# Quercetin reverses TNF-α induced osteogenic damage to human periodontal ligament stem cells by suppressing the NF-κB/NLRP3 inflammasome pathway

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**Abstract.** Quercetin (Quer) is a typical antioxidant flavonoid from plants that is involved in bone metabolism, as well as in the progression of inflammatory diseases. Elevated levels of tumor necrosis factor-α (TNF-α), a typical pro-inflammatory cytokine, can affect osteogenesis. In the present study, TNF-α was used to establish an *in vitro* model of periodontitis. The effects of Quer on, as well as its potential role in the osteogenic response of human periodontal ligament stem cells (hPDLSCs) under TNF-α-induced inflammatory conditions and the underlying mechanisms were then investigated. Within the appropriate concentration range, Quer did not exhibit any cytotoxicity. More importantly, Quer significantly attenuated the TNF-α induced the suppression of osteogenesis-related genes and proteins, alkaline phosphatase (ALP) activity and mineralized matrix in the hPDLSCs. These findings were associated with the fact that Quer inhibited the activation of the NF-κB signaling pathway, as well as the expression of NLRP3 inflammation-associated proteins in the inflammatory microenvironment. Moreover, the silencing of NLRP3 by small interfering RNA (siRNA) was found to protect the hPDLSCs against TNF-α-induced osteogenic damage, which was in accordance with the effects of Quer. On the whole, the present study demonstrates that Quer reduces the impaired osteogenesis of hPDLSCs under TNF-α-induced inflammatory conditions by inhibiting the NF-κB/NLRP3 inflammasome pathway. Thus, Quer may prove to be a potential remedy against periodontal bone defects.

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## Introduction

Periodontitis is a chronic inflammatory disease that can cause irreversible damage to the tissues that support the teeth, such as the periodontal ligament, cementum and alveolar bone, thereby leading to the loss of the affected tooth (1). Due to the importance of the periodontium, periodontal therapy is aimed at suppressing disease progression and promoting the regeneration of the affected periodontal tissue or the structures that support the tooth. Studies have reported the significance of stem cell-based tissue engineering technology in repairing and regenerating damaged tissues and maintaining a highly orderly internal environment (2-4). Human periodontal ligament stem cells (hPDLSCs) are mesenchymal stem cells (MSCs) obtained from human periodontal ligament tissue in a normal or periodontitis environment (5). *In vivo* and *in vitro* experiments have verified that hPDLSCs have good proliferative, self-renewal and multidirectional differentiation potentials (6,7). The osteogenic differentiation ability of hPDLSCs is superior to that of other odontogenic stem cells, such as dental pulp cells, gingival mesenchymal stem cells and dental follicle stem cells (8,9). However, the biological behavior of stem cells is closely associated with the tissue microenvironment. For instance, an inflammatory microenvironment may alter the differentiation ability of stem cells, and weaken their osteogenic differentiation and tissue regeneration ability (5,10). Therefore, an effective strategy is required to modulate the differentiation potential of hPDLSCs during inflammation.

Tumor necrosis factor-α (TNF-α) is the main regulator of several pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6 that are released by monocytes and macrophages, as well as by endothelial cells in periodontitis (11). It is associated with osteoclastogenesis, the absorption of alveolar bone and the inhibition of osteogenesis.

Nuclear factor (NF)-κB is an important nuclear transcription factor that regulates cell apoptosis, inflammatory responses

and osteoblast differentiation. Elevated TNF-α levels negatively affect the osteogenic differentiation of mesenchymal stem cells via the activation of the NF-κB signaling pathway when it binds its receptor (12,13). Moreover, NF-κB is a priming signal for the activation of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome that is involved in the expression of NLRP3 protein (14). The NLRP3 inflammasome is a protein complex composed of a receptor protein (NLRP3), adaptor protein (ASC) and effector protein (procaspase-1). It plays a vital role in the innate immunity of infections, inflammatory and chronic diseases, such as Alzheimer's disease, type 2 diabetes and osteoporosis (15). The assembly of the NLRP3 inflammasome, followed by the activation of caspase-1, and the maturation of IL-1β and IL-18, trigger an inflammatory cascade response (16). Advanced glycation end products significantly upregulate the expression of NLRP3 in hPDLSCs through the NF-κB signaling pathway (17). Studies have demonstrated that NLRP3 activation increases adipogenesis and inhibits the osteogenesis of human umbilical cord stem cells, while the silencing of NLRP3 alleviates estrogen depletion-induced osteoporosis in mice (18,19). Most importantly, previous clinical studies have reported that gene polymorphisms of NLRP3 are associated with susceptibility to periodontitis and the expression levels of NLRP3 inflammasome-associated proteins are particularly enhanced in periodontitis (20,21). However, whether NLRP3 associates with NF-κB to inhibit the osteogenic differentiation of hPDLSCs needs to be elucidated in a model of TNF-α-induced periodontitis.

Quercetin (Quer; 3,3',4',5,7-pentahydroxyflavone), a flavonoid derived from common vegetables and fruits, such as apples, onions and blueberries, is known to exhibit antitumor, anti-inflammatory and antioxidant properties (22). Quer has been reported to reduce periodontitis damage in rats and to improve the osteogenic differentiation abilities of bone marrow-derived MSCs (BMMCs) during inflammation to alleviate osteoporosis symptoms (23,24). Furthermore, it has been demonstrated that Quer can reverse lipopolysaccharide (LPS)-induced osteoblast apoptosis and restore the impaired osteogenic differentiation ability of MC3T3-E1 cells (25). However, it has not yet been established whether Quer reduces osteogenic damage to hPDLSCs during TNF-α-induced inflammation. It has also been shown that Quer decreases osteoclast formation by inhibiting IL-17-induced receptor activator of nuclear factor κB ligand (RANKL) expression and inhibits bone resorption in rheumatoid arthritis via the suppression of NF-κB (26). Recent studies based on rat models *in vivo*, have demonstrated that Quer treatment reduces NLRP3 inflammasome-related protein expression and inflammatory cytokine levels (27,28). In addition, Quer has been shown to exert protective effects against endoplasmic reticulum stress-related endothelial cell damage and isoniazid-induced L02 cell apoptosis by suppressing NLRP3 inflammasome activation (29,30).

Based on these findings, it was hypothesized that Quer could attenuate the suppression of the osteogenic differentiation of hPDLSCs in TNF-α-induced inflammation and that the underlying mechanisms may be associated with the NF-κB/NLRP3 inflammasome pathway.

