to the PBS group. However, there was no significant difference between the rhPGRN group and the anti-TNF-α group (Fig. 6a, b).

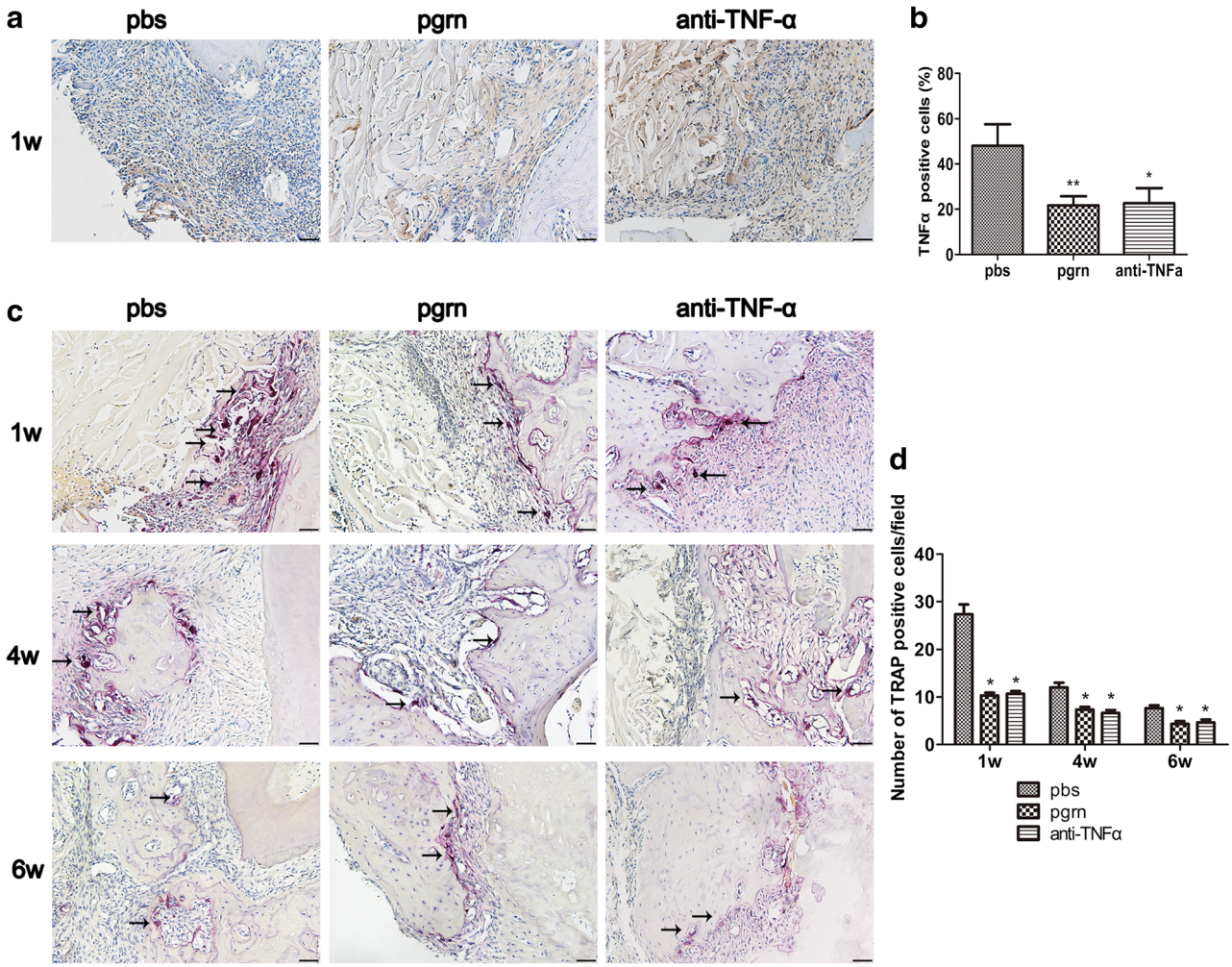

Fig. 6. Immunohistochemical analysis of the expression of TNF-α at 1 week and TRAP staining of the multinucleated osteoclasts to evaluate the osteoclastogenesis activity at 1, 4, and 6 weeks among three groups. **a** TNF-α positive cells with brown-stained nuclei in the defect area. **b** Semi-quantitative analysis of the number of TNF-α-positive cells. **c** Multinucleated osteoclasts with red staining in the bone defect regions. **d** Statistical analysis of the number of TRAP-positive cells. *Black arrows* TRAP-positive osteoclasts. $*P < 0.05$ *vs* the control group, $**P < 0.01$ *vs* the control group; scale bars, 50 μm.

