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ORIGINAL RESEARCH

Salidroside Improves Periodontitis by Mitigating Inflammatory Reactions and Enhancing Osteogenic Differentiation of Human Periodontal Ligament Stem Cells

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Purpose: Salidroside (Sal), a significant bioactive compound found in *Rhodiola rosea*, is documented to possess various pharmacological properties. This study investigated the effects of Sal in alleviating periodontitis.

Methods: The rat periodontitis model was utilized to assess the therapeutic impact of Sal on periodontitis. Human periodontal ligament stem cells (hPDLSCs) were used to investigate the effect of Sal on lipopolysaccharide (LPS)-inhibited osteogenic differentiation. RNA sequencing (RNA-seq), and Western blot were employed to analyze the genes and proteins impacted by Sal treatment.

Results: Sal significantly alleviated the alveolar bone loss and gingival inflammation in rats periodontitis model. Sal demonstrated a dose-dependent pattern of promoting osteogenesis on hPDLSCs. A concentration of 0.5 μM Sal could effectively counteract the impact of LPS on osteogenic differentiation. Mechanically, Sal inhibited the ratios of phospho-IκBα(p-IκBα)/IκBα and phospho-p65 (p-p65)/p65 in Nuclear Factor kappa-B (NF-κB) pathway and reduced the expressions of interleukin-6 (IL-6) and interleukin-8 (IL-8). Sal increased the expression of lymphoid enhancer-binding factor 1 (LEF1).

Conclusion: Sal promoted the osteogenic differentiation by inhibiting the activation of the NF-κB pathway and increasing the expression of LEF1.

Keywords: periodontitis, salidroside, osteogenesis, the human periodontal ligament stem cells

Introduction

Periodontitis is a chronic inflammatory disease of periodontal tissues, with clinical characteristics including red, swollen and bleeding gingiva, deep periodontal pocket, loose teeth and oral malodor.[1] Epidemiological investigations show that periodontitis was the sixth most common human disease, with severe periodontitis affecting 11.2% of the world's population.[2] Its late stage is characterized by the loss of periodontal ligament and destruction of surrounding alveolar bone.[3] Moreover, periodontitis is associated with multiple systemic diseases, such as diabetes,[4] cardiovascular diseases,[5] Alzheimer's disease,[6] and various types of cancer.[7] Therefore, preventing periodontitis is pressing challenges that we must address.

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The initiation of periodontitis originates from the local colonization of periodontal pathogens in periodontal pocket and the release of virulence factors, which is the main prerequisite of sub-gingival inflammatory response.[8] Among them, LPS is a cell wall component of Gram-negative bacteria, widely existing in periodontitis-causing pathogens such as *Porphyromonas gingivalis (P. gingivalis)*, and is one of the main toxic factors inducing periodontal inflammatory reactions.[9] Studies have shown that LPS can be specifically recognized by host cells, triggering the host immune response to secrete a large number of proinflammatory cytokines, including IL-6, IL-8, and tumor necrosis factor-α (TNF-α).[10] Simultaneously, the homeostasis between bone formation and bone resorption is disrupted by the over expression of inflammatory factors, with osteoclast activity activated, while osteoblast function inhibited, leading to alveolar bone resorption.[11,12]

Sal, the primary glycoside belonging to the phenylpropanoid tyrosol family, is isolated from *Rhodiola rosea* and exhibits various pharmacological activities, including anti-inflammatory,[13] antioxidant, antifatigue, antitumor,[14] antidiabetic,[15] improving osteoporosis,[16] and protecting cardiovascular and central nervous systems.[17,18] The anti-inflammatory activity of Sal has been confirmed to be beneficial in treating multiple diseases, such as arthritis,[19] colitis,[20] and vascular inflammation.[21] Compared to conventional clinical anti-inflammatory agents, Sal may exhibit relatively moderate anti-inflammatory effects. However, it demonstrates superior systemic regulation and tissue-protective properties, offering distinct advantages for long-term therapeutic applications.[13] Simultaneously, Sal can effectively improve postmenopausal osteoporosis in rats,[16,22] and promote the proliferation and osteogenic differentiation of osteoblasts[23,24] and human dental pulp stem cells.[25] However, to date, there has been few research into the effect of Sal in periodontitis. This study aims to reveal the performance of Sal in periodontitis treatment, as well as to explore the underlying mechanisms.

Materials and Methods

Construction of Rat Periodontitis Models

The required experimental animal size was calculated according to the resource equation method. Detailed methodology can be found in Supplementary Material 1. All experiments involving the rats adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Shandong University (No. 20221146). Twenty-one male Wistar rats (Vital River, Beijing, China) free of specific pathogens were selected for the experiment and housed in Specific-Pathogen-free (SPF) facilities. After a one-week acclimation period, the rats were anesthetized by an intraperitoneal (IP) administration of pentobarbital sodium (40 mg/kg) and 0.2 mm stainless steel ligatures were inserted around the first molar of right maxillary to induce periodontitis. The ligation process lasted for 5 to 10 minutes. The rats received local gingival injections of Phosphate Buffered Saline (PBS) or 25 mg/kg of Sal every other day for 14 consecutive days. Rats were randomly divided into the following three groups with 7 rats in each group. Rats in Control group were injected with PBS without orthodontic wire. Rats in periodontitis (PD) group were injected with PBS with periodontitis as described above. Rats in PD+Sal group were injected with Sal with periodontitis as described above.

Micro-Comupted Tomography (Micro-CT) Analysis

The rat's right maxillas were scanned using micro-CT (Quantum GX2, PerkinElmer, USA) with the following settings: voltage of 90 kV, current of 88 μa, and scan thickness of 36 μm. The image information was imported into Mimics Research 21.0 software. To assess alveolar bone resorption around the first molar, the linear distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured, as well as the bone volume/tissue volume (BV/TV) fraction.

Hematoxylin and Eosin (H&E) Staining, Masson Staining, and Immunohistochemistry (IHC) Staining Assay

The rat maxillary bone samples were decalcified in 10% ethylene diamine tetraacetic acid (EDTA) solution for 3 months. Following gradient dehydration and transparency, the samples were embedded in paraffin and sectioned into 5 μm-thick continuous sagittal slices. H&E staining kits and Masson staining kits (Servicebio, Hubei, China) were employed to stain the paraffin sections, allowing for the observation of morphological changes in periodontal tissues surrounding the first molar. For IHC staining, the samples were first treated with hydrogen peroxide to block endogenous peroxidase activity,

then incubated with primary antibodies including type I collagen (COL1, Proteintech, 67288-1-Ig, 1:2500), TNF-α (Proteintech, 26405-1-AP, 1:200) overnight at 4°C. Subsequently, secondary antibodies were added, and immune reactions were detected using a Diaminobenzidine (DAB) detection kit (Zhongshan Goldenbridge Biotechnology, Beijing, China). All sections were counterstained with hematoxylin.

Cultivation and Identification of hPDLSCs

This research was undertaken in compliance with the Declaration of Helsinki and received approval from the School of Stomatology Ethics Committee at Shandong University (No. 20221005). The main focus was on collecting the premolars or third molars that were extracted due to orthodontic needs. Inclusion criteria: (1) Under 18 years old; (2) Healthy dental and periodontal tissues. Exclusion criteria: (1) Dental caries; (2) Periodontitis; (3) Have a history of bisphosphonate medication; (4) Have systemic diseases related to bone metabolism. Informed consent was obtained from each participant for all surgeries. Informed consent was obtained from each participant for all surgeries. The periodontal ligament was scraped from the middle third of the root surface of the extracted teeth, cut into 1.0 mm^3 fragments using a sterile scalpel, and cultured in α-MEM (Hyclone, Logan, UT, USA) containing 20% FBS (Biological Industries, Israel). The fragments were then incubated under conditions of 37°C and 5% CO_2. To characterize the stem cell properties of hPDLSCs, cells were co-incubated with antibodies (CD34, CD44, CD45, and CD105, Elabscience, USA), and data was analyzed using a flow cytometer (Beckman Coulter, USA) and FlowJo® software. hPDLSCs at passages 3–5 were used for all experiments to ensure consistent differentiation potential.