## Materials and methods

*Cells and cell culture*. The approval for the present study was provided by the Committee on Ethics of the Stomatology Hospital of Shandong University (protocol no. GR201806). From April, 2019 to September, 2019, freshly extracted teeth were collected from orthodontic volunteers aged 16-22 years at the Stomatology Hospital of Shandong University. All participants provided signed informed consent in accordance with the Helsinki Declaration. The method of hPDLSC isolation from teeth was similar to a previously reported one (31). Briefly, the extracted teeth were placed in a 15 ml centrifuge tube with α-MEM (Biological Industries) amended with 5% antibiotics (100 U/ml penicillin, 10 mg/ml streptomycin) on ice. They were transferred to an ultra-clean workbench as soon as possible for use in further experiments. Each tooth was washed thrice in phosphate-buffered saline (PBS; Biological Industries) containing 5% antibiotics. Periodontal ligament tissue from the middle third of the root surface was gently scraped with a sterile surgical blade and cut into tiny fragments, which were then seeded into culture dishes (25 cm). hPDLSCs were grown at 37˚C in medium supplemented with α-MEM, 20% fetal bovine serum (FBS; Biological Industries) and 1% antibiotics in a 5% $CO_2$ incubator. Following inoculation 3-4 h, the culture dish (25 cm) was turned over to ensure that the hPDLSCs touched the culture medium. The medium was refreshed after every 3 days until the cells grew out from the tissue sections. The cells were passaged at 80-90% confluence and the cells at passages 3 to 5 were used for further experiments.

*Colony-forming assay*. The hPDLSCs were cultured in a 10-cm diameter culture plate (1,000 cells per plate) in α-MEM supplemented with 10% FBS [common medium (CM)]. After 7 days, the hPDLSCs were rinsed thrice using PBS, then fixed in 4% polyformaldehyde, after which they were stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 15 min. The cell clones were observed under a microscope (Olympus Corp.), and aggregates of >3 mm were considered as clones.

*Flow cytometric analysis of hPDLSC surface marker phenotypes*. Passage 3 cells were digested using trypsin, followed by washing using PBS. A total of 100 µl of prepared cell suspension was then incubated with antibodies (FITC mouse anti-human CD90, 5 µl, PE mouse anti-human CD44, 5 µl, PerCP-Cy™5.5 mouse anti-human CD105, 5 µl, APC mouse anti-human CD73, 5 µl, PE hMSC isotype control negative cocktail, 20 µl, PE hMSC negative cocktail, 20 µl, hMSC isotype control positive cocktail, 20 µl, hMSC positive cocktail, 20 µl, #562245, BD Biosciences) conjugated with a monoclonal fluorescent dye in the dark for 20 min at 4˚C. Cells were detected using flow cytometry, as per the manufacturer's protocol (#660519, BD Biosciences). The MSC-associated positive cocktail, including CD105-PerCP-Cy, CD73-APC, CD44-PE, CD90-FITC and MSC-associated negative cocktail that included CD34 PE, CD45 PE, CD19 PE, HLA-DR PE and CD11b PE (#562245, BD Biosciences) were used. Respective negative and positive isotype control cocktails were used as

systemic controls. Final database analysis was carried out using FlowJo software (Beckman coulter, Inc.).

*Multi-lineage differentiation assays of hPDLSCs*. The hPDLSCs were incubated in 6-well dishes at $1x10^5$ cells/well with CM. At 80-90% density, the corresponding culture medium was replaced to examined osteogenesis and adipogenesis.

For osteogenesis, cells were exposed to an osteogenic induction medium (OIM) containing α-MEM with 10% FBS, β-glycerophosphate (10 mM), dexamethasone (10 nM) and ascorbic acid (50 mg/l; Beijing Solarbio Science & Technology Co., Ltd.) for 21 days. The cells were then incubated with Alizarin Red solution (Sigma-Aldrich; Merck KGaA) at 22˚C for 10 min to observe the mineralization.

For adipogenesis, cells were treated in an adipogenic induction medium containing α-MEM with 10% FBS, insulin (10 mg/l), dexamethasone (1 $\mu$M), indomethacin (0.2 mM) and isobutyl-methylxanthine (0.5 mM; Beijing Solarbio Science & Technology Co., Ltd.) for 28 days. Subsequently, the hPDLSCs were incubated with Oil Red O solution at 22˚C for 20 min to observe lipid droplets.

*Cell proliferation assay*. According to the manufacturer's instructions, the cell counting kit-8 (CCK-8; MedChemexpress Co., Ltd.) kit was utilized to detect the proliferative ability of hPDLSCs. A total of 4,000 cells/well were plated in a 96-well plate and cultured at 37˚C for 24 h with CM. The hPDLSCs were then subjected to various concentrations (0, 0.01, 0.1, 1, 5, 10, 50 and 100 $\mu$M) of Quer (Beijing Solarbio Science & Technology Co., Ltd.) for 3 days. Cell viability was examined at 24, 48 and 72 h. Moreover, the hPDLSCs were stimulated with various concentrations (0, 1, 10, 20, 30, 50 ng/ml) of TNF-α (cat. no. 300-01A Peprotech Inc.) for 4 days. CCK-8 reagent (10 $\mu$l) was mixed with 90 $\mu$l complete culture medium per well and incubated at 37˚C with the cells 1 h later. The absorbance at 450 nm was measured with the SPECTROstar plate reader (BMG Labtech Inc.).

*Alkaline phosphatase (ALP) activity and ALP staining assay*. Following 7 days of osteogenesis, the ALP activity of the cells was assessed using the ALP activity kit (Nanjing Jiancheng Bioengineering Institute) as per the producer's protocols. For ALP staining, the hPDLSCs were fixed in paraformaldehyde (4%), then stained with an ALP staining kit (Beyotime Institute of Biotechnology) at room temperature for 10 min after 7 days. Cells were observed and examined using an inverted microscope (Olympus Corp.).

*Alizarin Red staining (ARS) assay*. Following 21 days of osteogenic induction, the cells were rinsed 3 times then fixed in paraformaldehyde (4%), after which they were stained using an Alizarin Red solution (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at 22˚C.

*Gene expression analysis*. TRIzol reagent (Qingdao Haosail Science & Technology Co., Ltd.) was utilized to isolate total RNA from cells as per producer's suggestions. Subsequently, 1 $\mu$g RNA was reverse transcribed as the template strand using the HiScript® III Reverse Transcriptase kit (#R323-01, Nanjing Vazyme Biotech Co., Ltd.) to obtain cDNA. RT-qPCR was performed by The Roche Light Cycler® 480II in a 10 $\mu$l reaction volume with the SYBR qPCR Master Mix (Nanjing Vazyme Biotech Co., Ltd.). The PCR cycling conditions were as follows: Initial denaturation at 95˚C for 30 sec followed by 45 cycles of denaturation at 95˚C for 5 sec and annealing 65˚C for 30 sec. Changes in target gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method (32). The primers used in this assay were as follows: GAPDH forward, 5'-GCACCGTCAAGG CTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGG AALP-3'; collagen I (COL1) forward, 5'-GCTGATGATGCC AATGTGGTT-3' and reverse, 5'-CCAGTCAGAGTGGCA CATCTTG-3'; ALP forward, 5'-GTGAACCGCAACTGG TACTC-3' and reverse, 5'-GAGCTGCGTAGCGATGTCC-3'; runt-related transcription factor 2 (RUNX2) forward, 5'-GTT TCACCTTGACCATAACCGT-3' and reverse, 5'-GGGACA CCTACTCTCATACTGG-3'; NLRP3 forward, 5'-ACGACT GCGTCTCATCAAGG-3' and reverse, 5'-CATCGGGGT CAAACAGCAAC-3'; caspase-1 forward, 5'-GTGCAGGAC AACCCAGCTAT-3' and reverse, 5'-TGCGGCTTGACTTGT CCATT-3'; IL-1β forward, 5'-GTACCTGTCCTGCGTGTT GA-3' and reverse, 5'-GGGAACTGGGCAGACTCAAA-3'; IL-6 forward, 5'-CCTTCGGTCCAGTTGCCTTCT-3' and reverse, 5'-CAGTGCCTCTTTGCTGCTTTC-3'.

