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Original Article

Nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5 affects the progression of periodontitis by regulating the function of periodontal membrane cells

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KEYWORDS
NLRC5;
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Abstract *Background/purpose:* Nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5 (NLRC5) plays a regulatory role in innate and adaptive immunity. However, its role in periodontitis remains unclear. This study investigated the effects of NLRC5 on periodontitis and the underlying mechanism.
Materials and methods: Experimental periodontitis models of wild-type and Nlrc5 knockout mice were established to detect alveolar bone loss. The inflammatory environment was established with *Porphyromonas. gingivalis* lipopolysaccharide (*P. gingivalis* LPS). The expression of NLRC5 in periodontal ligament stem cells (PDLSCs) were detected with P. gingivalis LPS stimulated. After knocking-down or overexpressing the NLRC5 expression level, the inflammatory cytokine level and osteogenic ability of PDLSCs were detected.
Results: The Nlrc5 knockout mice exhibited greater alveolar bone loss in periodontitis. In the presence of *P. gingivalis* LPS, the expression of NLRC5 decreased. Downregulating NLRC5 increased the expression of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α). Upregulated NLRC5 inhibited nuclear factor kappa-B (NF-κB) signaling and inhibited the expression of those proinflammatory factors. NLRC5 had a positive regulatory effect on the osteogenic differentiation of PDLSCs. When NLRC5 was knocked down, the ALP activity and the number of mineralized nodules in PDLSCs decreased. Conversely, overexpression of NLRC5 enhanced the osteogenic differentiation ability of PDLSCs. Overexpression of NLRC5 increased

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the osteogenic differentiation of PDLSCs in inflammatory environments.
Conclusion: NLRC5 affects the progression of periodontitis by regulating the function of PDLSCs. NLRC5 reduced the expression of inflammatory factors by inhibiting NF-κB, and had a positive regulatory effect on the osteogenic differentiation of PDLSCs.

Introduction

Periodontitis remains one of the most common oral diseases.[1,2] An imbalanced immune response between microorganisms and the host leads to reduced periodontal tissue repair ability and ultimately tissue destruction.[3] However, the molecular mechanism of attachment loss in periodontitis is still unclear and needs to be further explored. Periodontal ligament stem cells (PDLSCs), which are essential for maintaining periodontal homeostasis and regeneration,[4,5] exhibit decreased osteogenic differentiation potential in patients with periodontitis.[6] Recent studies have reported that PDLSCs also possess immunomodulatory abilities, attracting immune cells to clear invading pathogens while playing an immunosuppressive role through the secretion of factors such as tissue growth factor-beta 1 (TGF-β1), hepatocyte growth factor (HGF) and indoleamine 2,3-dioxygenase (IDO).[7–9] PDLSCs promote the differentiation of immunosuppressive cells such as M2 macrophages and regulatory T cells, preventing extensive tissue destruction caused by excessive immune inflammatory responses and promoting the initiation of tissue repair.[10–13] Abnormal PDLSC function may disrupt periodontal homeostasis by aggravating the host immune response, promoting abnormal angiogenesis, enhancing osteoclast activity and alveolar bone resorption.[14]

Pattern recognition receptors (PRRs), known as damage-associated molecular patterns (DAMPs), play a crucial role in host defense by identifying pathogen-associated molecular patterns (PAMPs) and nonpathogenic danger signals from the body.[15] Our group has been paying attention to PRRs for a long time and has performed much research on their role in the pathogenesis of periodontitis. The results showed that the expression levels of the nucleotide-binding oligomerization domain-like receptor family (NLRs) in periodontitis patients' gingival tissues were significantly different from those in the healthy group.[16,17] Recombinant nucleotide binding oligomerization domain containing protein 1 (NOD1) and NOD2 may be involved in regulating the expression of the adhesion molecules intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), the proinflammatory factors interleukin (IL)-6 and IL-8 mediated by *Porphyromonas. gingivalis* (*P. gingivalis*) in periodontal membrane fibroblasts and gingival fibroblasts, as well as promotes monocyte-fibroblast adhesion.[18,19] NLRs family pyrin domain containing 6 (NLRP6) is involved in the regulation of *P. gingivalis*-induced pyroptosis of periodontal cells, including gingival fibroblasts and gingival epithelial cells.[16,17]

Nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5 (NLRC5), the largest protein molecule in the NLRs family, is involved in regulating innate and adaptive immune responses, and plays an important role in tumor immunity and inflammatory diseases.[20] Recent studies have shown that NLRC5 also has functions beyond antigen recognition, such as participating in cell proliferation and differentiation by regulating various intracellular signaling pathways.[21,22] Periodontitis is also a chronic infectious inflammatory disease, however, the role of NLRC5 in the pathogenesis of periodontitis remains unclear. Previous studies by our group revealed that the expression of NLRC5 was lower in the periodontal tissue of patients with periodontitis.[23] These results suggested that changes in NLRC5 expression may be related to the development of periodontitis and the function of PDLSCs.

Previous studies have focused on the function of NLRC5 in immune cells and tumor cells. In this study, the role of NLRC5 in the development of periodontitis was clarified, and the molecular mechanism of NLRC5 affecting periodontal tissue destruction was elucidated based on the inflammatory activation and osteogenic differentiation ability of PDLSCs.

Materials and methods

Experimental periodontitis model

Wild-type (WT) C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and *Nlrc5* knockout (*Nlrc5*$^{-/-}$) mice were purchased from GenePharma Biotechnology Co., Ltd. (Suzhou, China). This study was approved by the Laboratory Animal Welfare Ethics Branch of the Biomedical Ethics Committee of Peking University, and the approval number is LA2020131. Male mice aged 8 weeks were given general anesthesia with 1% pentobarbital sodium at a dose of 100 mg/kg. The 4—0 thread was gently pressed into the proximal and distal parts of the second molar using a microscopic instrument and then knotted on the palatal side for fixation. Unligated mice served as healthy controls. One week after ligation, the mice were killed, and the maxillary bone tissue was fixed and analyzed by micro-computed tomography (micro-CT).