TRAP staining reflects the activity of multinucleated osteoclasts. Compared with the rhPGRN group and the anti-TNF-α group, the number of TRAP-positive osteoclasts was significantly much higher in the PBS group at 1, 4, and 6 weeks, and there was no obvious difference between the rhPGRN group and the anti-TNF-α group. Conspicuously, the number of TRAP-positive osteoclasts decreased sharply at 4 weeks. At 6 weeks, there were only a few TRAP-positive osteoclasts among three groups (Fig. 6c, d).

PGRN Bound with TNFRs and Promoted Expression of TNFR2 Rather Than TNFR1 in Inflammatory Periodontal Bone Defect

To examine the interaction of PGRN and TNFRs, the Co-IP assay was used. The added rhPGRN was demonstrated to effectively bind with TNFR1 and TNFR2 (Fig. 7). To explore the association of PGRN with TNFRs in periodontal bone regeneration, immunofluorescence staining for TNFR1 and TNFR2 were conducted for 1-week specimens. TNFR2-positive cells were significantly higher in the rhPGRN group

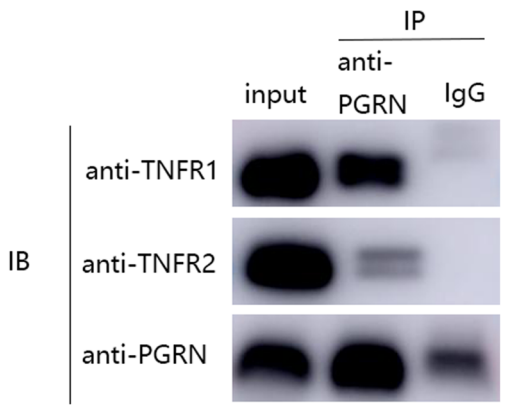

Fig. 7. Coimmunoprecipitation assay between PGRN and TNFRs in P-DLSCs. Cell lysates from PDLSCs stimulated by rhPGRN cultures were coimmunoprecipitated with anti-PGRN or with control IgG antibodies. Then, proteins were incubated with protein A/G PLUS-Agrose. Western blotting was performed using the TNFRI, TNFRII, and PGRN antibodies. Cell lysate was served as positive control, and the IgG group was served as negative control group.

than in the other two groups. However, there was no significant difference between the PBS group and the anti-TNF-α group (Fig. 8a, c). As for the expression of TNFR1, the number of TNFR1-positive cells slightly reduced in the rhPGRN group, but there was no significant difference among three groups (Fig. 8b, d).

DISCUSSION

It is well accepted that the expression of TNF-α increased in the course of periodontitis upon activation of monocytes and macrophages [28, 29] and increased inflammatory cytokines, such as TNF-α and interleukin 1 beta (IL-1β), would contribute to the soft and hard periodontal tissue destruction in the periodontitis progression [2]. TNF-α and TNFR signaling pathway was identified as the apex of proinflammatory cytokine cascade in the periodontitis progress, which reduced the capacity of osteogenic differentiation through activation of NF-κB signaling pathway [16, 30]. Therefore, anti-TNF therapy and TNFR blockade may benefit for inflammatory control and alveolar bone regeneration in periodontitis. In this experiment, we novelly showed that PGRN, a TNFR blockader, promoted periodontal bone regeneration in experimental periodontitis rats *via* inhibiting inflammatory response and osteoclastogenesis and promoting osteogenic differentiation.

TNF-α and IL-1, regarded as the pivotal factors in initiating inflammatory responses, can increase the expression of adhesion molecules by stimulating leukocytes and endothelial cells, stimulate the production of chemokines and recruitment of circulating leukocytes which could cause tissue destruction and activate resident cells to produce lytic enzymes [29, 31]. They can also magnify and maintain the inflammatory response by activating the cell populations of the innate and adaptive immune responses and induce the expression of some mediators, such as prostaglandins, which could accelerate the destruction of periodontal tissue [31, 32]. Therefore, antagonists of specific host mediators such as IL-1 and TNF may provide a potential treatment modality to combat periodontal disease process [29, 31]. In our previous study, administration of rhPGRN attenuated inflammatory cell infiltration, alveolar bone loss, and the production of TNF-α and IL-1β in periodontal tissues of rats with experimental periodontitis [33]. Here, we found that rhPGRN and anti-TNF-α therapy significantly reduced the number of TNF-α-positive cells during the regeneration process of periodontal bone defect.

