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Exendin-4 regulates Wnt and NF-κB signaling in lipopolysaccharide-induced human periodontal ligament stem cells to promote osteogenic differentiation

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ABSTRACT

A major feature of chronic periodontitis (CP) is the damage and destruction of alveolar bone. Periodontal ligament stem cells (PDLSCs) can differentiate into bone and improve CP. Exendin-4 (Ex-4) has been shown to have anti-inflammatory mechanisms and can promote bone regeneration. However, the effects of Ex-4 on the osteogenic differentiation of PDLSCs in the inflammatory microenvironment remains uncharacterized. In this study, we assessed the effects of Ex-4 on PDLSCs stimulated with lipopolysaccharide (LPS) to mimic the inflammatory environment. PDLSCs proliferation was assessed through CCK-8 assays and osteogenic differentiation was measured using Alizarin Red staining. The anti-inflammatory and osteogenic mechanisms of Ex-4 were assessed by western blot, RT-PCR, ELISA and immunofluorescence. We found that LPS treatment promoted the proliferative capacity of PDLSCs and inhibited their osteogenic differentiation. However, Ex-4 reversed these effects through suppressing PDLSCs proliferation and promoting osteogenic differentiation. Ex-4 increased Runx2, ALP, and Osx levels and decreased TNF-α and IL-6 expression. Ex-4 also reduced the expression of IκBα and p-IκBα, and inhibited the nuclear translocation of NF-κB/p65. The expression of β-catenin decreased in nucleus after co-treatment of Ex-4 with LPS. Taken together, these data demonstrate that Ex-4 promotes PDLSCs osteogenic differentiation in the inflammatory microenvironment through regulating NF-κB and Wnt signaling.

1. Introduction

Chronic Periodontitis (CP) is a destructive disease that invades gingiva and periodontal tissue. CP is characterized by the formation of periodontal pockets, inflammation of the pocket wall, alveolar bone absorption, and the gradual loosening of teeth. CP is the most common cause of tooth loss in adults [1]. The main pathological changes of periodontitis include the absorption of alveolar bone, widening of the periodontal membrane cavity, and the occurrence of inflammatory reactions. The pathological changes of periodontitis include the reaction to pathogenic bacteria and their toxic products, and cytokine production [2]. Amongst the various chronic inflammatory bone diseases, the expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) generally increase. These include osteoarthritis, rheumatoid arthritis, and periodontal disease [3,4]. Inflammatory cytokine expression is regulated by NF-κB, the activation of which suppresses osteogenic differentiation. Current periodontitis treatments involve supragingival scaling, flap surgery, and guided tissue regeneration (GTR). With the development of periodontal tissue regeneration technology, the use of stem cells represents a key advance in the reconstruction of damaged tissue.

Human periodontal ligament stem cells (PDLSCs) have similar characteristics to mesenchymal stem cells (MSCs). PDLSCs differentiate into osteoblasts and have been used to regenerate periodontal support tissue [5]. Periodontal tissue regeneration therapy aims to control inflammation and stimulate stem cell mediated regeneration. However, the differentiation potential of PDLSCs in CP is impaired due to the presence of inflammatory factors [6]. Exploring new methods to promote the osteogenic differentiation of PDLSCs in the inflammatory microenvironment is of great significance to CP treatment.

Exendin-4 (Ex-4) is a Glucagon-like peptide-1 (GLP-1) agonist that has similar properties to GLP-1. GLP-1 is one of incretins secreted by L-

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Abbreviations: CP, chronic periodontitis; PDLSCs, Periodontal ligament stem cells; Ex-4, Exendin-4; LPS, lipopolysaccharide; CCK-8, Cell counting kit-8; RT-PCR, real-time polymerase chain reaction; Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; Osx, osterix; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; NF-κB, nuclear factor-κB; IκBα, inhibitor of nuclear factor-κB; GSK3β, glycogen synthase kinase-3β

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cells into the intestine to inhibit glucagon secretion [7,8]. Recent studies highlight the ability of GLP-1 to promote bone growth and remodeling [9,10], and to reduce inflammation in both pancreatic islets and adipose tissue [11–13]. GLP-1 inhibits inflammatory processes through its suppression of cytokine expression and immune cell infiltration. GLP-1 has anti-inflammatory effects on the liver, brain, kidney, lung, testis, skin, and vascular system [14–19]. As an analogue of GLP-1, Ex-4 has positive effects on the osteogenic differentiation of mesenchymal stem cells (MSCs) [20,21]. Ex-4 has been shown to increase regulatory T-cell numbers and promote anti-inflammatory cytokine IL-10 secretion in NOD mice with type 1 diabetes [22]. Chaudhuri and colleagues [23] found that Ex-4 exerts rapid anti-inflammatory effects at both the cellular and molecular level. More recently, Ex-4 was shown to suppress oxidative stress and inflammatory mediators including isoprostane, CRP, and monocyte chemoattractant protein (MCP)-1 in type 2 diabetic models [24]. Thus, Ex-4 not only promotes bone formation, but has anti-inflammatory effects during chronic inflammatory disease.

Despite this knowledge, the effects of Ex-4 on PDLSCs in the inflammatory niche remain unclear. LPS is a thick layer of lipopolysaccharide located on the outer membranes of the cell wall of Gram-negative bacteria. Studies have indicated that LPS can inhibit the osteogenic differentiation of stem cells [25–27]. Recent studies have shown that prolonged treatment of PDLSCs with LPS enhances IL-6 production and inhibits osteogenic differentiation. PDLSCs were responsive to LPS at 10 μg/ml, whilst 1 μg/ml had fewer effects [28]. In this study, PDLSCs were treated with LPS (10 μg/ml) to stimulate the inflammatory microenvironment *in vitro* and the role of Ex-4 in PDLSC function was investigated.