Micro-CT analysis and measurement of alveolar bone loss

The maxillary bone from WT and *Nlrc5*$^{-/-}$ mice (n = 3) was scanned using a micro-CT instrument (Siemens Medical Solutions, Siemens AG, Germany). Following the scanning

process, three-dimensional images were reconstructed, and alveolar bone loss was measured utilizing the software provided by Inveon Research Workshop (Siemens Medical Solutions).

The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 predetermined locations on the palatal side of each mouse maxilla. As shown in Fig. 1. A, The height of the CEJ-ABC from the corresponding position of the maxillary first molar (disto-palatal groove and distal cusp), second molar (mesio-palatal cusp, palatal groove and disto-palatal cusp) and third molar (palatal cusp) was measured.[24]

Cell culture

Human PDLSCs were purchased from Beijing Jinxin Stem Cell Bank. PDLSCs were cultured in minimum essential medium α (αMEM) (Gibco, Grand Island, NY, USA, Catalog No: 12571063) supplemented with 10% fetal bovine serum (Gibco, Catalog No: 10099141C). PDLSCs at passages 4—6 were used for subsequent experiments.

NLRC5 knockdown model and small interfering RNA transfection

NLRC5 expression in PDLSCs was knocked down using small interfering RNA (siRNA) (Gemma Biotechnology, Suzhou, China). After the cells grew to 90% confluence, siRNA (50 nM) transfection was performed using the nucleic acid transfection reagent Rfect (BAIDAI, Changzhou, China, Catalog No:11012), and the transfection efficiency was detected. After 24 h of transfection with siNLRC5 or negative control (siNC), PDLSCs were stimulated with osteogenic induction to detect their osteogenic differentiation ability or stimulated with *P. gingivalis* lipopolysaccharide (*P. gingivalis* LPS) to detect inflammatory factor expression levels.

NLRC5 overexpression model establishment and adenovirus transfection

An NLRC5 overexpression model of PDLSCs was established using adenovirus type 5 virus containing the full length of NLRC5 (Adv5-NLRC5). Adv5-NLRC5 and Adv5-NC were purchased from Xibei Hongcheng Biotechnology (Beijing, China). Transfection was performed at a multiplicity of infection of 100:1 after the cells grew to 70%. The transfection efficiency was detected after 24 h, and after osteogenic induction, the osteogenic differentiation ability of the cells was detected. *P. gingivalis* LPS stimulation was used to detect inflammatory factor expression levels.

Osteogenic induction

As described above, PDLSCs (1×10^4 cells/well) were cultured in 24-well plates containing osteogenic medium

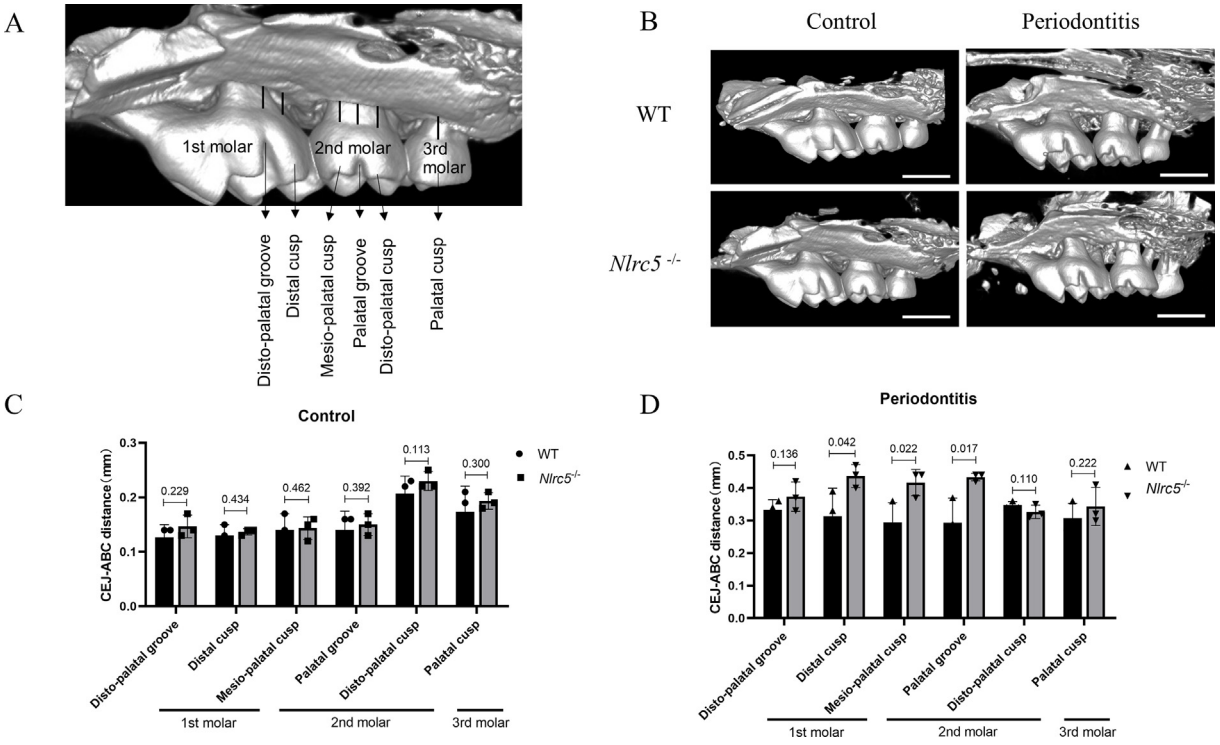

Figure 1 Absence of NLRC5 exacerbated alveolar bone loss (A) Measurement sites for bone loss in experimental periodontitis models. (B) Maxillary micro-CT results of WT and *Nlrc5*$^{-/-}$ mice. (C) CEJ-ABC distance of WT and *Nlrc5*$^{-/-}$ mice in the control group. (D) The CEJ-ABC distance in WT and *Nlrc5*$^{-/-}$ mice in the periodontitis group. WT, wild type; *Nlrc5*, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; CEJ, cemento-enamel junction; ABC, alveolar bone crest.

(α-MEM supplemented with 10% fetal bovine serum, 50 mg/L dexamethasone, 10 mmol/L β-sodium glycerophosphate, and 50 μg/mL ascorbic acid) (Sigma—Aldrich, Saint Louis, MO, USA).[25] The medium was refreshed every 2 days.