*Western blot analysis*. Proteins were extracted from the cells as previously described (7). Briefly, the hPDLSCs were rinsed in PBS, then lysed on ice in RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) comprising 1% phosphatase inhibitor (Boster Biological Technology Co., Ltd.) plus 1% PMSF (Beijing Solarbio Science & Technology Co., Ltd.). Following ultrasonic cracking and centrifugation (12,000 x g; 20 min; 4˚C), the concentration of proteins was determine using the BCA assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, the proteins from different groups were resolved on 10% SDS-PAGE and transferred onto a PVDF membrane. A total of 20 $\mu$g proteins from different groups were loaded on 10% SDS-PAGE and separated through electrophoresis. Subsequently, the separated proteins were eletro-blotted onto a PVDF membrane. Blocking was performed using 5% non-fat dry milk at 22˚C for 1 h, then probed with rabbit anti-human GAPDH polyclonal (1:20,000, cat. no. 10494-1-AP; ProteinTech Group, Inc.), rabbit anti-human RUNX2 monoclonal (1:1,000, cat. no. ab23981; Abcam), rabbit anti human-COL1 monoclonal (1:1,000, cat. no. #84336; Cell Signaling Technology, Inc.), rabbit anti human-ALP monoclonal (1:5,000, cat. no. ab108337; Abcam), rabbit anti human-NLRP3 polyclonal (1:1,000, cat. no. WL02635; Wanlei Biotech Co., Ltd.), rabbit anti human-procaspase-1 polyclonal (1:500, cat. no. WL02996; Wanlei Biotech Co., Ltd., China), rabbit anti human-caspase-1 polyclonal (1:500, cat. no. WL03450; Wanlei Biotech Co., Ltd.), rabbit anti human-phosphorylated (p-)p65 polyclonal (1:500, cat. no. WL02169; Wanlei Biotech Co., Ltd.), rabbit anti human-p-IκBα polyclonal (1:500, cat. no. WL02495; Wanlei Biotech Co., Ltd.), rabbit anti human-IκBα polyclonal (1:500, cat. no. WL01936; Wanlei Biotech Co., Ltd.), rabbit anti human-p65 monoclonal (1:1,000, cat. no. #59674; Cell Signaling Technology, Inc.) at 4˚C for 24 h. This was followed by incubation with a horseradish peroxidase-labeled goat

anti-rabbit IgG secondary antibody (1:20,000, cat. no. 7074S; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Protein detection was performed using a chemiluminescent HRP (EMD Millipore) and the expression levels of target proteins were analyzed using ImageJ 1.47V software (National Institutes of Health) and normalized to GAPDH expression.

*NLRP3 interfering*. The hPDLSCs were seeded into a 6-well plate with CM and transfected with siRNA targeting NLRP3 (si-NLRP3 forward, 5'-CCUCGGUACUCAGCACUAATT-3' and reverse, 5'-UUAGUGCUGAGUACCGAG-3'; 20 $\mu$M) or negative control (si-NC forward, 5'-UUCUCCGAACGUGUC ACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGA ATT-3'; 20 $\mu$M) (Shanghai GenePharma Technology Co., Ltd.) with Micropoly-transfecter$^{TM}$ cell reagent (#MT115; Nantong Micropoly Biotech Co., Ltd.) when the cells were at a density of 70-80%. Following 24 h of treatment, the proteins were extracted to examine the interference efficiency.

*Statistical analysis*. Data were analyzed using GraphPad Prism, v. 8.0. One-way analysis of variance (ANOVA) was employed to compare data between multiple groups. All post hoc analyses were performed using Tukey's test. The t-test was used to compare data between 2 groups. All tests were carried out in 3 replicates, and data are reported as the means ± SD. A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

*Cell culture and characterization of hPDLSCs*. hPDLSCs were obtained from periodontal ligament tissues by tissue block culture. They were mainly long, fusiform and fibroblast-like cells under an inverted microscope (Fig. 1A). Flow cytometry analysis revealed that passage 3 hPDLSCs positively expressed mesenchymal surface markers (CD90, CD105, CD73 and CD44), and negatively expressed hematopoietic or endothelial-specific markers (CD11b, CD34, CD19, HLA-DR and CD45) (Fig. 1E). Furthermore, the hPDLSCs exhibited their colony formation properties (Fig. 1B). In multi-lineage differentiation potential assays, osteogenic differentiation detected by ARS exhibited mineralized nodules (Fig. 1C), and adipogenic differentiation revealed lipid droplets following staining with Oil Red O solution (Fig. 1D).

*Low concentrations of Quer exert a non-toxic effect on hPDLSCs*. To evaluate cell viability, CCK-8 assay was performed to determine whether Quer exerts potential cytotoxic effects on the hPDLSCs. It was revealed that Quer did not affect hPDLSC viability at concentrations up to 10 $\mu$M at 24 and 48 h. However, at high concentrations, such as 50 and 100 $\mu$M, Quer significantly exhibited cellular toxicity against the hPDLSCs at the 3 time points examined (Fig. 2). Therefore, these 2 higher concentrations were excluded from further experiments.

*Optimum concentration of Quer exerts a protective effect against hPDLSCs osteogenic damage induced by TNF-α*. Relative to the control group, various concentrations of TNF-α did not exert an obvious effect on hPDLSC proliferation, as revealed by CCK-8 assay (Fig. 3A). Following osteogenic induction for 7 days, ALP activity revealed that TNF-α concentrations >20 ng/ml significantly decreased the hPDLSC osteogenic differentiation ability (Fig. 3B and C). When the hPDLSCs were cultured in OIM, Quer concentrations >0.01 $\mu$M, particularly those between 1-10 $\mu$M, reversed the TNF-α-induced inhibition of ALP activity (Fig. 3D). Therefore, the 1 $\mu$M concentration of Quer was used in the subsequent experiments.

*Quer inhibits the induction of IL-1β and IL-6 induced by TNF-α*. To investigate the mimicking of the inflammatory environment stimulated by TNF-α, the hPDLSCs were stimulated with Quer (1 $\mu$M), TNF-α (20 ng/ml), or their combination in CM. RT-qPCR analysis revealed that Quer + TNF-α significantly downregulated the mRNA expression levels of IL-1β and IL-6 in the hPDLSCs relative to the group stimulated with TNF-α alone. However, Quer alone did not affect the IL-1β and IL-6 gene expression levels (Fig. 4). Therefore, the *in vitro* inflammatory environment was successfully established, and 1 $\mu$M Quer had no significant pro-inflammatory effect.