2. Materials and methods

2.1. Cell culture

Periodontal ligament cells were collected from young orthodontic patients who underwent removal of the premolars. All patients signed informed consent. Premolars were rinsed in sterile phosphate-buffered saline (PBS) (hyclone, USA) and the periodontal membrane in the middle of the root was gently removed through scraping with a sterile scalpel. Membranes were cut into 1 mm^3 sections and digested with collagenase type I (3 mg/ml, Sigma-Aldrich, USA) in a constant temperature water bath at 37 °C for 30 min. Following digestion, cells were transferred into T25 cell culture flasks containing fetal bovine serum (FBS) (GEMINI, US) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 6 h of culture, 5 ml of α-minimum essential medium (α-MEM) (Sigma-Aldrich, USA) supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 mg/ml of streptomycin (Gibco BRL, USA) was added to the culture flasks and replenished every three days. The morphological and biological characteristics of the PDLSCs are shown in (Fig. 1).

2.2. Cell identification

We examined colony-formation efficiency, differentiation capabilities and phenotypic molecular markers of MSCs to confirm the stem cell characteristics of PDLSCs. Cells (400) were seeded into 6 cm^2 cell culture dishes for 14 days and the aggregation of more than 50 cells was identified as a colony. PDLSCs at passage 3 were induced to undergo osteogenic or adipogenic differentiation. The osteogenic induction medium consisted of α-MEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 1 μmol/l dexamethasone, and 3 mmol/l β-glycerophosphate (Sigma-Aldrich, USA). After 21 days, the degree of extracellular matrix calcification was assayed through Alizarin Red staining (Solabio, China). The adipo-inductive medium consisted of α-MEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μmol/l dexamethasone, 10 μmol/l insulin, 200 μmol/l indomethacin, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, USA). After 14 days, newly formed lipid droplets were detected by Oil Red O staining (Solabio, China). PDLSCs at passage 3 were seeded into 6-well plates containing coverslips (2×10^5 cells/well). MSC phenotypic molecular markers including STRO-1 and CD146 were detected by immunofluorescent staining.

2.3. CCK-8 assays

The Cell counting kit-8 (CCK-8) (Beyotime, China) was used to measure cell proliferation according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates (3×10^3 cells/well) and treated with LPS from porphyromonas gingivalis (Sigma-Aldrich, USA) (0–100 μg/ml) or Ex-4 (Sigma-Aldrich, USA) (1–100 nmol/l) in triplicate. Untreated cells were included as control group (N_1) and medium was changed every other day. Assays were performed on days 1, 3, 5, and 7 through the addition of 10 μl CCK-8 solution for 1 h at 37 °C. Absorbances were read at 450 nm.

2.4. Alizarin Red staining

Seeded PDLSCs at passage 3–5 in 6-well plates (2×10^5 cells/well) and differentiated into osteoblasts with osteogenic induction medium. Cells cultured with osteogenic induction medium only was the control group (N_2). Cells were cultured in osteogenic induction medium supplemented with LPS (10 μg/ml) or Ex-4(10 nmol/l). Each group was replaced with the same medium as before every three days. At day 21, the amount of mineralized nodules were measured by Alizarin Red staining (Solabio, China).

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

PDLSCs were cultured as described above. At day 7 and 14, osteogenic genes including Runx2, ALP and Osx were analyzed by qRT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, USA). Total RNA (1000 ng) was reverse transcribed into cDNA using the Prime Script™ RT reagent Kit with gDNA Eraser (Takara, Japan). Quantitative real-time PCR reactions were performed using the SYBR Premix Ex Taq II kit (Takara, Japan) on a BIO-RAD CFX Manager software program Connect PCR detection system. TNF-α and IL-6 mRNA expression were measured after 24 h of LPS treatment (10 μg/ml) in the presence or absence of Ex-4 (10 nmol/l) and normalized to β-actin. Primers used in the study are listed in Table 1.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded into 12-well plates (1×10^5 cells/well) and treated with LPS (10 μg/ml) or Ex-4 (10 nmol/l) in triplicate for 24 h. Cells in the control group were cultured in media alone (N1). According to the manufacturer's protocol, soluble TNF-α and IL-6 in culture supernatants were measured with Human Tumor necrosis factor α (TNF-α) ELISA kits and Human Interleukin 6 (IL-6) ELISA kits (mlbio, China) respectively. Absorbances were read at 450 nm. The concentrations of TNF-α and IL-6 were determined by comparison of the OD values to standard curves.

2.7. Western blot analysis

Nuclear and cytoplasmic proteins were separated using commercially available Nuclear Extraction Kit (Millipore, USA) according to the manufacturer's protocol. Protein concentrations in the cell lysates were measured *via* BCA assay (Beyotime, China). Samples were mixed in a 4:1 ratio with 5 × loading buffer and boiled for 15 min. Proteins (50 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes

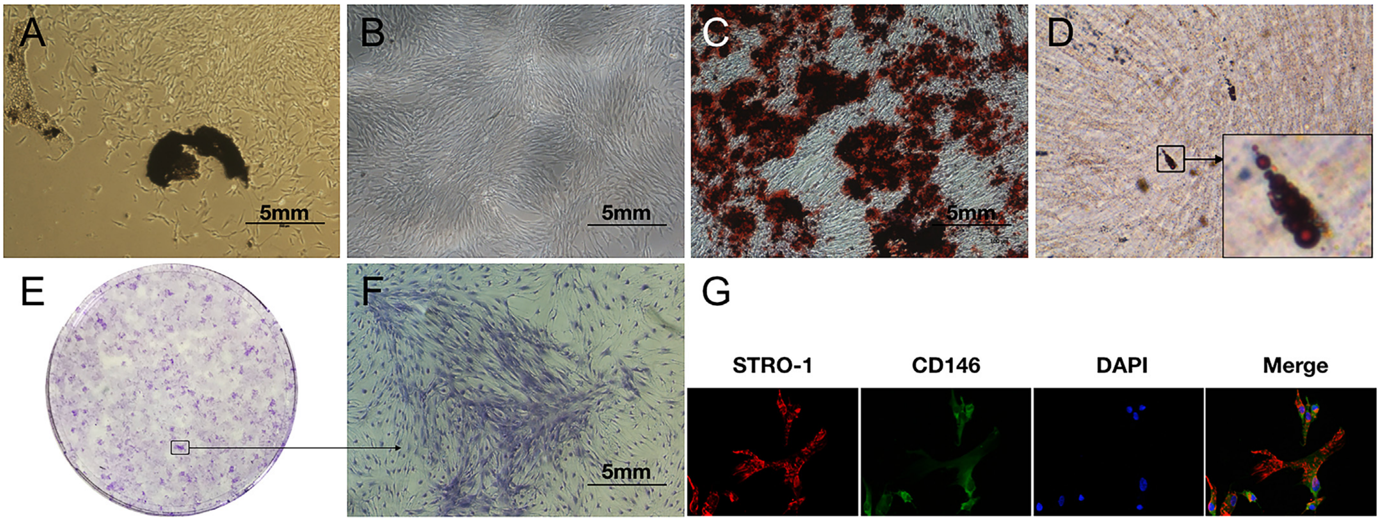

Fig. 1. PDLSCs morphology and characterization. (A) Primary cells typically grow around the tissue mass and multiply. (B) Morphology of PDLSCs. (C) Following osteogenic induction, PDLSCs formed a mineralized extracellular matrix as assessed by Alizarin Red staining. (D) Following adipogenic induction, PDLSCs formed lipid droplets as assessed by Oil Red O staining. (E–F) Colonies formed by PDLSCs were stained with crystal violet after 14 days of culture. (G) Cells expression STRO-1 (red) and CD146 (green). Scale bar: A, B, C, D, F = 5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Specific primer sequences used for real time-PCR. Genes associated with osteogenesis were detected as follows: Runx2 (runt-related transcription factor 2); ALP (alkaline phosphatase); Osx (osterix). Genes associated with inflammation were detected as follows: TNF-α (tumor necrosis factor α); IL-6 (interleukin 6). Values were normalized to β-actin.

Specific primer sequences used for real time-PCR.

Primers	Sequences
Runx2	Forward (5′–3′): GTCTCACTGCCTCTCACTTG
	Reverse (5′–3′): CACACATCTCCTCCCTTCTG
ALP	Forward (5′–3′): CCATACAGGATGGCAGTGAAGG
	Reverse (5′–3′): TTGACCTCCTCGGAAGACACTC
Osx	Forward (5′–3′): TGAGGAGGAAGTTCACTATGG
	Reverse (5′–3′): TTCTTTGTGCCTGCTTTGC
TNF-α	Forward (5′–3′): AGCCCATGTTGTAGCAAACC
	Reverse (5′–3′): CTTGAAGAGGACCTGGGAGT
IL-6	Forward (5′–3′): AGCCCACCGGGAACGA
	Reverse (5′–3′): GGACCGAAGGCGCTTGT
β-Actin	Forward (5′–3′): CGCGAGAAGATGACCCAGAT
	Reverse (5′–3′): GAGGCGTACAGGGATAGCAC

were blocked in 5% skimmed milk in Tris-buffered saline and 0.2% Tween for 1 h at room temperature. Membranes were probed overnight at 4 °C with primary antibodies for: β-actin, GAPDH, TNF-α, IL-6, IκBα, phospho-IκBα (p- IκBα), β-catenin, GSK-3β, phospho-GSK-3β (p-GSK-3β) (Servicebio, China) and labeled with HRP-labeled Goat Anti-Rabbit IgG (H + L) (Servicebio, China) for 1 h at room temperature. Proteins were detected using Hypersensitive ECL Chemiluminescence kits (Beyotime, China) according to the manufacturer's protocol. Band intensities were quantified using ImageJ.

2.8. Immunofluorescent staining

PDLSCs at passage 3–5 were seeded into 6-well plates containing coverslips (2×10^5 cells/well) and treated with LPS (10 μg/ml) ± Ex-4 (10 nmol/l) for 24 h. Cells in the control group were cultured in media alone (N_1). Following treatment, cells were fixed in 4% paraformaldehyde (PFA) for 30 min and washed with PBS at least 3 times. Cells were permeabilized in 0.5% Triton X-100 for 20 min at room temperature and washed with PBS 3 times. Cells were blocked in 1% BSA for 30 min at room temperature, labeled with anti-NF-κB/p65, antibodies (Servicebio, China) overnight at 4 °C, and stained with Cy3 conjugated Goat Anti-rabbit IgG (H + L) (Servicebio, China) at room temperature for 2 h. Nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 5 min. Coverslips were mounted onto slides and imaged on a Leica fluorescence microscope.

2.9. Statistical analysis

Prism software (version 7.0) was used to perform statistical analyses. A Student's *t*-test was used to analyze statistical significance between the groups. A one-way ANOVA was used to assess statistical significance. A P-value < 0.05 indicated statistical significance. All experiments were performed on a minimum of 3 occasions.

3. Results

3.1. PDLSCs morphology and characterization

Primary cells were obtained and proliferated around the tissue mass (Fig. 1A). The PDLSCs had a morphology similar to fusiform or fibroblasts with regularity and directionality (Fig. 1B). PDLSCs were assessed in osteogenic differentiation assays after 21 days of osteogenic induction. Colony-forming assays were performed after 14 days of culture. Alizarin Red staining showed calcified nodules, confirming the ability of PDLSCs to differentiate into osteoblasts (Fig. 1C). Oil Red O staining showed lipid droplet formation (Fig. 1D). The isolated cells showed high colony-forming ability (Fig. 1E–F). The mesenchymal stem cell phenotypic molecular markers STRO-1 and CD146 were expressed on the PDLSCs (Fig. 1G).