Oil Red O Staining Assay

To induce adipogenesis, hPDLSCs were co-cultured with adipogenic induction medium (α-MEM containing 10% FBS, 10 mM insulin, 0.5 M hydrocortisone, 500 mM isobutylmethylxanthine, and 60 mM indomethacin). After 28 days, lipid droplets were stained with oil red O staining solution (Beyotime, Shanghai, China) at room temperature.

Alizarin Red Staining and Calcium Quantitative Assay

To induce osteogenesis, hPDLSCs were co-cultured with osteogenic induction medium (α-MEM containing 10% FBS, 0.01μM dexamethasone, 10mM β-glycerophosphate, and 50 μg/mL ascorbic acid). After 28 days, the calcium nodules were stained with Alizarin red staining solution (OriCell, Guangzhou, China). Then, 10% CHClN (cetylpyridinium chloride, Beyotime, Shanghai, China) was added to the stained wells, and the absorbance was recorded at a wavelength of 562 nm using a microplate reader (Thermo Fisher Scientific, USA).

Cell-Counting Kit-8 (CCK-8) Assay

To investigate the effect of Sal (Macklin, 10338–51-9, purity≥98%) on the proliferation activity of hPDLSCs, hPDLSCs were co-cultured with 10% FBS α-MEM containing Sal (0, 0.1, 0.5, 1, 5, and 10 μM, Solarbio, Beijing, China). At day 1, 3, and 5, the culture medium was replaced with 10% CCK-8 reagent (Beyotime, Shanghai, China), and the absorbance was recorded using a microplate reader at 450 nm.

Alkaline Phosphatase (ALP) Staining and ALP Activity Assay

hPDLSCs were co-cultured with osteogenic induction medium containing Sal and/or 1μg/mL LPS (from *P. gingivalis*, Solarbio, L8810, Beijing, China). The negative control group consisted of hPDLSCs cultured in osteogenic induction medium without LPS and Sal. ALP activity of each sample was measured using an ALP activity assay kit (Jiancheng, Nanjing, China), and absorbance was recorded at 520 nm. ALP activity was normalized to protein concentration. Similarly, hPDLSCs were cultured and subjected to ALP staining using an ALP staining kit (Beyotime, Shanghai, China).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from tissues or cells using the Trizol method, and RNA was reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (Yeasen, Shanghai, China). Each reaction system

consisted of three replicate wells. After design analysis, the primer sequences for this experiment were presented in Table 1. The obtained data was analyzed using the $2^{-\Delta\Delta Ct}$ method to relatively quantify gene expression.

Western Blot Assay

The protein concentration of samples was determined using a bicinchoninic acid kit (BCA, Solarbio, Beijing, China). The protein samples were separated using SDS-PAGE (SparkJade, Shandong, China) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany). Blocking was performed using rapid blocking solution (Servicebio, Hubei, China) at room temperature, followed by overnight incubation at 4°C with primary antibodies including GAPDH (Proteintech,10494-1-AP,1:8000), ALP (Proteintech,11187-1-AP,1:2000), Runt-associated transcription factor 2 (RUNX2, Proteintech, 20700-1-AP, 1:1000), COL1 (Proteintech, 67288-1-Ig, 1:6000), IL-6 (abcam,ab233706,1:1000), LEF1 (Abways, CY5350,1:1000), p65 (Cell Signaling Technology, 8242S,1:1000), p-p65 (Cell Signaling Technology, 3033S,1:1000), IκB-α (Cell Signaling Technology, 4814S,1:1000), p-IκB-α (Cell Signaling Technology, 9246S,1:1000). The samples were then incubated with secondary antibodies including HRP-conjugated Goat Anti-Rabbit IgG(H+L) (Proteintech, SA-00001-2, 1:10000) and HRP-conjugated Goat Anti-Mouse IgG(H+L) (Proteintech, SA-00001-1, 1:10000) for 1 hour at room temperature. The bands were detected using an ultrasensitive luminescent reagent kit (Biosharp, Beijing, China).

Immunofluorescence (IF) Staining Assay

The hPDLSCs were seeded onto a confocal dish and fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100 (Solarbio, Beijing, China) and treatment with 5% bovine serum albumin (BSA, Solarbio, Beijing, China), the samples were incubated overnight with the primary antibody of COL1(*Proteintech, 67288-1-Ig, 1:200*) at 4°C. Then, cells were co-incubated with the fluorescein (FITC)-conjugated affinity pure goat anti-rabbit IgG (H + L) (1:200, SA00013-2), labeled with 4',6-diamidino-2-phenylindole (DAPI, Solarbio, Beijing, China), and observed under a laser confocal microscope (Olympus, Japan).

RNA-Seq Analysis

hPDLSCs were divided into LPS group and LPS+Sal group, and osteogenic induction differentiation was carried out for 48 h. Each group was repeated 3 times, and a total of 6 samples were collected. Total RNA was isolated using the Trizol Reagent (Invitrogen Life Technologies), after which the concentration, quality and integrity were determined using a NanoDrop spectrophotometer (Thermo Scientific). mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. According to the protocol of the MRNA-SEQ sample preparation kit (Illumina, San Diego, USA), the mRNA was segmented and reverse-

Table 1 Specific Primers for Control and Target Genes

Genes	Accession	Forward	Reverse
Homo-*GAPDH*	P04406	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
Homo-*IL6*	P05231	ATAACCACCCCTGACCCAAC	CCCATGCTACATTTGCCGAA
Homo-*IL8*	P10145	TCAGAGACAGCAGAGCACAC	GGCAAATGCACTTTCACACA
Homo-*ALP*	B2R6Q2	GGCGGTGAACGAGAGAATGT	GGACGTAGTTCTGCTCGTGG
Homo-*RUNX2*	Q13950	GGAGTGGACGAGGCAAGAGT	AGGCGGTCAGAGAACAAACT
Homo-*COL1*	P02452	TAAAGGGTCACCGTGGCTTC	GGGAGACCGTTGAGTCCATC
Rat-*Gapdh*	P04797	TCTCTGCTCCTCCCTGTTCT	ATCCGTTCACACCGACCTTC
Rat-*Tnf-α*	P16599	ATGGGCTCCCTCTCATCAGT	GCTTGGTGGTTTGCTACGAC
Rat-*Il-1β*	Q63264	GGGATGATGACGACCTGCTA	ACAGCACGAGGCATTTTTGT

transcribed to create the final cDNA library. The average insert size for the paired-end libraries was 400bp-500bp. Finally, the mixed library was diluted and quantified step by step, and PE150 mode sequencing was performed on an Illumina sequencer. Transcriptome analysis was conducted via R software (version R-4.3.0). PCA analysis was implemented using the R function "prcomp", while differentially expressed gene (DEGs) analysis employed the R package "limma" (version 3.58.1), with screening criteria set at $p < 0.01$ and $|logFoldChange| > 0.585$. Enrichment analysis was accomplished through the R package "clusterProfiler" (version 4.10.1).

To gain deeper insights into the biological functions and pathways associated with differentially expressed genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using the "compareCluster" function from the "clusterProfiler" package (version 4.10.1) in R. The analyses were conducted based on the annotation database "org.Hs.eg.db". GO enrichment included three categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). To eliminate redundancy among enriched terms, the "simplify" function was applied. GO and KEGG enrichment analyses were conducted separately for all DEGs, as well as for the upregulated and downregulated gene subsets. For Gene Set Enrichment Analysis (GSEA), all expressed genes were ranked based on the log2 fold change obtained from differential expression analysis using the "limma" package, and enrichment was performed using the "gseGO" or "gseKEGG" functions from the "clusterProfiler" package. The enrichment analysis results are provided in Supplementary Table 1–6.

Small Interfering RNA (siRNA) Assay

After discarding the old medium, the cells were gently washed with PBS and then fresh culture medium was added. Lipofectamine™ 2000 Transfection Reagent was mixed with 500 μL of Opti-MEM (GenePharma, Suzhou, China) and allowed to stand at room temperature for 5 minutes. Small interfering RNA targeting *LEF1* (*siLEF1*) was added to 500 μL of Opti-MEM, mixed, and then combined with the aforementioned solution. The mixture was incubated at room temperature for 20 minutes before being added to the cells. The small interfering sequences used in this study are presented in Table 2.