*Quer reverses the inhibitory effects of TNF-α on the osteogenic differentiation of hPDLSCs*. Osteogenic differentiation was observed in order to ascertain the effects of Quer on osteogenesis under normal conditions or in a TNF-α-induced inflammatory microenvironment. Following 7 days of osteogenic induction, western blot analysis revealed that the protein levels of COL1, ALP and RUNX2 were downregulated in the TNF-α group, while the levels of these osteogenic differentiation-related proteins were evidently upregulated following the addition of Quer (Fig. 5A and B). RT-qPCR revealed that the gene expression levels of COL1 and RUNX2 were markedly suppressed in the TNF-α group relative to the control group. However, the suppression of these osteogenesis-associated genes was significantly reversed in the Quer + TNF-α group (Fig. 5D). Following 7 days of osteogenic induction, ALP activity and ALP staining assay revealed a decreased and improved osteogenic ability of the hPDLSCs in TNF-α group and in the Quer + TNF-α group, respectively (Fig. 5C and E). After 21 days of osteogenic induction, ARS detected more mineralized nodules in the Quer + TNF-α group than in the TNF-α group (Fig. 5F). These findings suggested that Quer reversed the inhibitory effect of TNF-α on the osteogenesis of hPDLSCs.

*Quer disrupts the TNF-α-induced activation of the NF-κB/NLRP3 inflammasome pathway in hPDLSCs*. To clarify the possible mechanisms through which Quer attenuated the TNF-α-induced suppression of the osteogenesis of hPDLSCs, the levels of NF-κB pathway-associated proteins and its downstream molecule, NLRP3, were examined by RT-qPCR and western blot analysis. As shown in Fig. 6A and B, the levels of p-p65 and p-IκBα were markedly increased in the TNF-α group compared to the control group. By contrast, the levels of p-p65 and p-IκBα were significantly decreased (Fig. 6A and B) following treatment with Quer. These results revealed that Quer disrupted the NF-κB pathway in the model of TNF-α-induced periodontitis. At the same time, western blot analysis was performed to

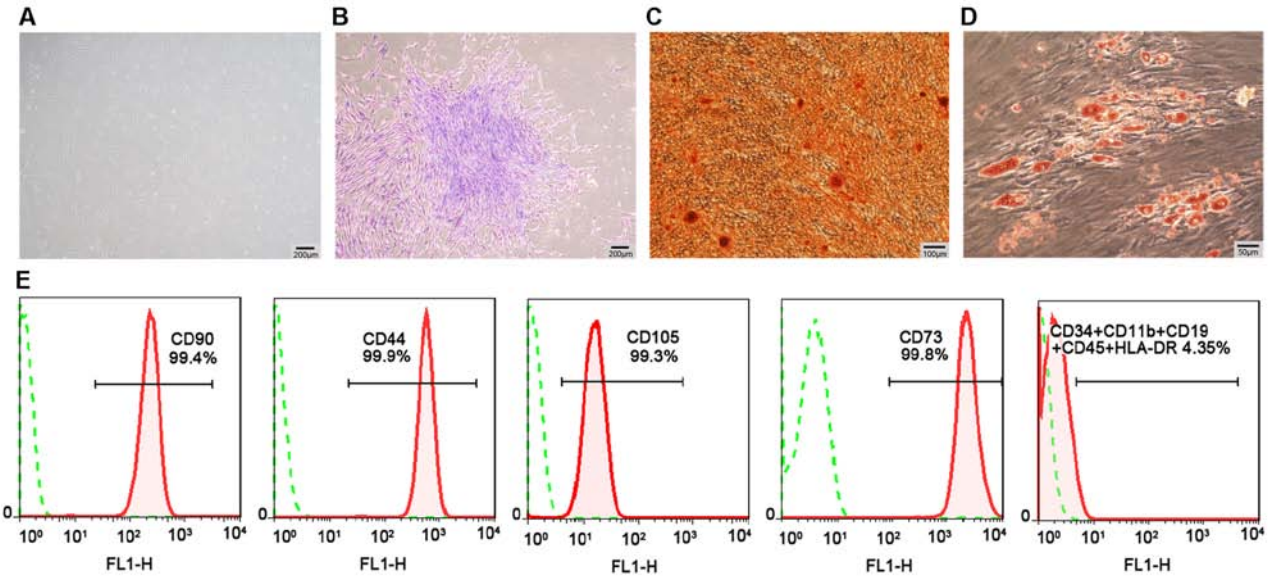


Figure 1. Cell culture and characterization of hPDLSCs. (A) hPDLSCs (P3) displaying spindle fibroblast-like morphology under a phase-contrast microscope (scale bar, 200 $\mu$m). (B) Detection of single clones of hPDLSCs (scale bar, 200 $\mu$m). (C) Osteogenic differentiation ability of hPDLSCs assayed by ARS (scale bar, 100 $\mu$m). (D) Adipogenic differentiation ability of hPDLSCs assayed by Oil Red O staining (scale bar, 50 $\mu$m). (E) Negative expression of CD11b, CD34, CD19, CD45 and HLA-DR, and positive expression of CD90, CD105, CD44, and CD73 in hPDLSCs assayed by flow cytometric analysis. hPDLSCs, human periodontal ligament stem cells; P3, passage 3; ARS, Alizarin Red staining.

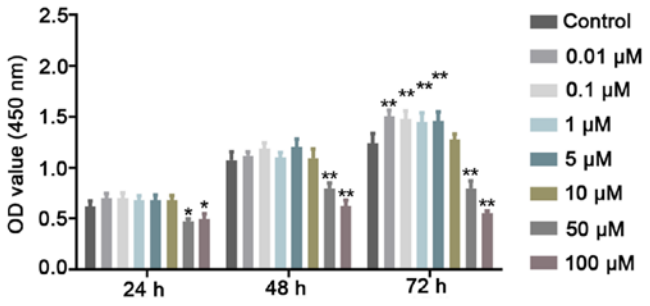


Figure 2. Concentration screening of Quer. hPDLSCs were cultured with various concentrations (0.01, 0.1, 1, 5, 10, 50 and 100 $\mu$M) of Quer for 72 h, and cell viability were examined at 24, 48 and 72 h using the CCK-8 assay kit. Quer at 50 and 100 $\mu$M markedly decreased the viability of hPDLSCs at 48 and 72 h. $^{*}$P<0.05 vs. the control; $^{**}$P<0.01 vs. the control. hPDLSCs, human periodontal ligament stem cells; Quer, quercetin.

observe the expression levels of NLRP3, procaspase-1 and caspase-1, which were found to be significantly elevated in the TNF-α group. In the Quer + TNF-α group, the levels of these proteins were found to be downregulated when compared to the TNF-α group (Fig. 6C and D). Additionally, NLRP3 inflammasome-associated gene expression was consistent with the results obtained for protein expression (Fig. 6E). These findings indicated that Quer suppressed the activation of NLRP3 stimulated by TNF-α in hPDLSCs.