In periodontitis, the inflammatory environment can activate an array of cells to stimulate pro-inflammatory cytokines, such as TNF-α, which could be activated by RANKL to induce the differentiation and activation of osteoclasts from the preosteoclasts [34]. In addition, with the increased concentration of RANKL produced by activated T lymphocytes, TNF-α could stimulate the differentiation of monocytes and macrophages to the preosteoclasts [35]. Graves et al. demonstrated that the amount of osteoclasts and mononuclear cells reduced notably through the injection of TNF blocker in experimental periodontitis [36, 37]. In our experiment, similarly to the anti-TNF-α group, administration of recombinant PGRN significantly reduced the number of TRAP-positive cells compared with the control group. This result, in line with that reported by Tang et al. [22], demonstrated that PGRN exerted the inhibition effect on osteoclastogenesis.

In addition to pro-inflammatory and osteoclastogenic activity, TNF-α and TNFR signaling pathway is also involved in the modulation of osteogenic differentiation. TNF-α can reduce the capacity of osteogenic differentiation through activation of NF-κB signaling pathway and inhibit the expression of Runx2 [15, 16, 32, 37]. PGRN has been demonstrated to bind with TNFRs, which could effectively block the activity of NF-κB signal pathway in Ti-induced inflammatory condition [22, 38]. Some experiments had illustrated that local administration of recombinant PGRN promoted bone healing in radial bone defect model [23]. In the present experiment, histological and