Enzyme Linked Immunosorbent Assay (ELISA)

According to the same method as above, hPDLSCs were co-cultured with LPS and / or Sal, and the cell supernatant was taken. The levels of inflammatory cytokines IL-6 and IL-8 in the supernatant of each group were detected according to the ELISA kit instructions (Biolegend, USA).

Statistical Analysis

Each experiment was independently repeated at least five times. The results were presented as mean ± standard deviation (SD) of all data obtained from five independent experiments. For the purpose of statistical analysis, one-way or two-way analysis of variance (ANOVA) was conducted using GraphPad Prism software (version 8, MacKiev Software, Boston, MA, USA). The Tukey's test was used for post hoc analysis. Statistical significance was considered at the level of $P < 0.05$.

Table 2 List of Forward and Reverse Primers

Genes	Forward	Reverse
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
GAPDH control	UGACCUCAACUACAUGGUUTT	AACCAUGUAGUUGAGGUCATT
siLEF1 1	CCCGAGAACAUCAAAUAAATT	UUUAUUUGAUGUUCUCGGGTT
siLEF1 2	GUUGCUGAGUGUACUCUAATT	UUAGAGUACACUCAGCAACTT
siLEF1 3	CUCAGCAUGAACAGAGAAATT	UUUCUCUGUUCAUGCUGAGTT
siLEF1 4	CAGAUGUCAACUCCAAACATT	UGUUUGGAGUUGACAUCUGTT

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Results

Sal Alleviated Periodontal Inflammation and Alveolar Bone Resorption

As shown in Figure 1A, Wistar rats were separated into Control, periodontitis (PD), and PD+Sal groups. After 14 days of modeling process, periodontal bone tissue in each group was assessed using micro-CT and histomorphological analysis as introduced in the Methods. The results showed that the PD group had a significant amount of alveolar bone loss and tooth root exposure in the first molars in comparison to the control group. However, Sal treatment significantly decreased alveolar bone resorption and exposure of tooth roots in the PD+Sal group (Figure 1B), with a notable decrease in the linear distance from the CEJ to ABC (Figure 1C). Microstructural indices of BV/TV also demonstrated that Sal treatment effectively reduced alveolar bone destruction (Figure 1D). H&E and Masson staining showed significant destruction of gingival papillae and recession, along with disorganized and sparse fiber alignment in the periodontal tissues, leading to a decrease in alveolar crest height extending to the mid-root region in the PD group (Figure 1E and F). The rats in the PD+Sal group exhibited a notable improvement in the morphology of their gingival papillae, characterized by an increase in the thickness of the epithelial cell layer within the periodontal tissue, a reduction in the quantity of inflammatory cells, a more organized and dense arrangement of connective fibers, as well as an enhancement in the development of the alveolar bone (Figure 1E and F).

The immunohistochemical staining of the periodontal ligament showed that, in comparison to Control group, the PD group contained a higher number of TNF-α immunoreactive cells and a lower amount of COL1 cells (Figure 1G and H). While rats in PD+Sal group showed observable decrease of TNF-α and increase of COL1 (Figure 1G and H). qRT-PCR assay showed that Sal treatment significantly decreased the expression of TNF-α and interleukin-1β(IL-1β) induced by periodontitis (Figure 1I). These results indicate that Sal can effectively reduce the inflammatory responses in periodontal tissue.

Sal Enhanced the Growth and Osteogenesis of hPDLSCs

We isolated and cultured hPDLSCs from the extracted teeth of healthy volunteers as introduced in the Methods. Flow cytometry analysis showed that hPDLSCs had negative expression of CD34 and CD45 on their surface, but positive expression of CD44 and CD105 (Figure 2A). After 14 days of culture, hPDLSCs crawled out from the surface of the tissue explants and exhibited typical spindle-shaped morphology (Figure 2B). Lipid droplets were visible under a microscope following 28 days of adipogenic induction, while mineralized nodules were also observed after four weeks of osteogenic induction (Figure 2C). These results demonstrate that the hPDLSCs that have been isolated and cultured exhibit traits similar to mesenchymal stem cells and the ability to differentiate into multiple cell types.

The CCK-8 cell viability test showed that Sal did not display any notable toxicity to hPDLSCs at concentrations from 0.1μM to 10μM, while 0.5μM Sal could significantly enhance cell growth on the fifth day (Figure 2D). The results of the MTT assay are also reflected in Supplementary Figure 1, which shows the same outcome. To study whether Sal could promote the osteogenic differentiation of hPDLSCs, the cells were grown in osteogenic induction medium with varying concentrations of Sal (0, 0.1, 0.5, 1, 5, and 10μM). ALP staining and alizarin red staining showed that Sal at concentrations of 0.1 and 0.5μM significantly enhanced the osteogenic differentiation of hPDLSCs, while this effect reduced as the concentration of Sal increase (Figure 2E and F). qRT-PCR and Western blot assay showed that ALP, RUNX2 and COL1 were significantly increased by treatment with Sal at 0.1 and 0.5μM, whereas their expression levels were gradually decreased by Sal at concentrations of 1, 5 and 10μM (Figure 2G and H). These findings indicate that an optimal dose of Sal can enhance the osteogenic differentiation of hPDLSCs.

Sal Reversed LPS-Induced Inhibition of Osteogenic Differentiation of hPDLSCs

To study whether Sal could attenuate the suppression of osteogenesis of hPDLSCs induced by LPS, we divided hPDLSCs into three groups (Control, LPS, and LPS+Sal group) as introduced in the Methods. ALP staining and alizarin red staining showed that LPS could dramatically reduce ALP staining and ALP activity, and decreased calcified nodule formation compared to the Control group (Figure 3A and B), while the hPDLSCs of LPS+Sal group were effectively attenuated the impairment of osteogenesis induced by LPS (Figure 3A and B).