*Silencing of of NLRP3 reverses TNF-α-induced osteogenic damage to hPDLSCs.* To explore the effects of NLRP3 protein on TNF-α-induced osteogenic damage to hPDLSCs, si-NLRP3 was used to decrease NLRP3 expression. The NLRP3 protein level was markedly decreased in the si-NLRP3, but not in the si-NC group (Fig. 7A). Additionally, the changes in the levels of NF-κB signaling pathway-associated proteins were examined following the silencing of NLRP3 in the TNF-α-induced inflammatory microenvironment. Western blot analysis revealed that the expression levels of p-p65 and p-IκBα were markedly upregulated in the TNF-α + si-NC group and the TNF-α + si-NLRP3 group. No significant differences were observed between the 2 groups (Fig. 7B and C). That is, the TNF-α-induced activation of the NF-κB signaling pathway was not inhibited by the use of NLRP3 siRNA. hPDLSCs osteogenesis was then induced and the effects of NLRP3 silencing on TNF-α-induced osteogenic damage were investigated. After 7 days of osteogenic differentiation, western blot analysis revealed that the levels of osteogenic differentiation-associated proteins, including COL1, ALP and RUNX2 were downregulated in the TNF-α + si-NC group. However, the silencing of of NLRP3 reversed the TNF-α-induced inhibition of osteogenic differentiation ability. The effect was consistent with the Quer + TNF-α + si-NC group (Fig. 7D and E). Moreover, RT-qPCR, ALP staining and ALP activity assay revealed a notably upregulated osteogenic ability in the Quer + TNF-α + si-NC or the TNF-α + si-NLRP3 groups, compared to the TNF-α + si-NC group (Fig. 7F-H). Taken together, these results demonstrated that the silencing of NLRP3 protected against TNF-α-induced osteogenic damage to hPDLSCs, consistent with the effects of Quer.

## Discussion

Periodontitis is a severe oral disease that results in the defection of periodontal supporting tissue, long-term inflammatory cytokines stimulation, and can cause teeth loss (33). Unlike traditional treatment methods, such as scaling and root planning for periodontitis, tissue regenerative technology, which utilizes combination treatment with drugs and stem cells, has more potential therapeutic properties (34,35). The selected drug should inhibit inflammatory factor secretion, while stem cells have high proliferation and multiple differentiation

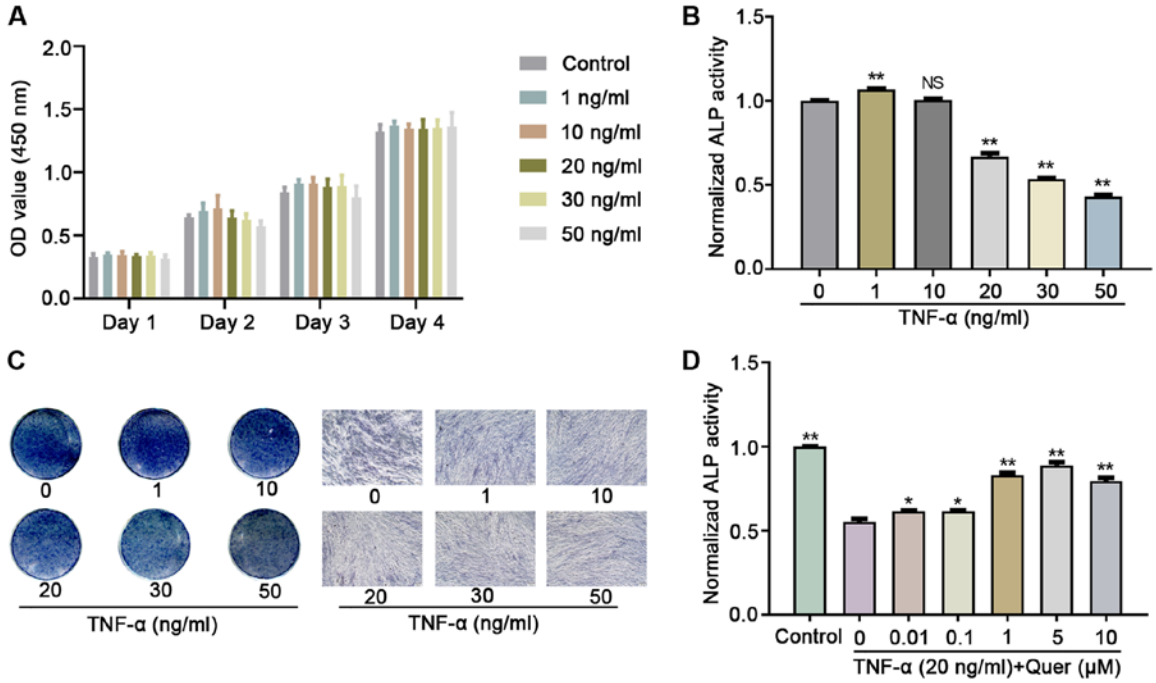


Figure 3. The optimum concentration of Quer exerts a protective effect against TNF-α-induced hPDLSC osteogenic damage. (A) hPDLSCs were cultured with various concentrations (1, 10, 20, 30 and 50 ng/ml) of TNF-α for 4 days, and cell proliferation was examined on days 1-3 and 4 using the CCK-8 assay kit. (B) hPDLSCs were exposed in OIM with 0, 1, 10, 20, 30 and 50 ng/ml TNF-α, and ALP activity was detected after 7 days. $^{**}$P<0.01 vs. the control, NS (no significant difference) vs. control. (C) Illustrative images of ALP staining in OIM with 0, 1, 10, 20, 30 and 50 ng/ml TNF-α for 7 days (scale bar, 100 $\mu$m). (D) hPDLSCs were exposed in OIM containing 20 ng/ml TNF-α with various concentrations of Quer (0, 0.01, 0.1, 1, 5, and 10 $\mu$M), and ALP activity was detected after 7 days. $^{*}$P<0.05 vs. TNF-α group (without Quer); $^{**}$P<0.01 vs. TNF-α group (without Quer). hPDLSCs, human periodontal ligament stem cells; Quer, quercetin; OIM, osteogenic induction medium; TNF-α, tumor necrosis factor-α; ALP, alkaline phosphatase.

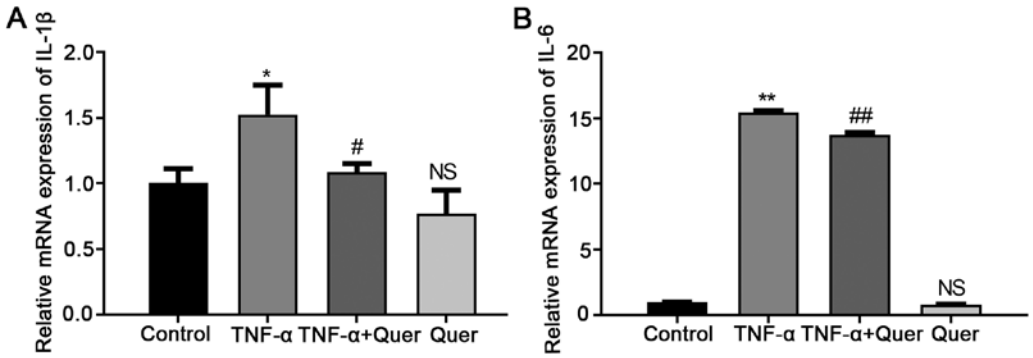


Figure 4. Downregulation of the classical inflammatory cytokines, IL-1β and IL-6, by Quer. Quer (1 $\mu$M) significantly downregulated the TNF-α (20 ng/ml)-induced elevated expression of IL-1β and IL-6 in hPDLSCs. Quer alone did not affect IL-1β and IL-6 mRNA expression levels. (A) mRNA expression level of IL-1β was examined by RT-qPCR. $^{*}$P<0.05 vs. the control; $^{\#}$P<0.05 vs. TNF-α, NS (no significant difference) vs. control. (B) mRNA expression level of IL-6 was detected by RT-qPCR. $^{**}$P<0.01 vs. the control; $^{\#\#}$P<0.01 vs. TNF-α, NS (no significant difference) vs. control. hPDLSCs, human periodontal ligament stem cells; Quer, quercetin; TNF-α, tumor necrosis factor-α; IL, interleukin.