3.2. Effects of Ex-4 on the proliferation of PDLSCs induced by LPS

CCK-8 assays were used to investigate the effects of Ex-4 on PDLSCs proliferation in the LPS-induced inflammatory microenvironment. Compared to the control group (N_1), increasing LPS concentrations led to enhanced proliferation rates of the PDLSCs on the third day, which decreased on the fifth day. On day 7, the proliferative capacity of the PDLSCs was significantly reduced (Fig. 2A). We next explored the effects of Ex-4 on the proliferation of PDLSCs in the LPS-induced inflammatory microenvironment. LPS (10 μg/ml) and Ex-4 co-treatment had no significant effects on the proliferative capacity of PDLSCs

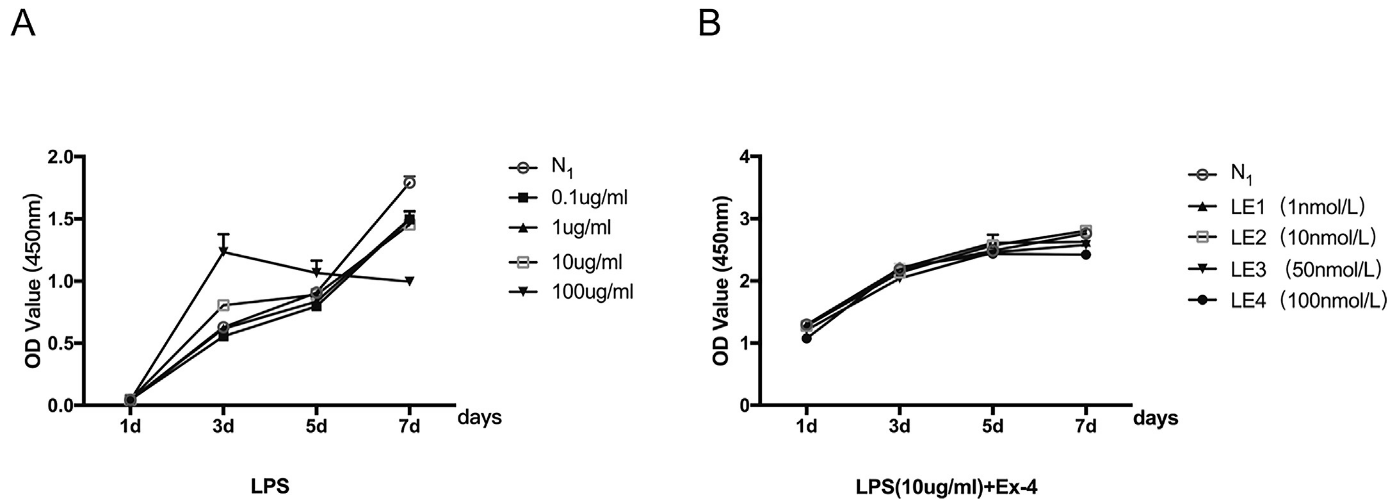

Fig. 2. Effects of Ex-4 on the proliferation of PDLSCs induced by LPS. (A) Growth curves of PDLSCs following LPS (0.1–100 μg/ml) intervention from days 1 to 7. (B) Growth curves of PDLSCs after LPS (10 μg/ml) and Ex-4 (1–100 nmol/l) treated from days 1 to 7. Data are expressed as the mean (SD) for triplicate samples from experiments.

compared to the control group (N_1) (Fig. 2B).

3.3. Effects of Ex-4 on the osteogenic differentiation of PDLSCs induced by LPS

To investigate the effects of Ex-4 on the osteogenic differentiation of PDLSCs in an inflammatory microenvironment, we cultured PDLSCs in osteogenic induction medium and treated them with LPS (10 μg/ml) or Ex-4 (10 nmol/l) for 21 days. Alizarin Red staining showed that Ex-4 treatment stimulated bone formation from the PDLSCs in a time-dependent manner. The formation of mineralized nodules strongly decreased in the LPS treatment group compared to the control group (N_2). Following Ex-4 treatment, mineralized nodules of the PDLSCs significantly increased in the presence of LPS, particularly at days 7, 14 and 21 (Fig. 3A–B). Quantitative real time PCR (qRT-PCR) showed that the expression of genes related to osteogenic differentiation (including Runx2, ALP and Osx) significantly increased in the Ex-4 treatment groups at day 14 compared to the control group (N_2). LPS treatment decreased the expression of the osteogenic genes that were recovered by Ex-4 co-treatment (Fig. 3C).

3.4. Effects of Ex-4 on NF-κB signaling in PDLSCs induced by LPS

Western blot analysis showed that both IL-6 and TNF-α expression significantly increased after 24 h of LPS (10 μg/ml) induction compared to N_1. However, the expression of IL-6 and TNF-α significantly decreased following LPS (10 μg/ml) and Ex-4 (10 nmol/l) treatment compared to LPS alone (Fig. 4A). qRT-PCR and ELISA analysis showed comparable results (Fig. 4B–C). To explore the anti-inflammatory mechanism(s) of Ex-4 on LPS-stimulated PDLSCs, we investigated the expression of IκBα and p-IκBα after 24 h of treatment. As shown in Fig. 5A, IκBα expression increased after 24 h of LPS induction. In contrast, IκBα accumulation was significantly lower in the Ex-4 treatment groups. LPS significantly induced IκBα phosphorylation that was inhibited by Ex-4. In addition, immunofluorescent analysis revealed that the NF-κB/p65 complex localized to the cytoplasm of untreated PDLSCs, but translocated to the nucleus after 24 h of LPS induction. Ex-4 treatment partially blocked this translocation (Fig. 5B).