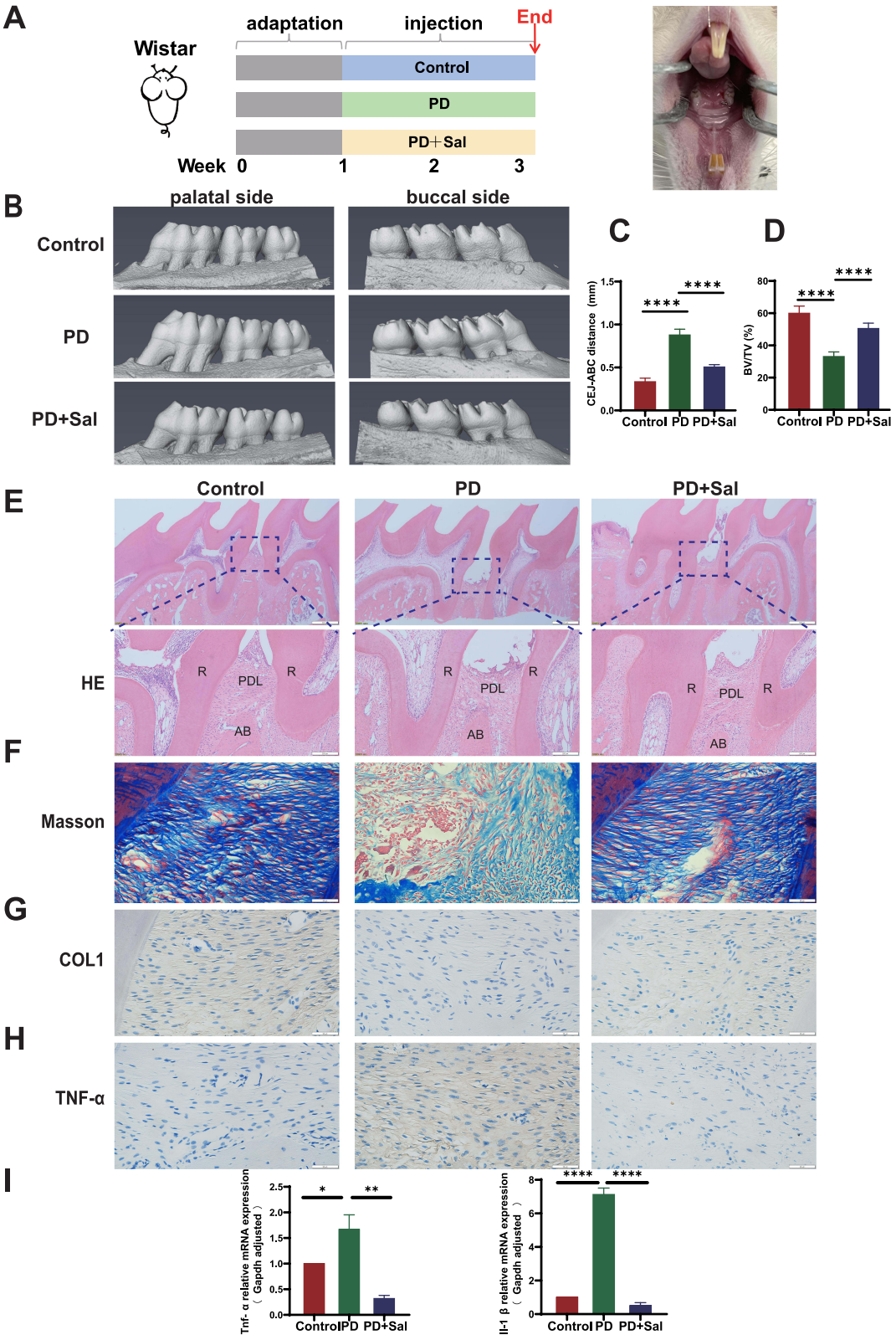

Figure 1 Sal alleviated periodontal inflammation and alveolar bone resorption. (**A**) Wistar rats were separated into Control, PD, and PD+Sal groups,and rat models of periodontitis were established.(**B**) Representative 3D micro-CT. (**C**) The straight-line distance (mm) from the CEJ to the ABC. (**D**) The percentage of BV/TV. (**E**) Representative H&E staining. Scale bar: 500 μm and 200 μm. R, root; PDL, periodontal ligament; AB, alveolar bone. (**F**) Representative Masson staining. Scale bar: 50 μm. (**G**) IHC staining of COL1.Scale bar: 50 μm. (**H**) IHC staining of TNF-α.Scale bar: 50 μm. (**I**) Relative expression of TNF-α and IL-1β mRNA.Error bars stand for mean ± SD (n=7). (*P < 0.05,**P < 0.01,****P < 0.0001.).

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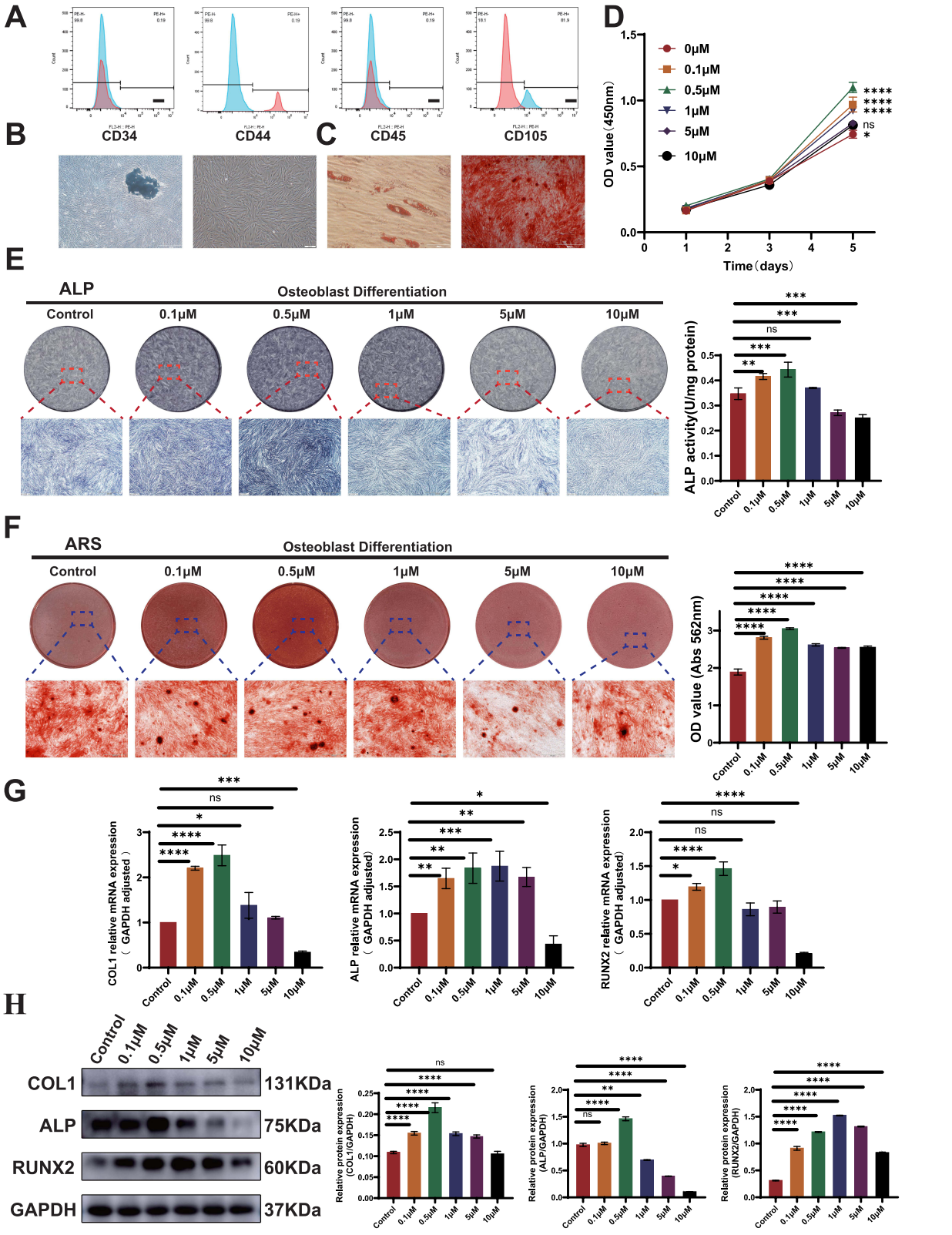

Figure 2 Sal enhanced the growth and osteogenesis of hPDLSCs. (**A**) hPDLSCs were characterized by flow cytometry for CD34, CD44, CD45, CD105 surface markers. (**B**) hPDLSCs climbed out of the tissue block and showed a long spindle shape and arranged in feathers. Scale bar: 500 μm and 200 μm. (**C**) Lipogenic induction staining and osteogenic induction staining. Scale bars: 500 μm. (**D**) The cytotoxicity of Sal on hPDLSCs determined at diverse concentrations using the CCK-8 assay. (**E**) ALP staining and ALP activity. Scale bar: 500 μm. (**F**) Alizarin red staining and calcium quantitative assay.Scale bar: 500 μm. (**G**) Relative expression of COL1, ALP and RUNX2 mRNA. (**H**) The expression level of COL1, ALP and RUNX2 protein using Western blot assay and semi-quantitative analysis.Error bars stand for mean ± SD. Each experiment was repeated five times.(*P < 0.05,**P < 0.01,***P < 0.001,****P < 0.0001).

At the gene expression level, qRT-PCR showed a significant decrease of osteogenic-related genes, ALP, COL1, and RUNX2 in LPS group, while their expression were dramatically reversed in LPS+Sal group (Figure 3C). Western blot confirmed that Sal up-regulated the expression of osteogenic proteins that were down-regulated by LPS (Figure 3D). If assay of COL1 proved that Sal markedly attenuated the down-regulation of COL1 induced by LPS (Figure 3E). These findings indicate that Sal can reverse LPS impaired osteogenic potentiality of hPDLSCs.