Alkaline phosphatase staining and alizarin red staining

After 7 days of osteogenic induction, PDLSCs were stained for ALP using a BCIP/NBT alkaline phosphatase (ALP) staining kit (Beyotime, Guangzhou, China, Catalog No: C3026), and ARS was performed using a 1% alizarin red staining (ARS) solution (Solarbio, Beijing, China, Catalog No: G1452) after 14 days of osteogenic induction. Subsequently, the cells were washed, and images were captured using an inverted microscope. The intracellular ALP activity was quantified using an ALP detection kit (Jiancheng, Nanjing, China, Catalog No: A059-2-2) in accordance with the manufacturer's instructions. To quantify ARS, mineralized nodules were dissolved in a 10% cetylpyridinium chloride solution (Solarbio, Catalog No: C9890), and the absorbance was measured at 560 nm.

Inflammatory environment

P. gingivalis LPS (Thermo Fisher Scientific, Waltham, MA, USA) was added to the culture medium to establish the periodontal inflammatory environment. After the knockdown or overexpression of NLRC5, PDLSCs were cultured in *P. gingivalis* LPS medium at a final concentration of 5 μg/mL for 24 h, total RNA and protein were extracted, or osteogenic induction was continued.

Total RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Catalog No: 15596018) according to the manufacturer's instructions.[26] cDNA was obtained using a reverse transcription kit (ABclonal, Catalog No: PK20429). Real-time quantitative polymerase chain reaction (real-time PCR) was performed using SYBR Green Reagent (ABclonal, Catalog No: RK21203). The primer sequences are shown in Table 1. The relative expression of the target gene was calculated using the $2^{-\triangle\triangle CT}$ method after normalization to β-actin as the standard.

Western blotting analysis

The protein levels of NLRC5 (ABclonal, Catalog No: A16710), runt-related transcription factor 2 (Runx2) (ABclonal, Catalog No: A2581), phospho-nuclear factor kappa-B (NF-κB) p65 (Ser536) (Cell Signaling Technology, Boston, MA, USA, Catalog No: 3033) and NF-κB p65 (Cell Signaling Technology, Catalog No: 6956) were detected by western blotting, as described previously.[26] Primary antibodies against NLRC5, Runx2, pp-65 and p-65 were diluted at a ratio of 1:1000, and the proteins were incubated with PVDF membranes overnight at 4 °C. After incubation with secondary antibodies (ABclonal, Catalog No: AS014/AS003), the protein bands were visualized using enhanced chemiluminescence (ECL) luminescence. The protein expression levels were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Table 1 Sequences of primers used for real-time PCR.

Genes	Primer sequences (5′ to 3′)
β-actin	Forward: TGGCACCCAGCACAATGAA
	Reverse: TTCAACCACTGGGCCACTATTTC
IL-1β	Forward: AGCTCGCCAGTGAAATGATG
	Reverse: GCCCTTGCTGTAGTGGTGGT
IL-6	Forward: GATTCAATGAGGAGACTTGCC
	Reverse: TGTTCTGGAGGTACTCTAGGT
TNF-α	Forward: CCTCTCTCTAATCAGCCCTCTG
	Reverse: GAGGACCTGGGAGTAGATGAG
IL-8	Forward: CTCTTGGCAGCCTTCCTGATTT
	Reverse: TGGGGTGGAAAGGTTTGGAGTA

IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 statistical software (version 23.0; IBM Corp., Armonk, NY, USA). All experiments were repeated at least 3 times, and the data are expressed as the mean ± standard deviation (SD). After removing the extreme values by the Shapiro—Wilk normality test, the data that conformed to a normal distribution were compared between the two groups using an independent sample t test. If the data could not be adjusted to a normal distribution, the rank sum test was used. $P < 0.05$ was considered to indicate a statistically significant difference between the groups, and "ns" indicates no statistically significant difference.

Results

Absence of NLRC5 exacerbated alveolar bone loss

To clarify the role of NLRC5 in periodontitis bone loss, the CEJ-ABC distances were measured at six sites on the palatal surface of the mouse maxilla (Fig. 1A). The micro-CT results in Fig. 1B show that there was no significant difference in the distance traveled by CEJ-ABC between the WT and *Nlrc5*$^{-/-}$ mice in the control group at any of the 6 sites (Fig. 1C), indicating that NLRC5 deletion had no effect on healthy periodontal supporting tissue. However, in the experimental periodontitis model, the distances of the CEJ-ABC in the distal cusp of the first molar, mesio-palatal cusp and palatal groove of the second molar were significantly greater in the *Nlrc5*$^{-/-}$ mice than in the WT mice (Fig. 1D), indicating that NLRC5 loss leads to increased alveolar bone loss in mice.

NLRC5 is downregulated in inflammatory environments and regulates the expression of inflammatory factors

As a PRR, NLRC5 can regulate the inflammatory response, and bone loss is also affected by the level of inflammatory factors.

First, we found that the expression level of NLRC5 decreased when PDLSCs were stimulated with 5 μg/mL *P. gingivalis* LPS for 4 h (Fig. 2A). After stimulation with *P. gingivalis* LPS for 24 h, the mRNA expression levels of IL-1β, TNF-α, and IL-6 in the siNLRC5 group were significantly greater than those in the siNC group ($P = 0.001$, 0.030, 0.001, respectively) (Fig. 2B, C and D). However, there was no significant change in the expression level of IL-8 in the siNLRC5 group ($P = 0.397$) (Fig. 2E).

The above results indicated that NLRC5 was downregulated in inflammatory environments and that NLRC5 deficiency led to increases in IL-1β, TNF-α, and IL-6 in PDLSCs. This suggested that the decreased expression of NLRC5 in the inflammatory environment may be a potential mechanism for the development of periodontal inflammation.

NLRC5 inhibited the secretion of inflammatory factors by inhibiting the NF-κB pathway

In the inflammatory environment, changes in NLRC5 expression affect the expression of inflammatory factors; therefore, we further explored the signaling pathways involved. The NF-κB pathway is a classic proinflammatory signaling pathway. PRRs recognize PAMPs and activate NF-κB signals, which then leads to the release of a large number of inflammatory factors through cascade amplification.