3.5. Effects of Ex-4 on Wnt signaling in PDLSCs induced by LPS

One of the major signal transduction pathways involved in stem cell osteogenic differentiation is the Wnt signaling pathway. To investigate the effects of Ex-4 (10 nmol/l) on Wnt signaling, we assessed β-catenin expression and distribution after 7 days of LPS (10 μg/ml) treatment. The cytoplasm and nuclear fractions of PDLSCs were extracted and β-catenin expression in both fractions increased following Ex-4 treatment. In the LPS group, β-catenin levels were significantly higher in the nuclear fraction, with decreased cytoplasmic expression. Interestingly, co-treatment of Ex-4 with LPS significantly decreased β-catenin expression in the nuclear fraction and increased its cytoplasmic expression (Fig. 6A). In addition, the phosphorylation of GSK-3β significantly decreased in the Ex-4 treatment group compared to the control group (N_2), whilst the expression of total GSK-3β increased. LPS treatment significantly promoted the phosphorylation of GSK-3β, but reduced total GSK-3β expression. LPS and Ex-4 co-treatment increased total GSK-3β expression and reduced p-GSK-3β levels compared to the LPS group (Fig. 6B). We also observed increased Runx2 expression in the Ex-4 treatment group and reduced Runx2 expression in the LPS treatment group compared to the control group(N_2). LPS and Ex-4 co-treatment led to significantly increased Runx2 expression levels compared to LPS treatment alone (Fig. 6C).

4. Discussion

To-date, the treatment strategy for CP is to delay inflammatory damage, but the lost bone cannot be regenerated. Regenerating the periodontal tissue is the ultimate goal of CP treatment. PDLSCs can differentiate into osteoblasts and play a dynamic role in periodontal homeostasis [29]. For these reasons, PDLSCs are important target cells for the reconstruction of periodontal tissue. Ex-4 is a peptide analogue of GLP-1 that possesses cytoprotective effects. Ex-4 displays similar efficacy to GLP-1. However, dipeptidyl peptidase-IV (DPP-4) rapidly degrades the active form of GLP-1 *in vivo*. Ex-4 is resistant to this degradation and has a longer plasma half-life [30]. The long duration of Ex-4 activity *in vivo* combined with its high potency makes it a potential pharmacological candidate for CP [31]. Recent studies showed that Ex-4 exerts rapid anti-inflammatory effects at both the molecular and cellular level. However, it remains unclear whether Ex-4 promotes the osteogenic differentiation of PDLSCs in the inflammatory microenvironment.

The inflammatory microenvironment promotes cellular proliferation and suppresses the differentiation potential of PDLSCs [32]. LPS can reduce the osteogenic potential of PDLSCs through NF-κB activation, which can be partially reversed by NF-κB inhibitors *in vitro* [33]. Our results showed that Ex-4 controls the abnormal proliferation of