Sal Mitigated Inflammation While Enhanced LEF1 Expression of hPDLSCs

RNA-seq analysis was performed on the LPS and LPS+Sal groups. Principal Component Analysis (PCA) indicated notable differences in the gene expression profiles between the two groups (Figure 4A). Compared to LPS group, the LPS +Sal group showed 325 genes that were significantly up-regulated while 293 genes down-regulated (Figure 4B). Gene ontology (GO) analysis showed an increase in genes involved in chromosome segregation, mitosis, DNA replication and cell cycle in the LPS+Sal group, with a corresponding decrease in genes related to apoptosis, toll-like receptor signalling, IL-6, acute inflammation and response to LPS. (Figure 4C–E). Likewise, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed an increase in osteogenesis-related pathways, such as the cell cycle and the MAPK signaling pathways, while a decrease of inflammation-related pathways, such as the NOD-like receptors pathway (Figure 4F). The Gene Set Enrichment Analysis (GSEA) from KEGG and BP showed that pathways related to cell differentiation, stem cell proliferation, osteoblast development, mitosis, and cell cycle were activated in the LPS+Sal group, while pathways associated with inflammatory factors, such as tumor necrosis factor production and response to LPS and NF−κB signaling pathway, were inhibited (Figure 4G and H). Meanwhile, fatty acid and cholesterol metabolism were also inhibited in the LPS+Sal group (Figure 4G and H). Notably, the LPS+Sal group significantly reduced the expression of the inflammatory factor *IL6* (Figure 4I) and increased the expression of *LEF1*, which is closely related to cell cycle and osteogenic differentiation (Figure 4J). This suggests that Sal may promote bone formation of hPDLSCs by means of inhibiting inflammatory response and increasing *LEF1* expression.

Sal Attenuated the LPS Induced Inflammatory Response of hPDLSCs

To validate the pro-inflammatory effect of LPS on hPDLSCs, we examined the activation of the NF-κB signalling pathway in hPDLSCs at 5, 15, 30 and 60 minutes after LPS stimulation. Western blot showed significant increase in p-p65/p65 ratio with duration of LPS stimulation (Figure 5A). Sal effectively reduced the p-IκBa/IκBa and p-p65/p65 ratios (Figure 5B). Correspondingly, Sal significantly reduced the expression of IL-6 and IL-8 in LPS+Sal group (Figure 5C–E).

Next, we used the inhibitor of the NF-κB signaling pathway, BAY 11–7082, to block the LPS-induced inflammatory response. Both BAY 11–7082 and Sal significantly ameliorated the LPS-induced reduction of osteoblast-related proteins COL1, ALP, and RUNX2 (Figure 5F). These results suggest that the effect of Sal on osteogenesis is related to alleviate inflammatory responses.

Sal Elevated the Expression of LEF1 to Promote Osteogenesis of hPDLSCs

Studies have shown that LEF1 is an important transcriptional factor that regulates osteoblast differentiation.[26] The RNA-seq data of LPS+Sal group also showed a significant up-regulation of *LEF1* in comparison of LPS group (Figure 4J). In order to confirm whether Sal could promote LEF1 (protein) expression, hPDLSCs were separated into Control, LPS, and LPS+Sal groups, and the expression of LEF1 were examined after 48 hours of culturing by Western blot. The results showed that LPS significantly suppressed the expression of LEF1, whereas its expression was significantly increased in the LPS+Sal group (Figure 6A).

To validate whether LEF1 is associated with Sal induced osteogenesis, siRNA was employed to knockdown *LEF1* (Figure 6B). Western blot showed that *siLEF1* significantly reduced the expression of osteogenic-related proteins COL1, ALP, and RUNX2 compared to the LPS+Sal group (Figure 6C). Subsequently, we used XAV-939 to inhibit LEF1 expression to validate these results, which also showed that inhibition of LEF1 led to the significant decrease of COL1, ALP, and RUNX2 in the LPS+Sal+XAV-939 group (Figure 6D). Both ALP staining and alizarin red staining showed that the LPS+Sal+XAV-939 group had a significant reduction in ALP staining compared to the LPS+Sal group, indicating

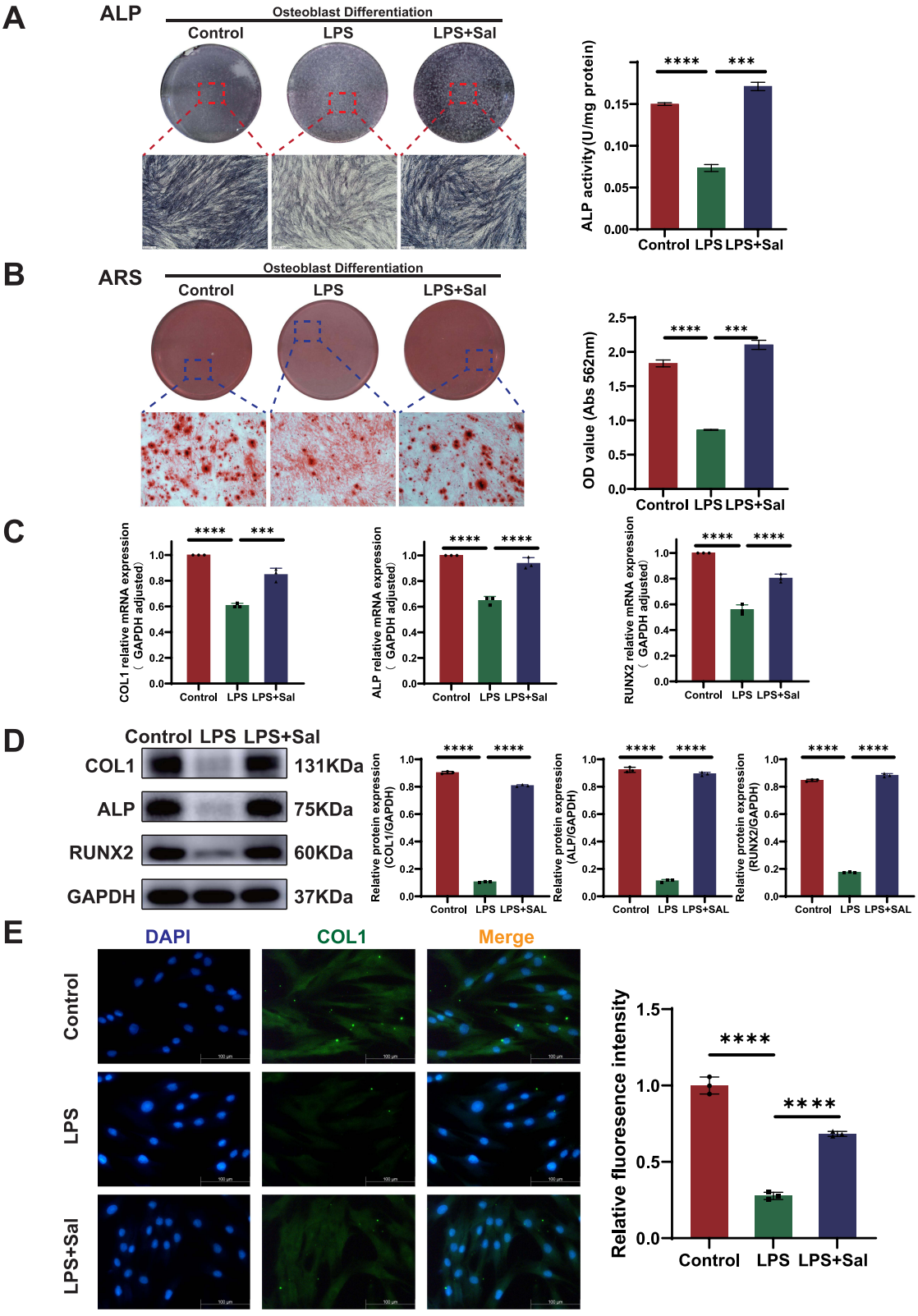

Figure 3 Sal reversed LPS-induced inhibition of osteogenic differentiation of hPDLSCs. (**A**) ALP staining and ALP activity. Scale bar: 200 μm. (**B**) Alizarin red staining and calcium quantitative assay.Scale bar: 200 μm. (**C**) Relative expression of COL1, ALP and RUNX2 mRNA. (**D**) The expression level of COL1, ALP and RUNX2 protein using Western blot assay and semi-quantitative analysis. (**E**) IF staining and relative fluorescence intensity of COL1.Scale bar: 100 μm.Error bars stand for mean ± SD. Each experiment was repeated five times.(***P < 0.001,****P < 0.0001).