We detected the level of phospho−NF−κB p65 (p-p65) in PDLSCs after overexpressing NLRC5. The results showed that the expression level of p-p65 in PDLSCs in the NLRC5-overexpressing group (NLRC OE) group was not significantly different from that in the NC group (Fig. 3A). In other words, the overexpression of NLRC5 alone did not affect the activation of NF-κB signaling in PDLSCs. Then both the NLRC5 OE group and the NC group were treated with *P. gingivalis* LPS for 15, 30, 60, 120, 240, or 480 min, and the expression level of p-p65 was detected at each time point. The results showed that from 15 min to 4 h after *P. gingivalis* LPS treatment, the p-p65 level in the NLRC5-overexpressing group was lower than that in the NC group (Fig. 3B). At 480 mins, the level of p-p65 in the overexpression group gradually increased, approaching the p-p65 expression level in the NC group.

After stimulation with *P. gingivalis* LPS for 24 h, the mRNA expression levels of the inflammatory factors IL-1β, TNF-α, and IL-6 in the NLRC5 OE group were significantly lower than those in the NC overexpression group ($P = 0.001$, 0.003, and 0.001, respectively) (Fig. 3C, D and E). However, there was no significant change in the expression level of IL-8 in the NLRC OE group ($P = 0.817$) (Fig. 3F).

The results showed that overexpression of NLRC5 in PDLSCs could inhibit the activation of NF-κB signaling in the inflammatory environment, thereby inhibiting the expression of the above inflammatory factors in PDLSCs. Increasing the expression level of NLRC5 in cells may help the host resist the stimulation of pathogenic microorganisms and tissue destruction caused by inflammation.

NLRC5 promoted osteogenic differentiation of periodontal ligament cells

After transfection with siRNA for 24 h, green fluorescence was observed in the cytoplasm of PDLSCs, indicating a high

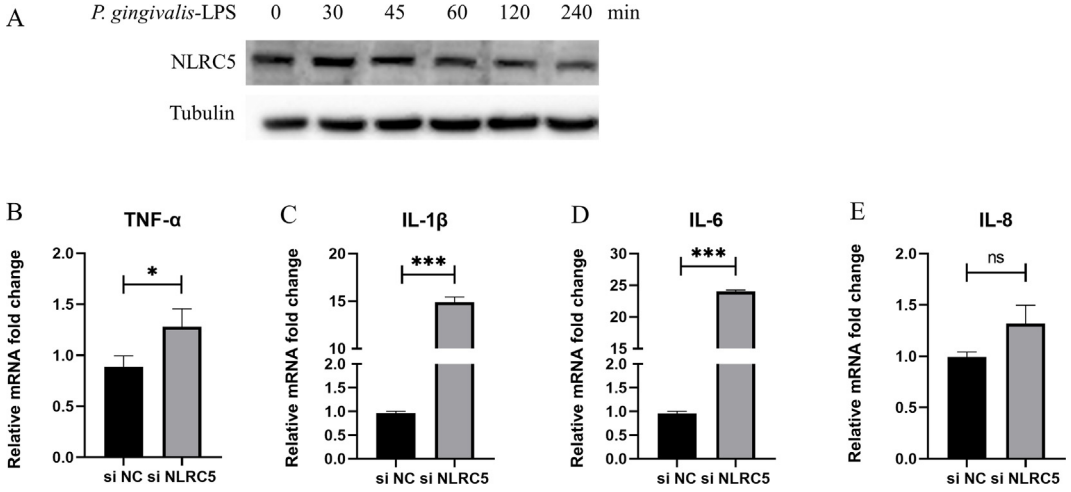

Figure 2 *P. gingivalis* LPS stimulation reduces NLRC5 levels, and NLRC5 knockdown promotes the expression of *P. gingivalis* LPS-induced inflammatory factors in PDLSCs. (A) The expression level of NLRC5 decreased when PDLSCs were stimulated with *P. gingivalis* LPS for 4 h. (B−D) The mRNA levels of IL-1β, IL-6, and TNF-α were further increased in the siNLRC5 group ($P = 0.001$, 0.001, and 0.030, respectively) of PDLSCs treated with *P. gingivalis* LPS. (E) There was no significant change in the expression level of IL-8 in the siNLRC5 group ($P = 0.397$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NLRC5, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; NC, negative control; *P. gingivalis* LPS, *Porphyromonas gingivalis* lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α.