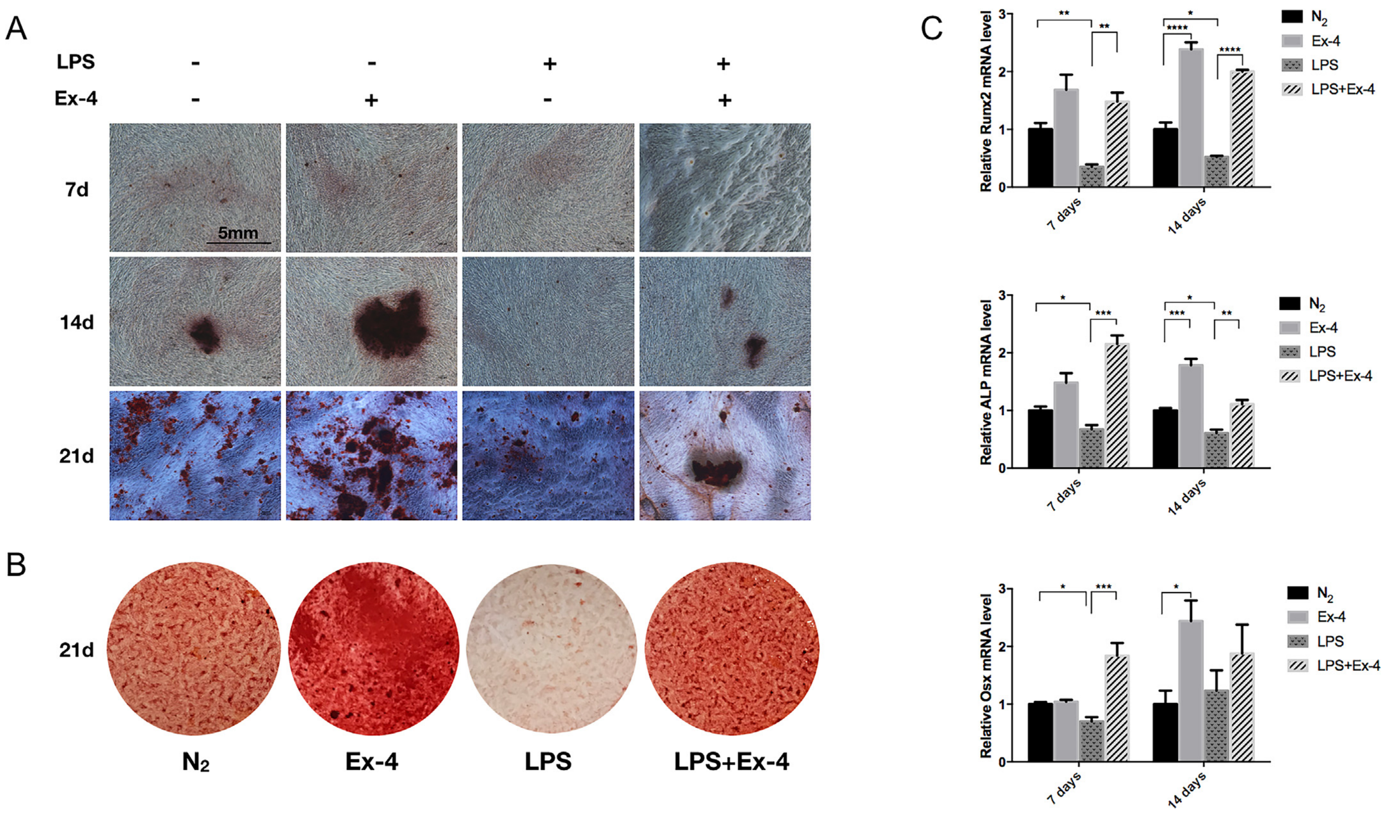

Fig. 3. Effects of Ex-4 on the osteogenic differentiation of PDLSCs induced by LPS. (A) Alizarin Red staining was performed on days 7, 14, and 21 to detect mineralized nodules. Nodules (40 ×) were visualized by a light microscopy. Scale bars = 5 mm. (B) Macroscopic images obtained by Alizarin Red staining 21 days after osteogenic induction of PDLSCs. (C) The expression of Runx2, ALP and Osx were detected by quantitative RT-PCR at 7 and 14 days after osteogenic induction. Expression levels were normalized to β-actin. Data are expressed as mean ± SEM from three independent experiments performed in triplicate. (*P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001. ****P ≤ 0.0001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

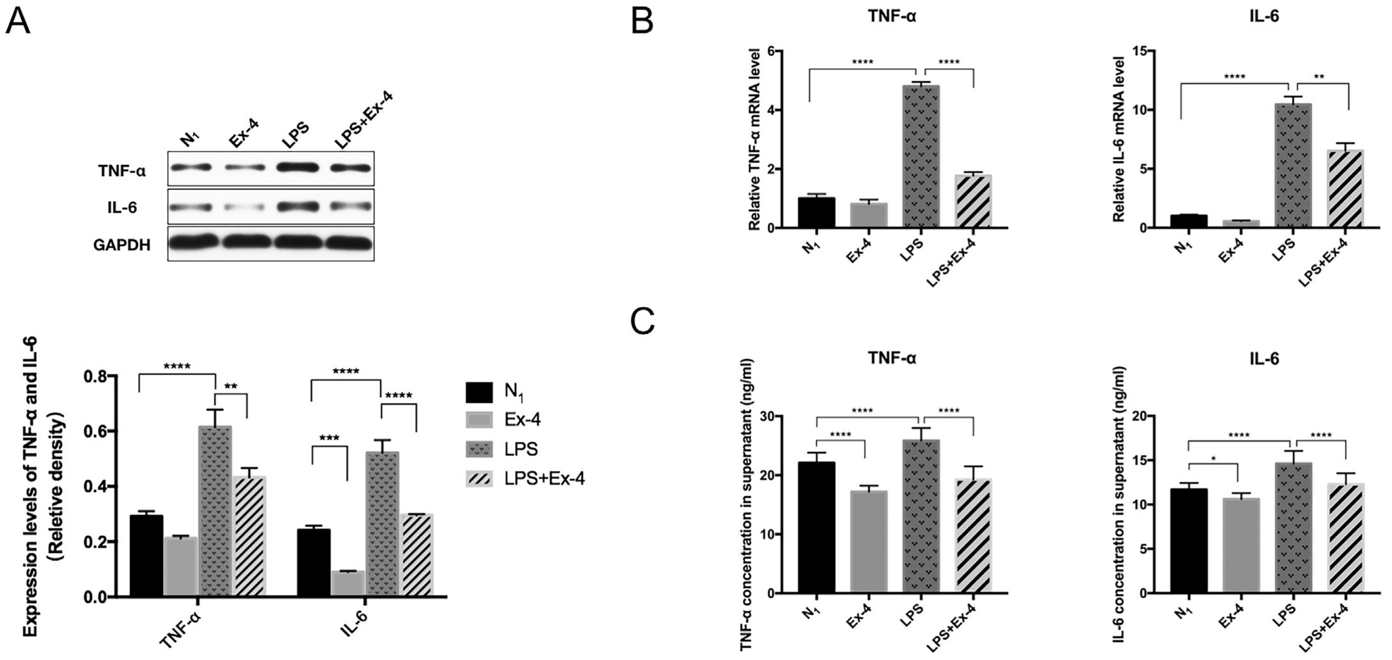

Fig. 4. Effects of Ex-4 on TNF-α and IL-6 expression of PDLSCs induced by LPS. (A) Western blots of TNF-α and IL-6 following LPS (10 μg/ml) or Ex-4 (10 nmol/l) treatment for 24 h. Expression levels were normalized to GAPDH. (B) Expression of TNF-α and IL-6 were analyzed by quantitative RT-PCR. (C) Expression of the soluble TNF-α and IL-6 in the medium supernatant were analyzed by ELISA. (*P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001. ****P ≤ 0.0001).