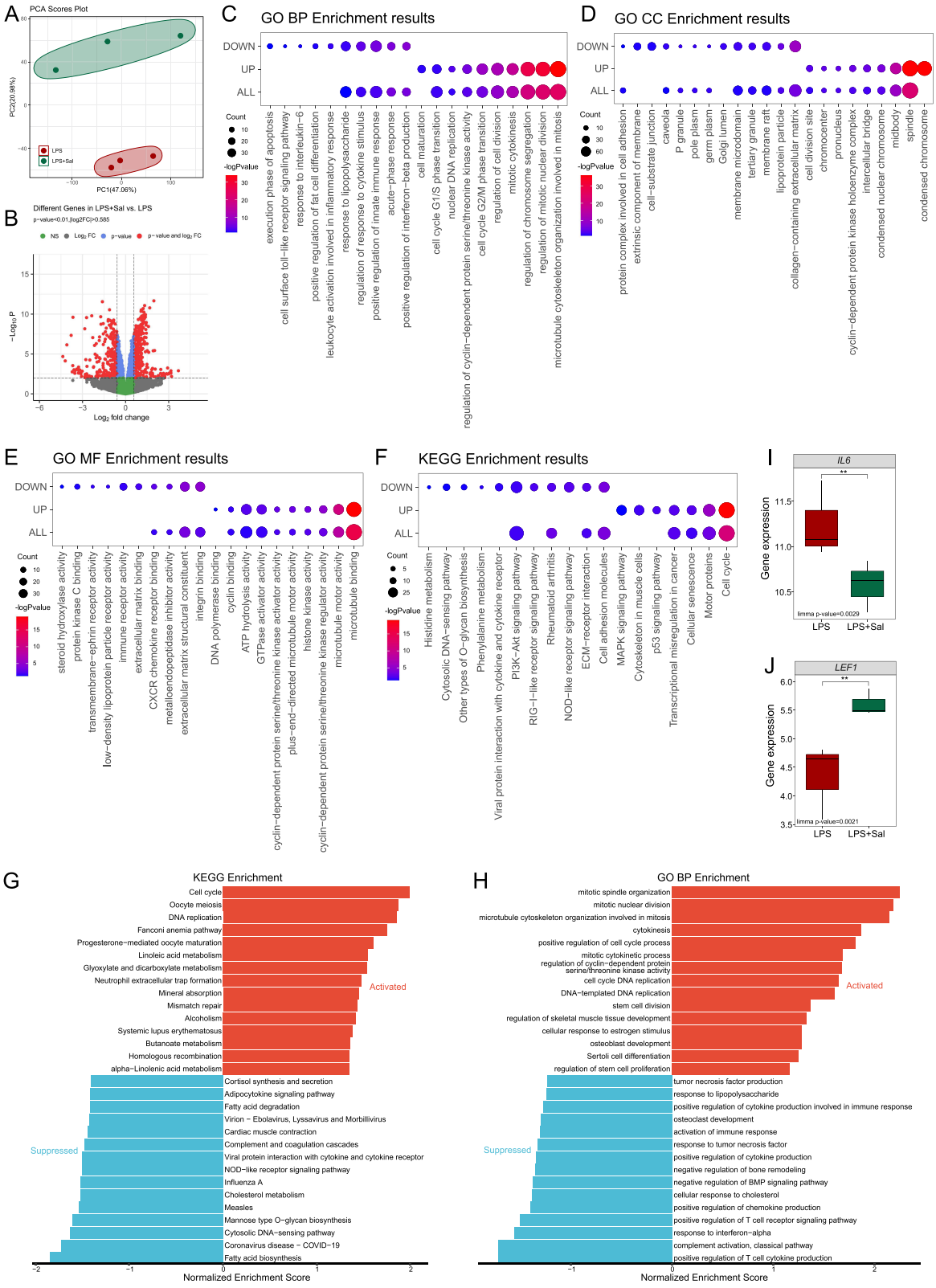

Figure 4 RNA-seq analysis was performed on the LPS and LPS+Sal groups. (**A**) Principal Component Analysis (PCA) of LPS and LPS+Sal groups. (**B**) Volcano plot of LPS and LPS +Sal groups. (**C-E**) Gene ontology (GO) analysis of Biological Process(BP), Cellular Component(CC), Molecular Function (MF) between LPS and LPS+Sal groups. (**F**) Kyoto Encyclopedia of Genes and Genomes(KEGG) enrichment analysis of LPS and LPS+Sal groups.(**G** and **H**) The Gene Set Enrichment Analysis (GSEA) of KEGG and GO BP between LPS and LPS+Sal groups. (**I** and **J**) Box-plot analysis of *IL6* and *LEF1* between LPS and LPS+Sal groups (**P < 0.01).

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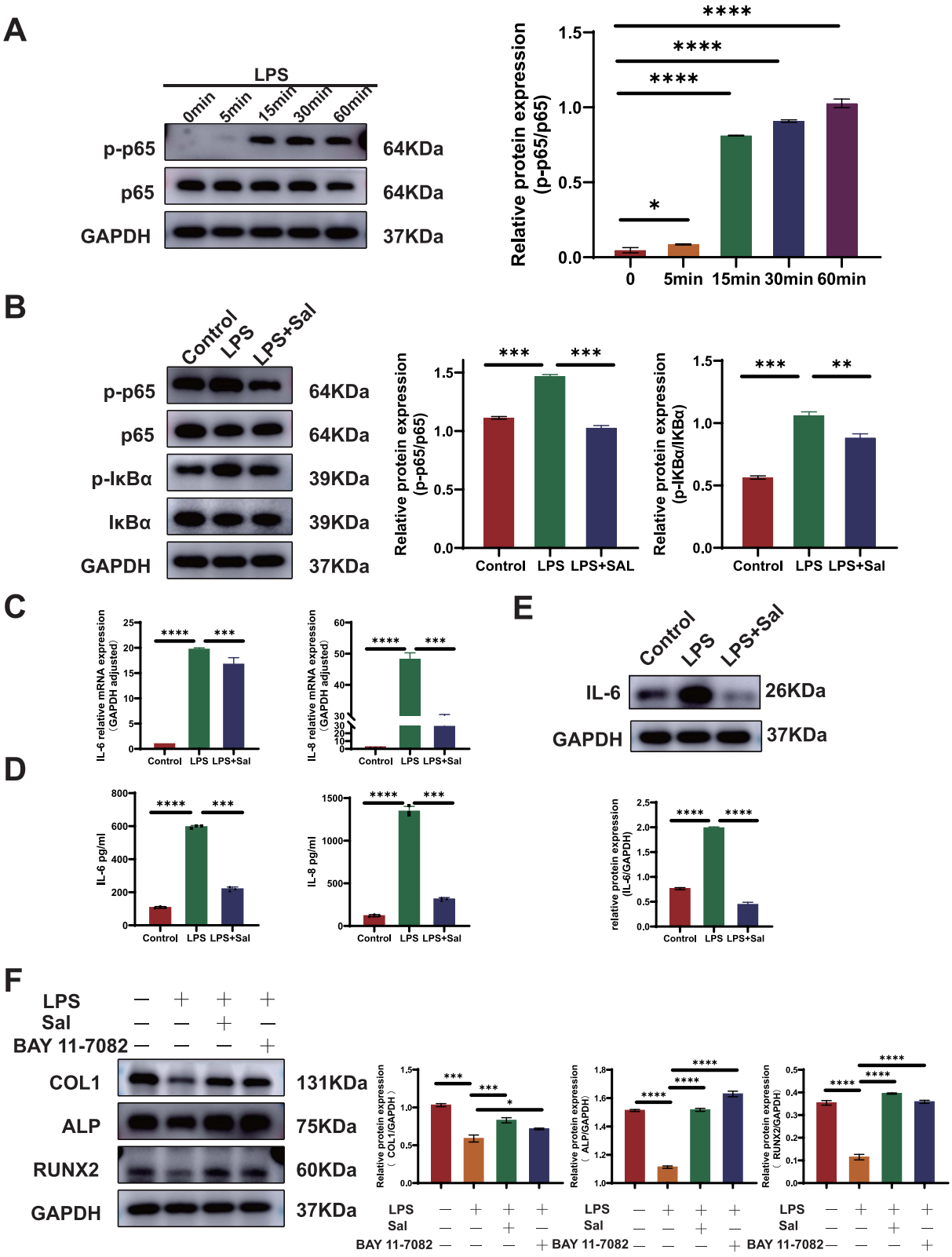

Figure 5 Sal attenuated the LPS induced inflammatory response of hPDLSCs. (**A**) The expression level of p-p65 protein after addition of LPS at different time points using Western blot assay and semi-quantitative analysis. (**B**) The expression level of p-p65, p65, p-IκBα and IκBα protein using Western blot assay and semi-quantitative analysis. (**C**)Relative expression of IL-6 and IL-8 mRNA. (**D**) Expression of IL-6 and IL-8 in the supernatant. (**E**) The expression level of IL-6 protein using Western blot assay and semi-quantitative analysis. (**F**) The expression level of COL1, ALP and RUNX2 protein with or without BAY117082 treatment using Western blot assay and semi-quantitative analysis.Error bars stand for mean ± SD. Each experiment was repeated five times.(*P < 0.05,**P < 0.01,***P < 0.001,****P < 0.0001).