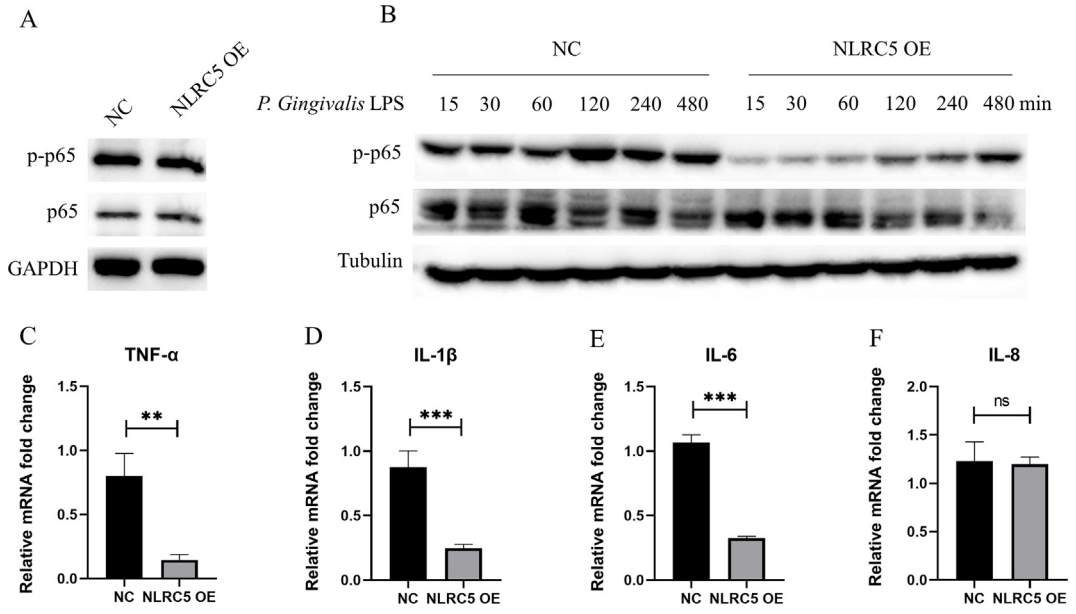

Figure 3 NLRC5 inhibited the secretion of inflammatory factors by inhibiting the NF-κB pathway. (A) Overexpression of NLRC5 alone did not affect the expression of NF-κB p-p65 in PDLSCs. (B) At 15 min to 8 h after *P. gingivalis* LPS treatment, the p-p65 level in the NLRC5-overexpressing group was significantly lower than that in the NC group. (C—E) The mRNA levels of IL-1β, IL-6, and TNF-α were further decreased in NLRC5-overexpressing PDLSCs ($P = 0.001$, 0.001, 0.003, respectively) following *P. gingivalis* LPS treatment. (F) There was no significant change in the expression level of IL-8 in the NLRC OE group ($P = 0.817$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NLRC5, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; NC, negative control; OE, overexpression group; *P. gingivalis* LPS, *Porphyromonas gingivalis* lipopolysaccharide; p-p65, phospho—NF—κB p65; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transfection efficiency (Fig. 4A). After osteogenic induction for 3 days, siNLRC5 significantly reduced the expression level of NLRC5 in PDLSCs ($P = 0.004$) (Fig. 4B). Moreover, the protein level of the osteogenic transcription factor Runx2 was also significantly reduced ($P = 0.020$) (Fig. 4B). ALP activity ($P = 0.014$) and mineralized nodules ($P = 0.002$) were significantly lower in the siNLRC5 group than in the siNC group (Fig. 4C and D).

After treatment with Adv-NLRC5 for 24 h, strong green fluorescence was observed in the PDLSCs of the NLRC5 OE,

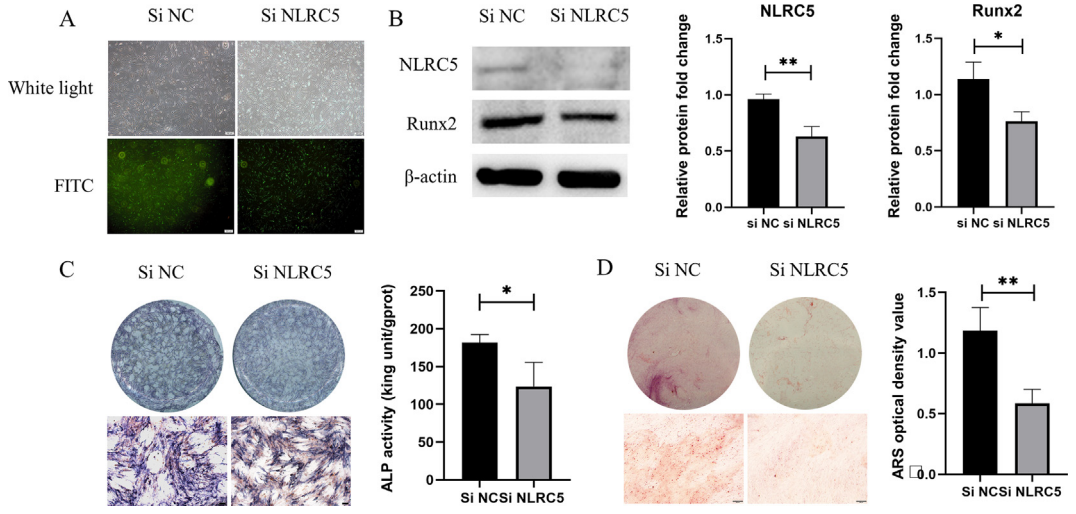

Figure 4 Silencing NLRC5 reduces the osteogenic differentiation ability of PDLSCs. (A) siRNA transfection efficiency; green fluorescence indicates successful transfection. (B) The expression of NLRC5 ($P = 0.004$) and Runx2 ($P = 0.020$) in PDLSCs was significantly decreased by si-NLRP5. (C) and (D) ALP activity ($P = 0.014$) and the number of mineralized nodules ($P = 0.002$) decreased in response to siNLRC5. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NLRC5, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; Si, small interfering RNA; NC, negative control; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; ARS, alizarin red staining; FITC, fluorescein isothiocyanate.

indicating that the NLRC5-overexpressing model of PDLSCs was successfully established (Fig. 5A). After osteogenic induction for 3 days, the protein expression levels of NLRC5 ($P = 0.001$) and Runx2 ($P = 0.004$) in the PDLSCs in the NLRC5 OE group were significantly increased (Fig. 5B). ALP activity ($P = 0.014$) and mineralized nodules ($P = 0.020$) were significantly greater in the NLRC OE group than in the NC group (Fig. 5C and D).

In previous experiments, we have demonstrated that changes in NLRC5 expression level have no significant effect on the cell viability of PDLSCs (Supplementary material 1), ruling out the effect of cell activity on the osteogenic differentiation of PDLSCs. These results indicated that NLRC5 deficiency inhibits the osteogenic differentiation of PDLSCs and that the overexpression of NLRC5 promotes the osteogenic differentiation of PDLSCs. The decrease of NLRC5 in the inflammatory environment may be the cause of the decreased osteogenic ability of PDLSCs. Regulating the expression level of NLRC5 in cells may be a potential target for promoting the osteogenic differentiation of PDLSCs.

Overexpression of NLRC5 enhanced the osteogenic differentiation of PDLSCs in inflammatory environments.

We also examined the effect of NLRC5 on the osteogenic ability of PDLSCs in an inflammatory environment. The results showed that the number of ALP-positive cells and mineralized nodules on PDLSCs in the NLRC5 OE group was greater than that in the NC group (Fig. 6A and B). ALP activity ($P = 0.001$) and ARS semi-quantification ($P = 0.001$) were also significantly greater in the NLRC5 OE group than in the NC group. These results suggested that NLRC5 improved the osteogenic ability of PDLSCs in an inflammatory environment. Improving the expression level of NLRC5 in periodontal tissue may enhance its ability to repair tissue under conditions of periodontal inflammation.