potentials to restore the balance of bone formation and resorption for periodontitis therapy. Studies have documented that hPDLSCs have more undifferentiated MSC characteristics and are suitable seed cells for bone tissue regeneration (6,7). In the present study, hPDLSCs were successfully isolated from volunteers with excellent stem cell properties.

The expression of TNF-α, as a key pro-inflammatory cytokine, is elevated during periodontal disease progression (36). The overexpression of TNF-α elevates osteoclast activity, resulting in bone destruction and a decreased osteoblast bone formation ability (37,38). As previously reported, various concentrations of TNF-α do not exert an obvious effect on hPDLSCs proliferation, while elevated concentrations of TNF-α exhibit a negative regulatory effect on osteogenesis (39). The present study found that a TNF-α concentration of 20 ng/ml was required to mimic an inflammatory microenvironment *in vitro*, which was demonstrated by decreased ALP staining and ALP activity.

To investigate a novel strategy for alleviating TNF-α-induced periodontal supportive tissue destruction, Quer was used. Quer is a natural flavonoid compound that exhibits anti-inflammatory, antioxidant and cardiovascular protective properties at an optimum concentration (40). Quer is a potential candidate for preventing various pathological

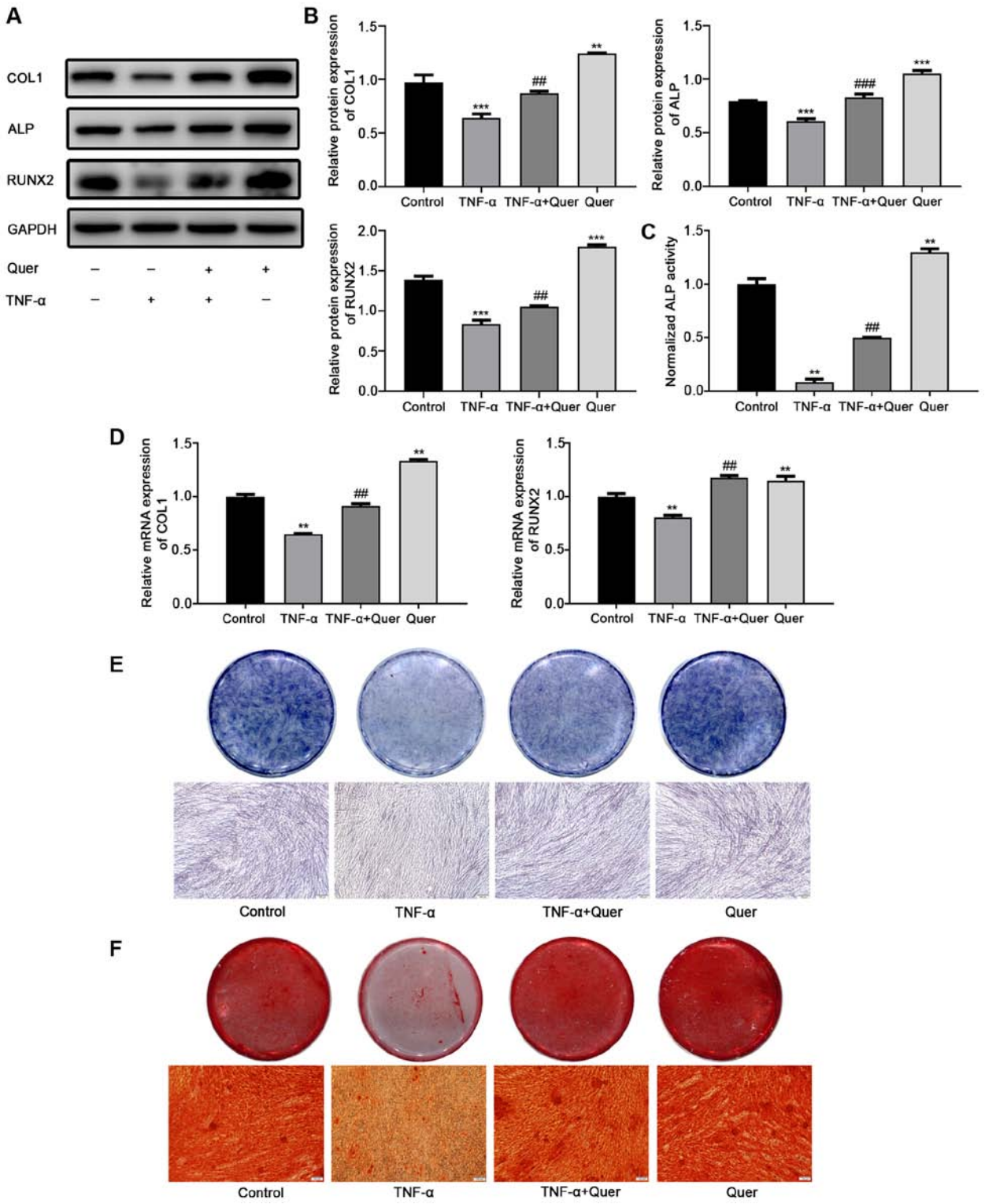


Figure 5. Reversing effect of Quer on TNF-α-induced suppression of osteogenesis. hPDLSCs were treated with Quer, TNF-α, or their combination for 7 days and/or 21 days in an OIM. (A) Protein levels of COL1, ALP and RUNX2 were measured by western blot analysis on day 7. (B) Band intensities were quantified using ImageJ software. $^{**}$P<0.01, $^{***}$P<0.001 vs. control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. TNF-α. (C) ALP activity detection following 7 days of osteogenic induction. $^{**}$P<0.01 vs. control; $^{\#\#}$P<0.01 vs. TNF-α. (D) mRNA expression levels of COL1 and RUNX2 were measured by RT-qPCR day 7. $^{**}$P<0.01 vs. control; $^{\#\#}$P<0.01 vs. TNF-α. (E) ALP staining detection following osteogenic induction for 7 days (scale bar, 100 $\mu$m). (F) Alizarin Red staining detection following 21 days of osteogenic induction (scale bar, 100 $\mu$m). hPDLSCs, human periodontal ligament stem cells; Quer, quercetin; OIM, osteogenic induction medium; TNF-α, tumor necrosis factor-α; ALP, alkaline phosphatase.