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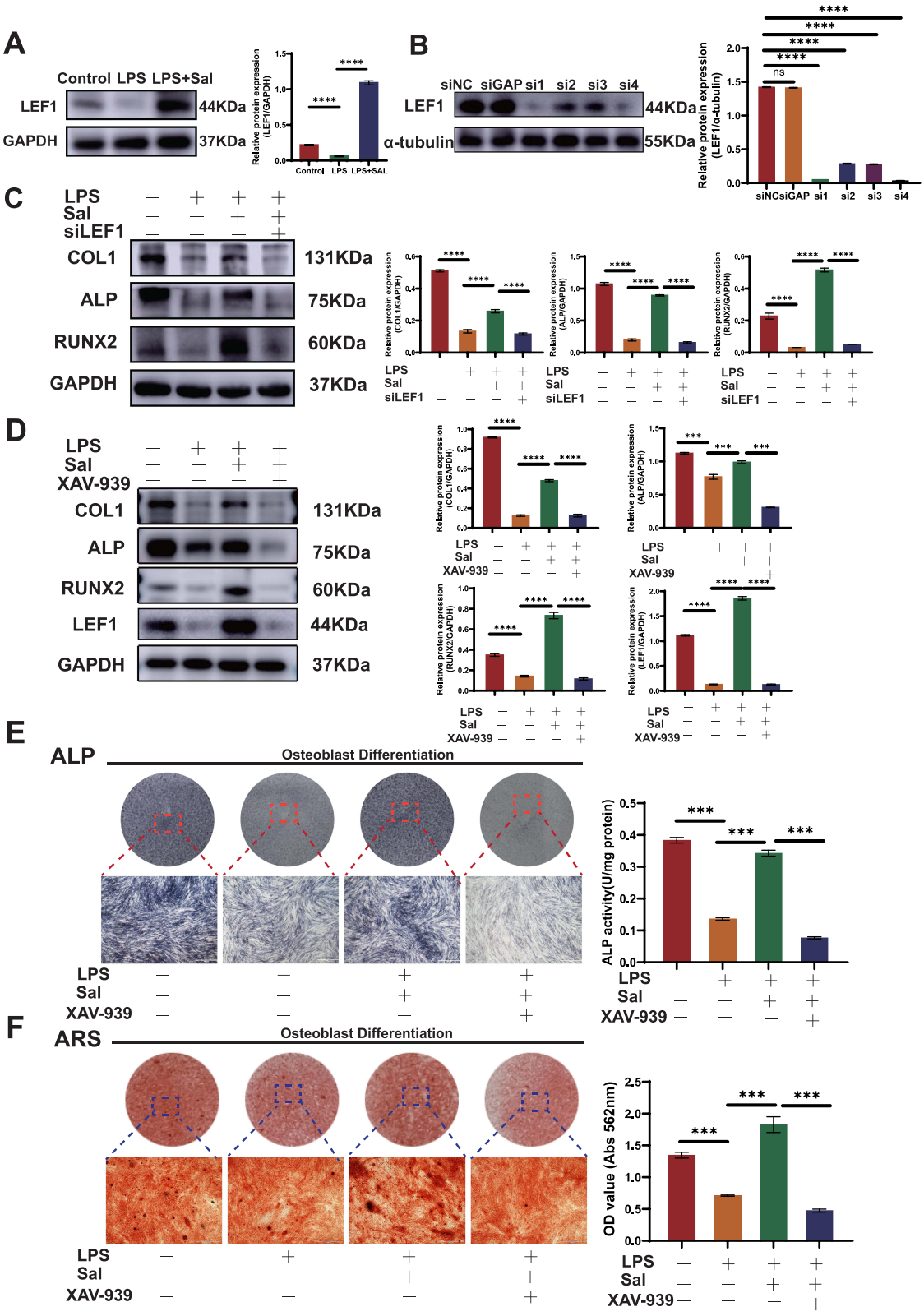

Figure 6 Sal elevated the expression of LEF1 to promote osteogenesis of hPDLSCs. (**A**) The expression level of LEF1 protein using Western blot assay and semi-quantitative analysis. (**B**) The expression level of LEF1 protein with *siLEF1* using Western blot assay and semi-quantitative analysis. (**C**) The expression level of COL1, ALP and RUNX2 protein with or without *siLEF1* treatment using Western blot assay and semi-quantitative analysis. (**D**) The expression level of COL1, ALP and RUNX2 protein with or without XAV939 treatment using Western blot assay and semi-quantitative analysis. (**E**) ALP staining and ALP activity. Scale bar: 500μm. (**F**) Alizarin red staining and calcium quantitative assay.Scale bar: 500μm. Error bars stand for mean ± SD. Each experiment was repeated five times.(***P < 0.001,****P < 0.0001).

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reduced ALP activity and a reduced amount of calcium nodules (Figure 6E and F). These results suggest that LEF1 is a crucial protein mediating the osteogenesis of Sal on hPDLSCs.

Discussion

In healthy individuals, periodontal tissues undergo continuous remodeling, where bone formation and bone resorption are tightly balanced. Pro-inflammatory cytokines (eg, IL-6, IL-8, TNF-α) and anti-inflammatory/antioxidant mediators counter-regulate each other, thereby maintaining dynamic homeostasis.[27,28] Periodontal lesions initiate as an acute inflammatory response, wherein endogenous anti-inflammatory and antioxidant defenses become insufficient to neutralize the excessive inflammatory mediators.[29] The prolonged exposure of periodontal tissues to this inflammatory microenvironment disrupts the balance between bone resorption and formation, favoring osteoclastic activity over osteogenesis.[30] This pathological cascade ultimately leads to periodontal pocket formation and alveolar bone loss. Previous studies have demonstrated that suppression of inflammatory cytokines can ameliorate periodontitis in rat models.[31] Consequently, there is an urgent need to develop effective anti-inflammatory agents for periodontitis management. In recent years, traditional Chinese medicine (TCM) has garnered increasing attention in therapeutic applications due to its stable, safe, and mild therapeutic profile.[32] The therapeutic potential of numerous TCM compounds, including curcumin[33] and resveratrol,[34] has been progressively elucidated in periodontitis treatment.

In periodontal therapy, achieving complete regeneration of damaged or lost periodontal tissues represents the ultimate clinical objective.[35] hPDLSCs have been extensively utilized in periodontal tissue engineering due to their multipotent differentiation capacity, particularly their remarkable osteogenic potential.[36] However, accumulating evidence demonstrates that inflammatory cytokines significantly impair the osteogenic differentiation capability of hPDLSCs.[11,37] Wang et al[38] reported that hPDLSCs derived from periodontitis patients exhibit substantially compromised osteogenic differentiation and regenerative capacity compared to those from healthy individuals. Consequently, successful stem cell-mediated bone regeneration requires not only mitigation of inflammatory responses but also reversal of inflammation-induced suppression of osteogenesis.[39]

Against this backdrop, we turned our attention to Sal, a bioactive phytochemical derived from the medicinal plant *Rhodiola rosea*. As a phenylethanoid glycoside, Sal represents the most pharmacologically active constituent of Rhodiola.[40] Accumulated evidence indicates that Sal exerts potent anti-inflammatory effects through suppression of pro-inflammatory signaling pathways and reduction of inflammatory cytokine production, thereby mitigating inflammatory damage in various pathological conditions.[41] Notably, emerging studies have demonstrated Sal's capacity to enhance osteogenic differentiation of stem cells and improve osteoporosis in postmenopausal rat models.[16,22–25,42] Despite its dual potential in both anti-inflammatory and pro-osteogenic activities, few studies have systematically evaluated the effects of Sal on hPDLSCs or its therapeutic potential for periodontitis. These knowledge gaps motivated our selection of Sal as the investigational compound in this study.