Discussion

This study identified the role of NLRC5 in the pathogenesis of periodontitis. A lack of NLRC5 leads to the aggravation of periodontitis-related alveolar bone loss, possibly because the absence of NLRC5 leads to the abnormal function of PDLSCs. NLRC5 expression in PDLSCs was decreased in *P. gingivalis* LPS induced inflammatory environment. Decreased expression of NLRC5 would lead to increased expression of pro-inflammatory factors in PDLSCs and decreased osteogenic differentiation ability. Overexpression of NLRC5 decreased the expression levels of IL-1β, IL-6 and TNF-α in PDLSCs by inhibiting the NF-κB signaling pathway, and enhanced the osteogenic differentiation ability of PDLSCs. This study further clarified the role of NLRC5 in the occurrence and development of periodontitis, expanded the understanding of pattern recognition receptors in the occurrence and development of periodontitis, and contributed to a deeper understanding of the pathogenic mechanism of periodontitis.

Due to the persistent presence of plaque microorganisms in the oral cavity, the host periodontal tissue is subject to bacterial invasion for a long time, and the dynamic balance between them maintains periodontal tissue homeostasis. Because of the presence of supragingival and subgingival plaque, the gingival epithelium and periodontal tissue around the gingival sulcus are also constantly challenged by bacteria, and a series of inflammatory reactions occur.[27,28] As a pattern recognition receptor, NLRC5 plays a regulatory role in both the innate and adaptive immune responses of the host.[20] The presence of NLRC5 may be an adaptive response of periodontal tissue to resist bacterial invasion and maintain a local immune steady state by inhibiting inflammatory reactions. Therefore, stable NLRC5 expression

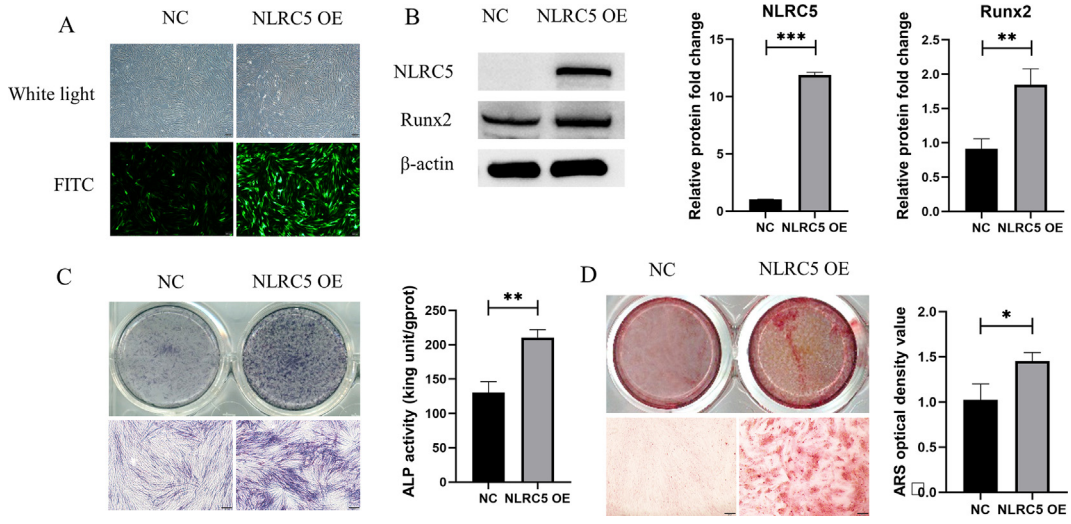

Figure 5 Overexpression of NLRC5 enhances osteogenic differentiation of PDLSCs. (A) Adv5-NLRC5 transfection efficiency; green fluorescence indicates successful transfection. (B) The expression of NLRC5 ($P = 0.001$) and Runx2 ($P = 0.004$) in PDLSCs was significantly increased by treatment with Adv5-NLRC5. (C) and (D) ALP activity ($P = 0.001$) and the number of mineralized nodules ($P = 0.021$) were increased by Adv5-NLRC5 interference. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NLRC5, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; NC, negative control; OE, overexpression group; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; ARS, alizarin red staining; FITC, fluorescein isothiocyanate.

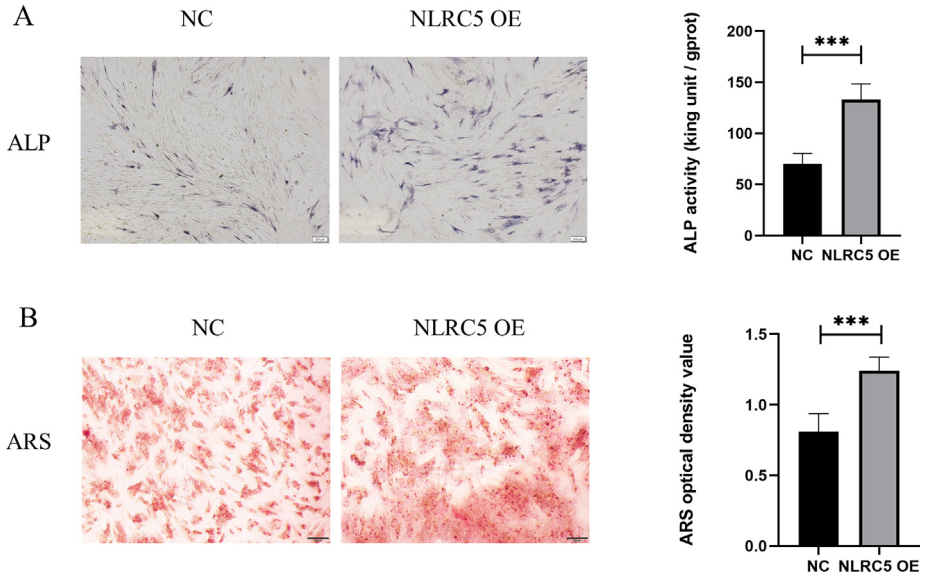

Figure 6 Overexpression of NLRC5 enhanced the osteogenic differentiation of PDLSCs in inflammatory environments. (A) ALP-positive cells and ALP activity were significantly greater in the NLRC5 OE group than in the NC group. ($P = 0.001$). (B) The mineralized nodules of PDLSCs and the ARS intensity ($P = 0.001$) in the NLRC5 OE group were also significantly greater than those in the NC group. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). NLRC5, nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain containing protein 5; NC, negative control; OE, overexpression group; ALP, alkaline phosphatase; ARS, alizarin red staining.

may be indispensable for maintaining a stable periodontal state.