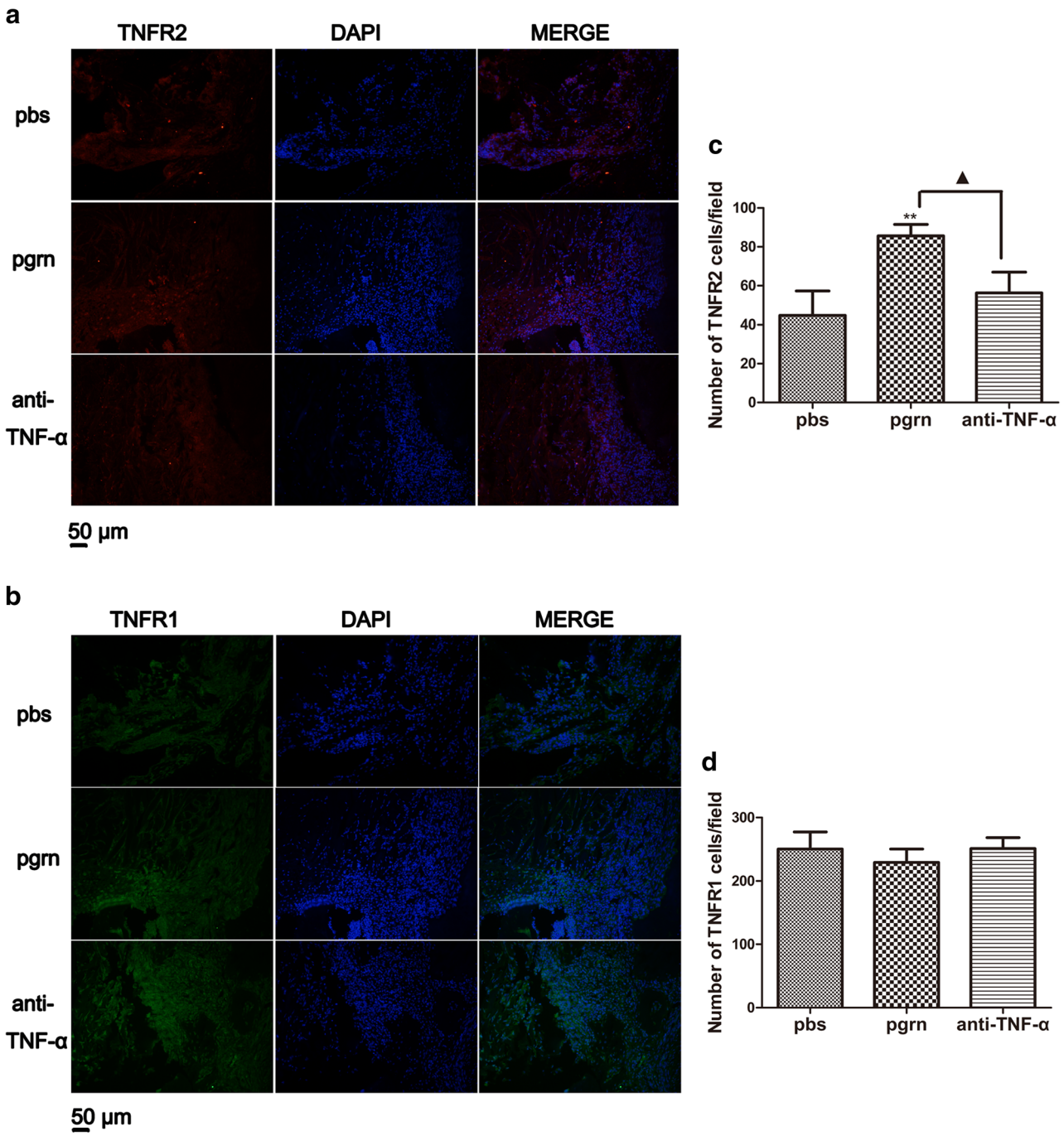

Fig. 8. Immunofluorescence analysis of the expressions of TNFR1 and TNFR2 at 1 week among three groups. **a** The expression of TNFR2 (red) among three groups. **b** The expression of TNFR1 (green) among three groups. **c** Semi-quantitative analysis of the number of TNFR2-positive cells. **d** Statistical analysis of the number of TNFR1-positive cells among three groups. The cell nuclei were stained with blue. $^{*}P < 0.05$ *vs* the control group, $^{▲}P < 0.05$ *vs* the anti-TNF-α group; scale bars, 50 μm.

micro-CT assay revealed that quantity and quality of newly formed bone in the rhPGRN group was significantly superior to the PBS group at 4 and 6 weeks after surgery. Immunohistochemical staining demonstrated that

expression of ALP and Runx2 protein significantly elevated in the rhPGRN group at 4 and 6 weeks compared with the PBS group. It indicates that PGRN may enhance osteogenic activity to promote the bone healing under inflammatory condition *via*, at least partly, binding to TNFRs and antagonizing the inhibition effect of TNF-α on osteogenic differentiation.

Receptors of TNF-α include TNFR1 and TNFR2. TNFR1 is expressed ubiquitously, whereas TNFR2 expression is tightly regulated and found predominantly in hematopoietic cells [22]. The accumulative data suggest that TNFR1 mediates activation of NF-κB signaling and inhibition of osteogenic differentiation by TNF-α stimulation [39], whereas TNFR2 activation may play a protective role in osteogenic differentiation [23]. Our previous study have revealed that the injection of human tumor necrosis factor receptorII:IgG Fc fusion protein (rhTNFR:Fc) could elevate the expressions of Runx2, OC, and BMP-2 in inflammatory mandibular bone healing in periodontitis rats [12]. Similarly, this study also revealed that anti-TNF-α therapy significantly reduced the number of TNF-α-positive cells as well as osteoclastogenesis and enhanced osteogenesis during the regeneration process of periodontal bone defect. However, quantity and quality of newly formed bone as well as osteogenic marker expression in the rhPGRN group were significantly superior to the anti-TNF-α group. Furthermore, as mentioned above, TNFR2 activation may play a protective role in osteogenic differentiation, whereas immunofluorescence staining demonstrated that the expression of TNFR2 was higher in the rhPGRN group than in the other two groups. These results imply that PGRN not only reverses inhibition effect of TNF-α on osteogenic differentiation through disturbing the interaction between TNF-α and TNFR1 but also directly enhances osteogenic differentiation *via* activating TNFR2 expression.

In conclusion, local administration of rhPGRN could effectively promote periodontal bone regeneration in periodontitis rats *via*, at least partly, anti-inflammation, osteoclastogenic inhibition, and osteogenic promotion. Considering the advantages of PGRN in promoting osteogenic differentiation and periodontal bone regeneration over TNF-α inhibitor, the importance of TNFR2 in the mechanism by which PGRN promote periodontal bone regeneration in periodontitis rats deserves to be explored.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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