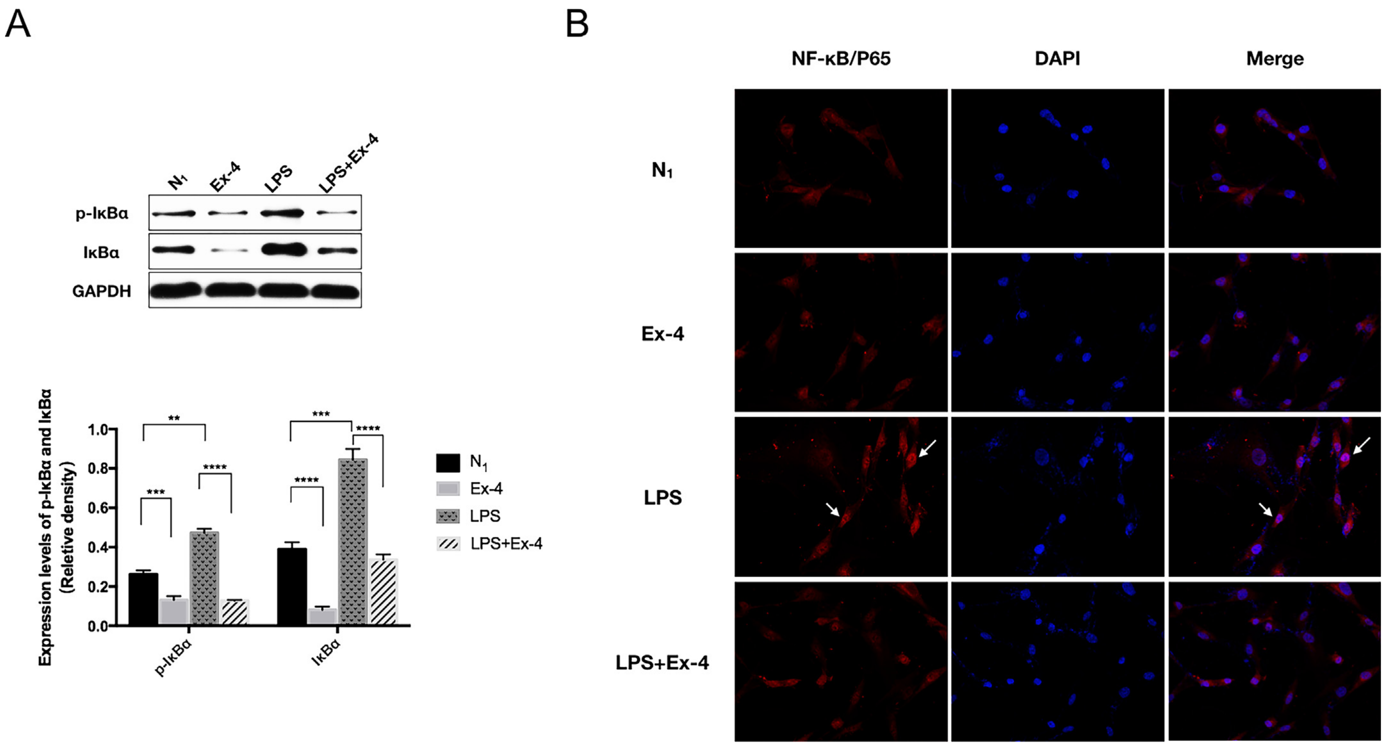

Fig. 5. Effects of Ex-4 on NF-κB signaling in PDLSCs induced by LPS. (A) Western blots showing the expression of IκBα and p-IκBα after treatment with LPS (10 μg/ml) or Ex-4 (10 nmol/l) for 24 h. Expression levels were normalized to GAPDH. (B) Localization of the NF-κB/p65 complex assessed through immunofluorescence microscopy (40 × magnification). NF-κB/p65 (red) accumulated in the nucleus following LPS (10 μg/ml) treatment. Ex-4 (10 nmol/l) treatment inhibited NF-κB/p65 accumulation in the nucleus. Arrows indicate nuclear localization. (*P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001. ****P ≤ 0.0001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PDLSCs caused by inflammation through alleviating the LPS mediated inhibition of PDLSCs.

An array of inflammatory factors promote bone absorption. Baker and coworkers [34] found that alveolar bone absorption in mice was significantly increased by IL-6, with the degree of IL-6 expression controlling lesion development and bone absorption. TNF-α also plays an important role in the development of periodontitis through its ability to increase the formation and activity of osteoclasts, and promote the absorption of alveolar bone. In this study, RT-PCR and western blot analysis revealed that Ex-4 treatment reduced the expression of TNF-α and IL-6, suggesting it has anti-inflammatory effects on PDLSCs in the LPS-inducted inflammatory microenvironment. This begged the question as to the nature of the Ex-4 anti-inflammatory activity. NF-κB is a transcription factor that regulates tissue immunity and function. NF-κB regulated genes include cytokines such as IL-6 and TNF-α [35]. Chang and coworkers [36] identified NF-κB as key factor during the inhibition of bone formation. IκBα is a direct target of NF-κB and is excessively phosphorylated in LPS treated PDLSCs, which in turn triggers the downstream translocation of p65 into the nucleus and the loss of osteogenesis. We found that the levels of IκBα and p-IκBα significantly increased following LPS treatment, which was inhibited by Ex-4. Immunofluorescence analysis showed that Ex-4 inhibited the nuclear translocation of the NF-κB/p65 complex, preventing its transcriptional activity, and reducing the LPS-induced phosphorylation of IκBα. Collectively, these data suggest that Ex-4 is inhibitory to NF-κB signaling.

Wnt signaling promotes osteogenic differentiation through a number of mechanisms that are dependent on the differentiation status of stem cells. GSK-3β is a key component of the Wnt canonical signaling pathway [37] which when phosphorylated, regulates the ubiquitination and degradation of β-catenin [38]. Runx2 is a transcription factor necessary for osteogenic differentiation and plays an important role in the formation and reconstruction of bone tissue. Runx2 is also a direct target for canonical Wnt signaling [39,40]. Wnt activation inhibits the degradation of β-catenin through GSK-3β phosphorylation, regulating the ability of stem cells to differentiate into bone [39,41,42]. Conversely, it has been suggested that Wnt signaling inhibits the osteogenic potential of hMSCs in lentiviral Wnt1 overexpression studies [43] [44]. The However, the absorption of alveolar bone during the course of periodontal disease is a complex process. Various inflammatory factors contribute to bone injury and regeneration in CP. Our results show that the inflammatory microenvironment inhibits the osteogenic differentiation of PDLSCs and increases β-catenin expression in the nucleus, consistent with previous studies [45]. The accumulation of β-catenin in the nucleus of PDLSCs under inflammatory conditions inhibits osteogenic differentiation. β-catenin contributes to canonical Wnt/β-catenin signaling and non-canonical Wnt/Ca^{2+} signaling, regulating the proliferation and osteogenic differentiation of PDLSCs under inflammatory conditions [6]. β-catenin expression in the cytoplasm and nucleus increased after Ex-4 treatment. We therefore assessed GSK-3β activity and found that Ex-4 partially reduced its phosphorylation in both normal and inflammatory microenvironments. We also found that LPS treatment significantly reduced Runx2 expression, whilst Ex-4 promoted Runx2 expression in both normal and inflammatory microenvironments, promoting PDLSCs osteogenic differentiation in the inflammatory microenvironment. Taken together, these data suggest that Ex-4 directly promotes osteogenesis through inhibiting GSK-3β phosphorylation and activating Wnt/β-catenin signaling, ultimately enhancing Runx2 expression. Given the effects of Ex-4 on both cytoplasmic and nuclear β-catenin expression, we speculate that Ex-4 also regulates the non-canonical Wnt/Ca^{2+} signaling pathway. The osteogenic differentiation of PDLSCs is complex, involving a large number of transcription factors, signaling molecules and signaling pathways. This study dissected only a subset of this network in the inflammatory state, and a complete analysis of the mechanisms of Ex-4 mediated osteogenic differentiation of PDLSCs requires further investigation.