In this study, we found that NLRC5 level in PDLSCs gradually decreased within 4 h after *P. gingivalis* LPS stimulation. It has also been reported that NLRC5 content in LPS-stimulated RAW cells decreased at 2 h.[29] Silencing NLRC5 in the mouse macrophage line RAW264.7 results in increased secretion of IL-6, TNF-α, the chemokine C-X-C motif ligand 5 and IL-1β while reducing the secretion of the anti-inflammatory cytokine IL-10.[29,30] Li et al. further confirmed the inhibitory effect of NLRC5 on inflammatory cytokines, showing that the silencing of NLRC5 in RAW264.7 macrophages induced by LPS stimulation resulted in increased secretion of IL-6 and TNFα.[31] Consistent with our results, the silencing of NLRC5 in PDLSCs induced by *P. gingivalis* LPS stimulation resulted in increased mRNA expression levels of IL-1β, IL-6 and TNFα. Consistent with the results of the experimental periodontitis model, the increased levels of inflammatory factors caused by NLRC5 deficiency may be the cause of increased bone loss in periodontitis. Conversely, overexpression of NLRC5 in PDLSCs decreased the increases in the mRNA expression levels of IL-1β, IL-6 and TNFα induced by *P. gingivalis* LPS stimulation. Our results demonstrated that *P. gingivalis* LPS induced a decrease in the expression of NLRC5 in PDLSCs, and the downregulation of NLRC5 further led to the abnormal immunomodulatory function of PDLSCs. Abnormal PDLSC function can aggravate periodontitis and cause persistent periodontal bone resorption, and the stable presence of NLRC5 in periodontal tissues helps the host maintain periodontal homeostasis and prevent the development of periodontitis.

NLRC5 inhibits the phosphorylation of IKK by directly interacting with IκB kinase-α (IKKα) and IKKβ, thereby inhibiting the activation of NF-κB.[29] *P. gingivalis* LPS is an agonist of TLR4 and can activate its downstream NF-κB signaling pathway.[32] This study also confirmed that the overexpression of NLRC5 can inhibit the activation of the NF-κB signaling pathway induced by *P. gingivalis* LPS. Interestingly, the overexpression of NLRC5 alone cannot inhibit the NF-κB signaling pathway. This may be due to differences in the function of NLRC5 in cells under different pathophysiological conditions.

This study showed that the regulation of NLRC5 expression directly affected the osteogenic differentiation ability of PDLSCs. Overexpression of NLRC5 directly improved the osteogenic differentiation ability of PDLSCs, and knockdown of NLRC5 inhibited the osteogenic differentiation of PDLSCs. The Wnt signaling pathway plays an indispensable role in osteoblast differentiation, bone development, and bone remodeling.[33] Several studies have shown that Wnt/β-catenin signal transduction is associated with PDLSC osteogenesis.[34,35] In addition, PI3K/AKT signaling also plays a central role in controlling the survival of whole-body cells, the expression of osteogenic transcription factors, and growth and differentiation.[36,37] Activation of the PI3K/AKT signaling pathway can also increase the osteogenic differentiation ability of PDLSCs.[38] Exosomes derived from human umbilical cord mesenchymal stem cells enhance the osteogenic differentiation of periodontal ligament stem cells under high glucose conditions through the PI3K/AKT signaling pathway.[39] Recent studies have shown that NLRC5-mediated activation of the Wnt/β-catenin and PI3K/AKT signaling pathways may promote tumor cell proliferation and invasion.[40] In this study, there was no significant changes in PDLSCs cell viability or cell proliferation induced by NLRC5 overexpression (Supplementary material 1). This may be caused by the different microenvironment between

tumor cells and normal tissue cells. Moreover, there is still no evidence that NLRC5 can enhance the osteogenic differentiation ability of PDLSCs through the above pathways. This study will also further explore the molecular mechanism by which NLRC5 regulates the osteogenic differentiation ability of PDLSCs.

The experimental results in this study were mainly from in vitro studies. Subsequent studies will further verify the effect of NLRC5 on the osteogenic differentiation of PDLSCs and its role in inhibiting the progression of periodontitis in animal models. The molecular mechanism by which NLRC5 regulates the osteogenic differentiation ability of PDLSCs will also be further explored.

In summary, this study demonstrated that NLRC5 affects the progression of periodontitis by regulating the function of PDLSCs. NLRC5 reduced the expression of inflammatory factors by inhibiting NF-κB in the inflammatory environment, and had a positive regulatory effect on the osteogenic differentiation of PDLSCs. The changes in the NLRC5 expression level and its effects on the function of PDLSCs in the inflammatory environment may be one of the mechanisms by which NLRC5 affects the development of periodontitis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.07.008.