diseases due to its extensive pharmacological properties. It has been shown that Quer can inhibit the IL-17-induced RANKL production and decrease IL-17-stimulated osteoclastogenesis in the bone destructive process (26). A previous study revealed that Quer decreased LPS-induced osteoclast formation, ligature-promoted periodontal inflammation and bone destruction with experimental periodontitis in rats (23). The present study found that Quer at concentrations of <10 $\mu$M did not exhibit

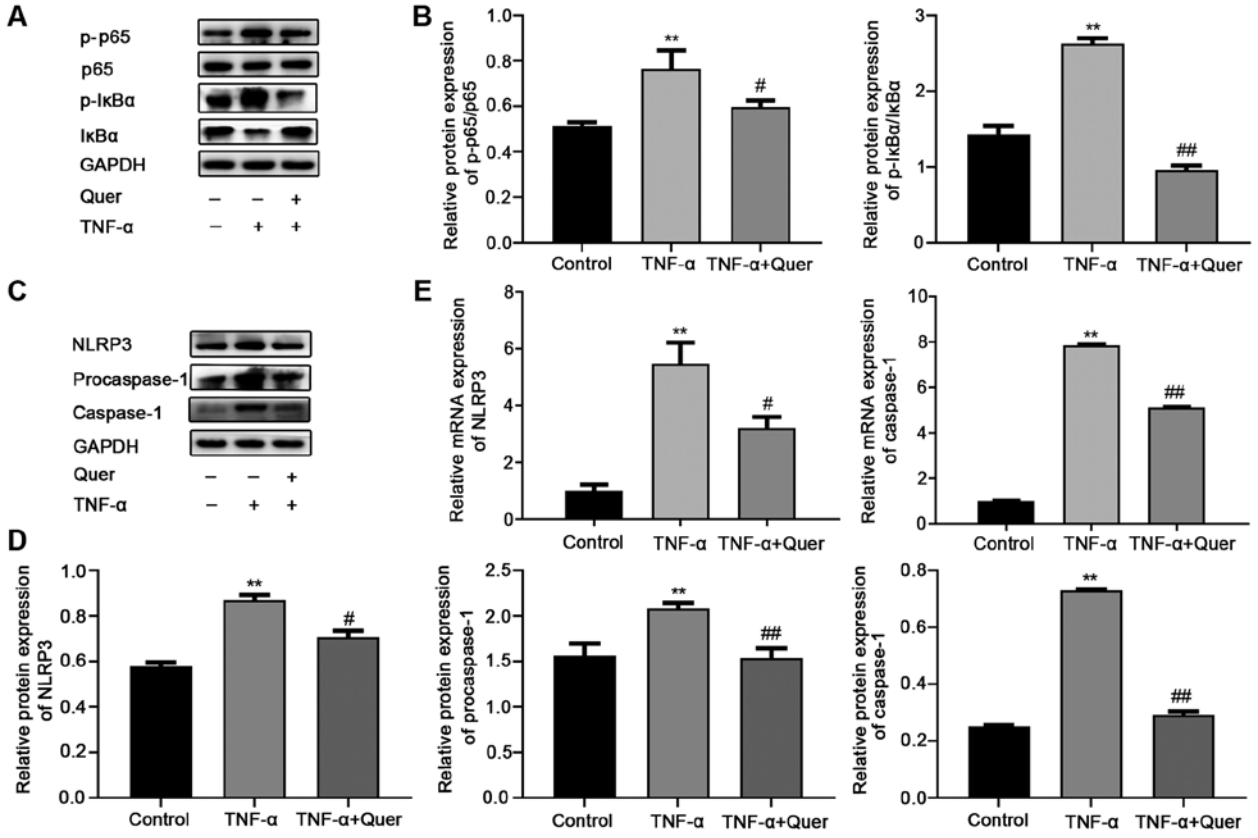


Figure 6. Effect of Quer on the activation of the NF-κB/NLRP3 pathway in TNF-α-induced hPDLSCs. hPDLSCs were treated with 1 $\mu$M Quer, 20 ng/ml TNF-α, or their combination for 24 h in a CM. (A) Protein expression levels of p-p65, p65, p-IκBα and IκBα. (B) Band intensities were quantified using ImageJ software. $^{**}$P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. TNF-α. (C) Protein expression levels of NLRP3, procaspase-1 and caspase-1. (D) Band intensities were quantified using the ImageJ software. $^{**}$P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. TNF-α. (E) mRNA expression levels of NLRP3 and caspase-1. $^{**}$P<0.01 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. TNF-α. hPDLSCs, human periodontal ligament stem cells; Quer, quercetin; TNF-α, tumor necrosis factor-α; NLRP3, NOD-like receptor family pyrin domain-containing protein 3.

any cytotoxicity, but significantly prevented TNF-α-induced osteogenic damage, particularly at the 1-10 $\mu$M concentrations.

In periodontal disease, the upregulation of IL-1β and IL-6 is closely associated with the pathophysiology of periodontitis and can accelerate the degeneration of inflammatory periodontal tissues (41,42). The present study used RT-qPCR to examine the mRNA expression levels of IL-1β and IL-6. Elevated IL-1β and IL-6 expression levels indicated that the model of inflammation was successfully mimicked by TNF-α stimulation *in vitro*. Quer treatment decreased the TNF-α-induced production of IL-1β and IL-6, and did not exert pro-inflammatory effects on hPDLSCs without TNF-α. Moreover, the gene level of IL-1β following the different treatments suggested that the NLRP3 inflammasome may be activated in the process. Subsequently, the levels of the osteogenic differentiation representative genes and proteins, COL1, ALP and RUNX2, were determined by RT-qPCR and western blot analysis, respectively. The results demonstrated that Quer (1 $\mu$M) significantly restored the osteogenic ability of the hPDLSCs which had been impaired by TNF-α. Furthermore, ALP staining and the results of ARS were in accordance with the obtained mRNA and protein expression levels. It has been previously demonstrated that Quer can promote mouse BMMC proliferation and osteogenic ability (43). Similarly, the present study found that Quer improved the osteogenic ability of hPDLSCs when compared to the control group without TNF-α. These findings revealed that Quer antagonized the TNF-α-induced inhibition of hPDLSC osteogenesis, which may be due to the suppression of inflammation and the pro-osteogenic effects on hPDLSCs.