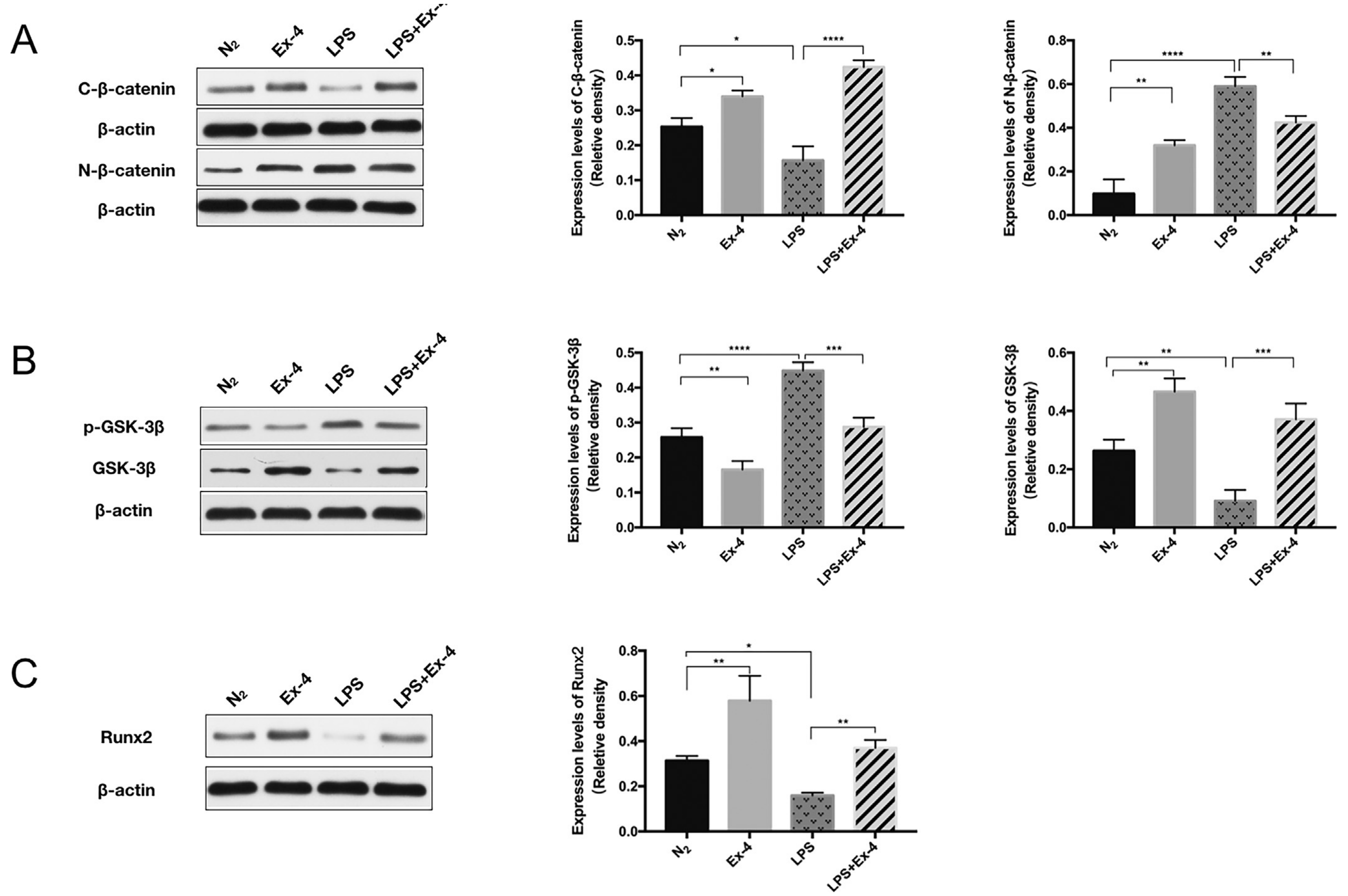

Fig. 6. Effects of Ex-4 on Wnt signaling in PDLSCs induced by LPS. (A) Cytoplasmic (C-β-catenin) and nuclear (N-β-catenin) β-catenin expression were assessed following the growth of PDLSCs in osteogenic medium containing LPS (10 μg/ml) or Ex-4 (10 nmol/l) for 7 days. (B) GSK-3β and p-GSK-3β levels were examined by western blot analysis. (C) Runx2 levels were examined by western blot analysis and normalized to β-actin. (*P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001. ****P ≤ 0.0001).

5. Conclusions

We show that LPS promotes PDLSCs proliferation and inhibits their osteogenic differentiation capacity. At the appropriate concentrations, Ex-4 alleviated the abnormal proliferation of PDLSCs in the inflammatory microenvironment, promoting osteogenic differentiation through regulating NF-κB and Wnt signaling. We thus conclude that Ex-4 has positive effects on periodontal tissue regeneration and remodeling under inflammatory conditions.

Funding

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Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

Declaration of competing interest

The authors declare no conflicts of interest related to this study.

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