References

1. Jiao J, Jing W, Si Y, et al. The prevalence and severity of periodontal disease in mainland China: data from the fourth national oral health survey (2015-2016). *J Clin Periodontol* 2021;48:168–79.
2. Eke PI, Borgnakke WS, Genco RJ. Recent epidemiologic trends in periodontitis in the USA. *Periodontol 2000* 2020;82:257–67.
3. Kinane DF, Demuth DR, Gorr SU, Hajishengallis GN, Martin MH. Human variability in innate immunity. *Periodontol 2000* 2007;45:14–34.
4. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;364:149–55.
5. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009;88:792–806.
6. Zheng W, Wang S, Wang J, Jin F. Periodontitis promotes the proliferation and suppresses the differentiation potential of human periodontal ligament stem cells. *Int J Mol Med* 2015;36:915–22.
7. Queiroz A, Albuquerque-Souza E, Gasparoni LM, et al. Therapeutic potential of periodontal ligament stem cells. *World J Stem Cell* 2021;13:605–18.
8. Lee JS, Lee JB, Cha JK, et al. Chemokine in inflamed periodontal tissues activates healthy periodontal-ligament stem cell migration. *J Clin Periodontol* 2017;44:530–9.
9. Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* 2009;219:667–76.
10. Obermajer N, Popp FC, Soeder Y, et al. Conversion of Th17 into IL-17A(neg) regulatory T cells: a novel mechanism in prolonged allograft survival promoted by mesenchymal stem cell-supported minimized immunosuppressive therapy. *J Immunol* 2014;193:4988–99.
11. Shin C, Kim M, Han JA, et al. Human periodontal ligament stem cells suppress T-cell proliferation via down-regulation of non-classical major histocompatibility complex-like glycoprotein CD1b on dendritic cells. *J Periodontal Res* 2017;52:135–46.
12. Ng J, Hynes K, White G, et al. Immunomodulatory properties of induced pluripotent stem cell-derived mesenchymal cells. *J Cell Biochem* 2016;117:2844–53.
13. Liu J, Wang H, Zhang L, et al. Periodontal ligament stem cells promote polarization of M2 macrophages. *J Leukoc Biol* 2022;111:1185–97.
14. Zhang Z, Deng M, Hao M, Tang J. Periodontal ligament stem cells in the periodontitis niche: inseparable interactions and mechanisms. *J Leukoc Biol* 2021;110:565–76.
15. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010;140:805–20.
16. Liu W, Liu J, Wang W, Wang Y, Ouyang X. NLRP6 Induces pyroptosis by activation of caspase-1 in gingival fibroblasts. *J Dent Res* 2018;97:1391–8.
17. Liu J, Liu W, Lv P, Wang Y, Ouyang X. Activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 6 by Porphyromonas gingivalis regulates programmed cell death in epithelium. *J Dent Sci* 2023;18:1867–75.
18. Liu J, Duan J, Wang Y, Ouyang X. Intracellular adhesion molecule-1 is regulated by porphyromonas gingivalis through nucleotide binding oligomerization domain-containing proteins 1 and 2 molecules in periodontal fibroblasts. *J Periodontol* 2014;85:358–68.
19. Liu J, Liu W, Xie Y, Wang Y, Ouyang X. Adhesion of monocytes to periodontal fibroblasts requires activation of NOD1/2- and TLR4-mediated LFA-1 and VLA-4. *Arch Oral Biol* 2015;60:834–44.
20. Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. *Nat Rev Immunol* 2012;12:813–20.
21. Neerincx A, Castro W, Guarda G, Kufer TA. NLRC5, at the heart of antigen presentation. *Front Immunol* 2013;4:397.
22. Benko S, Kovacs EG, Hezel F, Kufer TA. NLRC5 functions beyond MHC I regulation-what do we know so far? *Front Immunol* 2017;8:150.
23. Wang W, Liu W, Liu J, Lv P, Wang Y, Ouyang X. NLRC5 modulates bone metabolism and plays a role in periodontitis. *J Periodontal Res* 2022;57:891–903.
24. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. *J Immunol Methods* 2013;394:49–54.
25. Lv PY, Gao PF, Tian GJ, et al. Osteocyte-derived exosomes induced by mechanical strain promote human periodontal ligament stem cell proliferation and osteogenic differentiation via the miR-181b-5p/PTEN/AKT signaling pathway. *Stem Cell Res Ther* 2020;11:295.

26. Li Q, Liu J, Liu W, et al. LOX-1 regulates P. gingivalis-induced monocyte migration and adhesion to human umbilical vein endothelial cells. *Front Cell Dev Biol* 2020;8:596.
27. Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. *Periodontol 2000* 2006;40:11—28.
28. Moutsopoulos NM, Konkel JE. Tissue-specific immunity at the oral mucosal barrier. *Trends Immunol* 2018;39:276—87.
29. Cui J, Zhu L, Xia X, et al. NLRC5 negatively regulates the NF-kappaB and type I interferon signaling pathways. *Cell* 2010; 141:483—96.
30. Benko S, Magalhaes JG, Philpott DJ, Girardin SE. NLRC5 limits the activation of inflammatory pathways. *J Immunol* 2010;185: 1681—91.
31. Li L, Xu T, Huang C, Peng Y, Li J. NLRC5 mediates cytokine secretion in RAW264.7 macrophages and modulated by the JAK2/STAT3 pathway. *Inflammation* 2014;37:835—47.
32. Tong Y, Cui J, Li Q, Zou J, Wang HY, Wang RF. Enhanced TLR-induced NF-κB signaling and type I interferon responses in NLRC5 deficient mice. *Cell Res* 2012;22:822—35.
33. Vlashi R, Zhang X, Wu M, Chen G. Wnt signaling: essential roles in osteoblast differentiation, bone metabolism and therapeutic implications for bone and skeletal disorders. *Genes Dis* 2023;10:1291—317.
34. Liang L, Zhou W, Yang N, Yu J, Liu H. ET-1 promotes differentiation of periodontal ligament stem cells into osteoblasts through ETR, MAPK, and Wnt/β-catenin signaling pathways under inflammatory microenvironment. *Mediat Inflamm* 2016; 2016:8467849.
35. Cheng M, Zhou Q. Targeting EZH2 ameliorates the LPS-inhibited PDLSC osteogenesis via Wnt/β-catenin pathway. *Cells Tissues Organs* 2020;209:227—35.
36. Pramojanee SN, Phimphilai M, Chattipakorn N, Chattipakorn SC. Possible roles of insulin signaling in osteoblasts. *Endocr Res* 2014;39:144—51.
37. Chalhoub N, Baker SJ. PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 2009;4:127—50.
38. Zhao X, Sun W, Guo B, Cui L. Circular RNA BIRC6 depletion promotes osteogenic differentiation of periodontal ligament stem cells via the miR-543/PTEN/PI3K/AKT/mTOR signaling pathway in the inflammatory microenvironment. *Stem Cell Res Ther* 2022;13:417.
39. Yang S, Zhu B, Tian XY, et al. Exosomes derived from human umbilical cord mesenchymal stem cells enhance the osteoblastic differentiation of periodontal ligament stem cells under high glucose conditions through the PI3K/AKT signaling pathway. *Biomed Environ Sci* 2022;35:811—20.
40. Peng YY, He YH, Chen C, et al. NLRC5 regulates cell proliferation, migration and invasion in hepatocellular carcinoma by targeting the Wnt/beta-catenin signaling pathway. *Cancer Lett* 2016;376:10—21.