The NF-κB signaling pathway, as a significant downstream pathway of TNF-α, is activated when TNF-α binds TNF receptor 1 (TNFR1) to inhibit osteogenesis-associated gene transcription and regulate osteogenic differentiation (12,44,45). Studies have documented that various flavonoids, including Quer, can suppress LPS- or TNF-α-induced key protein expression of the NF-κB signaling pathway (46,47). These studies prompted us to determine whether the NF-κB pathway is a potential responsive mechanism through which Quer reverses the inhibitory osteogenic differentiation of TNF-α induced hPDLSCs. Consistent with previous studies (46,47), the results of the present study demonstrated that the 1 $\mu$M Quer concentration significantly inhibited the TNF-α-induced phosphorylation of p65 and IκBα in the hPDLSCs. Additionally, the NF-κB signaling pathway is a priming signal for activating the NLRP3 inflammasome (14). It was also found that the activation of the NF-κB signaling pathway was not significantly influenced by the silencing of of NLRP3 in the TNF-α-induced inflammatory microenvironment. Studies have revealed that Quer can attenuate diabetic encephalopathy

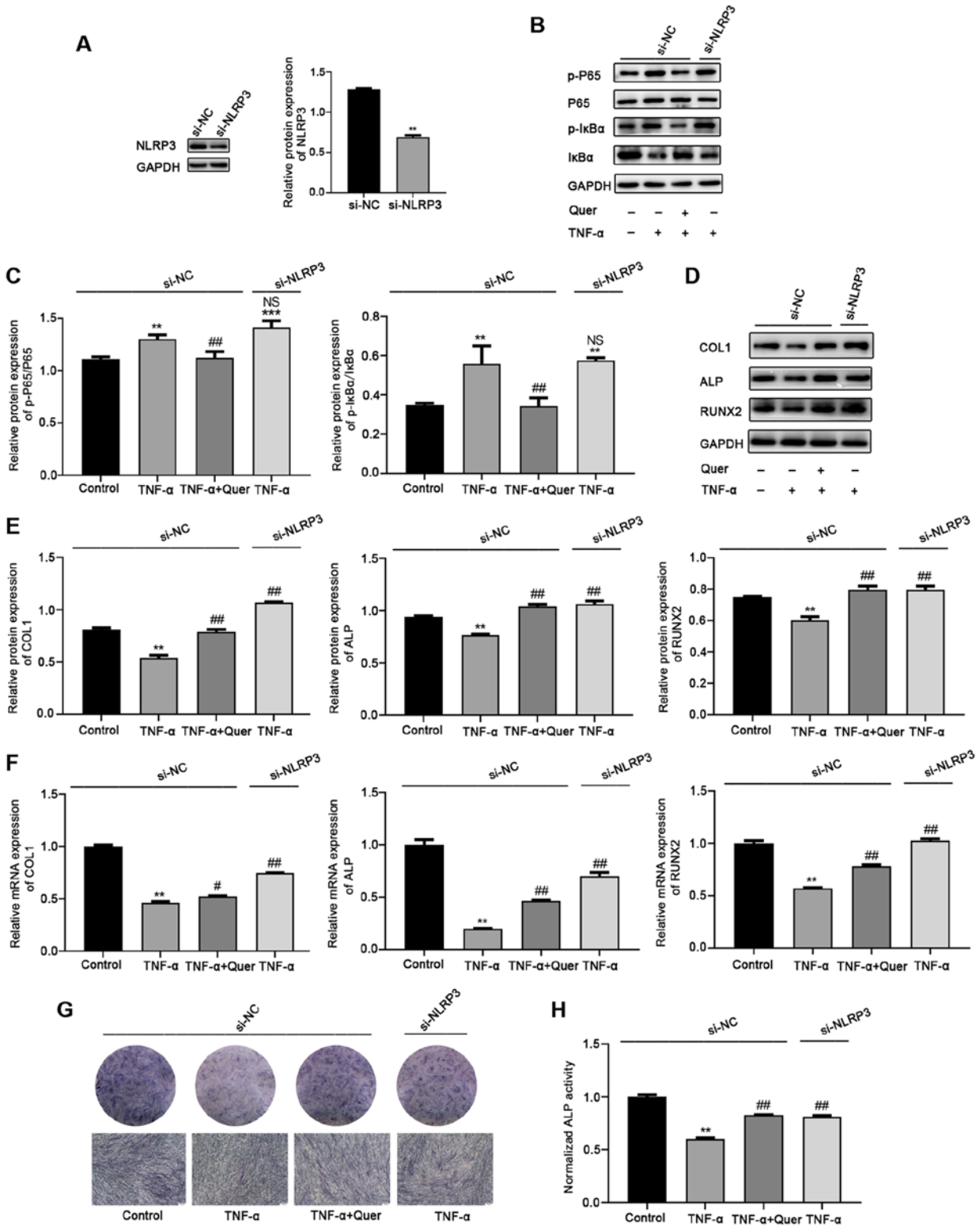


Figure 7. Antagonistic effect of si-NLRP3 on the inhibition of the osteogenesis of hPDLSCs by TNF-α. (A) Protein level of NLRP3 in hPDLSCs transfected with si-NLRP3 or si-NC was examined by western blot analysis. $^{**}$P<0.01 vs. si-NC. (B) Protein levels of p-p65, p65, p-IκBα and IκBα were measured by western blot analysis. (C) Band intensities were quantified using ImageJ software. $^{**}$P<0.01, $^{***}$P<0.001 vs. control + si-NC; $^{\#\#}$P<0.01 vs. TNF-α + si-NC, NS (no significant difference) vs. TNF-α + si-NC. (D) Protein levels of COL1, ALP and RUNX2 on day 7 of osteogenic induction. (E) Band intensities were quantified using ImageJ software. $^{**}$P<0.01 vs. control + si-NC; $^{\#\#}$P<0.01 vs. TNF-α + si-NC. (F) mRNA expression levels of COL1, ALP and RUNX2 following osteogenic induction for 7 days. $^{**}$P<0.01 vs. control + si-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. TNF-α + si-NC. (G) ALP staining following osteogenic induction for 7 days (scale bar, 100 $\mu$m). (H) ALP activity analysis on day 7 of osteogenic induction. $^{**}$P<0.01 vs. control + si-NC; $^{\#\#}$P<0.01 vs. TNF-α+si-NC.

and protect against isoniazid-induced hepatotoxicity by suppressing the NLRP3 pathway (30,48). Likewise, the present study determined that Quer downregulated the expression of NLRP3 inflammasome-associated proteins and genes treated in the hPDLSCs stimulated with TNF-α. Recent studies have revealed that NLRP3 inflammasome activation can suppress

the osteogenesis of MSCs and can contribute to the estrogen deficiency-induced suppression of osteogenesis in ovariectomized mice (18,19). However, few studies have demonstrated the effect of NLRP3 on the osteogenic differentiation of hPDLSCs stimulated with TNF-α. The present study indicated that NLRP3 silencing reversed TNF-α-induced osteogenic damage to hPDLSCs, similar to Quer treatment. Therefore, Quer treatment reversed the inhibition of osteogenic differentiation induced by TNF-α, which may be associated with the inhibition of the NF-κB/NLRP3 inflammasome pathway.

In conclusion, the present study demonstrated that Quer reversed the suppression of the osteogenesis of hPDLSCs in an *in vitro* model of TNF-α-induced periodontitis by inhibiting the relative targets involved in the NF-κB/NLRP3 inflammasome pathway. These findings provide a basis for the use of the drug and stem cell combinations as an effective treatment agent for bone regeneration under inflammatory conditions.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

**Authors' contributions**

XX designed the study. WZ, LJ and BZ performed the experiments. JL, YX and YNW analyzed the experimental data. WZ wrote the article. All authors listed have read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was authorized by the Medical Ethical committee of School of Stomatology, Shandong University (protocol no. GR201806). Signed informed consent was provided from every participant according to the Declaration of Helsinki.

**Patient consent for publication**

Not applicable.

**Competing interests**

All authors declare that they have no competing interests.

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