In our in vivo experiments, we observed that systemic administration of Sal to rats with experimental periodontitis resulted in significant thickening of the periodontal epithelial layer, marked reduction in inflammatory cell infiltration, improved organization and density of collagen fibers, and clinically measurable attenuation of root exposure and alveolar bone loss. IHC analysis revealed that Sal treatment downregulated pro-inflammatory cytokine TNF-α expression and upregulated osteogenic marker COL1 production. These findings collectively demonstrate that Sal exerts therapeutic effects through dual mechanisms of inflammation suppression and osteogenic promotion, effectively ameliorating periodontitis progression in the rat model.

ALP[43] is a crucial osteoblast differentiation marker, RUNX2[44] serves as the master transcriptional regulator of osteoblastogenesis, and COL1[45] constitutes the predominant fibrous component of bone extracellular matrix. These biomarkers represent specific and essential indicators of osteogenic differentiation, playing pivotal roles in both osteoblast maturation and physiological function. To evaluate the effects of Sal and/or LPS on the osteogenic differentiation of hPDLSCs, we used ALP activity and ALP staining, alizarin red staining, and calcium quantification at the cellular level. At the gene and protein levels, we mainly used qRT-PCR and Western Blot experiments to quantify the expression of ALP, RUNX2, and COL1. The research results showed that when Sal was used alone to stimulate hPDLSCs, 0.1–1 μM of Sal promoted osteogenic differentiation in a dose-dependent manner. Among them, 0.5 μM of

Sal had the most significant effect on osteogenic differentiation, and this concentration was used for subsequent experiments.

To investigate the effects of Sal on the osteogenic differentiation capacity of hPDLSCs under inflammatory conditions, we established an in vitro periodontal inflammatory microenvironment by co-culturing hPDLSCs with LPS and subsequently treated the cells with Sal to evaluate its therapeutic potential. The results showed that compared with the control group, LPS caused reduced ALP staining intensity, decreased ALP activity, and diminished mineralized nodule formation compared to the control group, indicating that LPS inhibited bone formation. However, Sal partially restored the compromised osteogenic capacity. qRT-PCR and Western blot analysis indicated that after LPS treatment, the expression levels of osteogenic genes and proteins including ALP, COL1 and RUNX2 significantly decreased. Similarly, Sal supplementation significantly upregulated these osteogenic markers. Therefore, we believe that Sal can effectively enhance the attenuated osteogenic potential of hPDLSCs in inflammatory microenvironments.

To elucidate the molecular mechanisms underlying the therapeutic effects of Sal, we performed RNA-seq analysis comparing hPDLSCs treated with LPS alone versus those co-treated with LPS and Sal. Our transcriptomic profiling revealed that Sal treatment attenuated LPS-induced inflammatory gene expression signatures while concurrently upregulating osteogenic differentiation markers. More specifically, Sal downregulated the inflammatory factor IL-6 and upregulated the osteogenic-related gene LEF1.

As is well-established, the NF-κB signaling pathway serves as a master regulator of diverse biological processes during mammalian development and adulthood, with its dysregulation implicated in a wide spectrum of pathological conditions including neoplasia, inflammatory/immune disorders, and metabolic diseases.[46,47] Under inflammatory conditions, NF-κB activation coincides with significant downregulation of osteogenic markers (ALP/RUNX2/OCN/COL1),[48] while specific inhibition of the IKK-NF-κB axis has been shown to partially restore the osteogenic capacity of PDLSCs.[49,50] These observations have led to growing consensus that targeted modulation of NF-κB signaling may offer dual therapeutic benefits by simultaneously promoting bone regeneration and suppressing inflammation. This was consistent with our experimental results. When hPDLSCs was stimulated by LPS, the protein level of p-p65 significantly increased within 15 minutes, accompanied by marked upregulation of IL-6 and IL-8 cytokine production, whereas subsequent treatment with Sal effectively suppressed the elevated levels of p-IκBα, p-p65, and these proinflammatory cytokines. Furthermore, pharmacological inhibition of NF-κB signaling using the specific inhibitor BAY-117082 partially restored the osteogenic differentiation capacity of PDLSCs, thereby corroborating the critical role of this pathway in mediating the observed effects.

LEF1 is known to act as a transcription factor to initiate osteogenic gene expression and regulate the expression of genes involved in cell proliferation, differentiation, migration and survival.[51] We found that Sal effectively reversed LPS-induced downregulation of LEF1. Using siRNA, we showed that while Sal increased the protein levels of COL1, ALP and RUNX2, which were inhibited by LPS, this effect was abolished by targeted silencing of LEF1. The LEF1 inhibitor XAV-939 also showed similar results, with reduced expression of osteogenic-related proteins, less ALP staining and fewer calcium nodules, suggesting that Sal may enhance osteogenic differentiation by increasing LEF1 expression.

This study systematically investigates Sal's anti-inflammatory and osteogenic effects using integrated in vivo and in vitro approaches. Utilizing RNA-seq, we elucidated potential mechanisms underlying its therapeutic effects, which were further validated using specific pathway inhibitors and targeted siRNA. Notably, These findings provide experimental evidence supporting Sal's significant therapeutic potential for periodontitis, thereby expanding its applicability in clinical practice. Nevertheless, the inflammatory microenvironment orchestrates a complex interplay of intracellular signaling pathways and cytokine networks, wherein Sal appears to exert multifaceted regulatory effects that warrant systematic investigation. Recognizing the limitations of the current study, we aim to address these gaps through the following future research directions: (1) Elucidating the precise role of canonical Wnt signaling and other potential pathways in mediating Sal-induced osteogenic differentiation of PDLSCs under inflammatory conditions; (2) Systematically characterizing Sal's immunomodulatory effects on other key periodontal cell populations, including gingival mesenchymal stem cells, osteoclasts, and macrophages, etc; (3) Evaluating long-term therapeutic outcomes, particularly regarding periodontal tissue regeneration and microbiome homeostasis; and (4) Developing optimized local delivery systems to enhance Sal's clinical applicability for periodontal therapy.

Conclusion

Our in vivo experiments showed that Sal effectively alleviated periodontal inflammation and alveolar bone resorption, demonstrating the therapeutic effect of Sal on periodontitis. In vitro analysis revealed that Sal enhanced osteogenesis of hPDLSCs by attenuating the activation of the NF-κB pathway to reduce inflammatory factor and increasing the expression of the osteogenic-related transcription factor LEF1. This study suggested that Sal may be an original and prospective agent for the clinical treatment of periodontitis.

Abbreviations

AB, alveolar bone; ABC, the alveolar bone crest; ALP, Alkaline phosphatase; BCA, bicinchoninic acid; BV/TV, bone volume/bone tissue volume; BP, Biological Process; BSA, bovine serum albumin; CC, Cellular Component; CCK-8, cell counting kit-8; CEJ, the cementoenamel junction; COL1, type I collagen; DAPI, 4',6-diamidino-2-phenylindole; DAB, Diaminobenzidine; DEGs, differentially expressed gene; ELISA, Enzyme linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, geneontology; H&E, hematoxylin and eosin; hPDLSCs, Human Periodontal Ligament Stem Cells; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-6; IHC, Immunohistochemistry; IP, intraperitoneal; KEGG, kyoto encyclopedia of genes and genomes; LEF1, lymphoid enhancer-binding factor 1; LPS, lipopolysaccharide; Micro-CT, micro-comupted tomography; MF, Molecular Function; NF-κB, nuclear factor kappa-B; OCN, osteocalcin; PBS, phosphate-buffered saline; PD, periodontitis; PDL, periodontal ligament; P. gingivalis, Porphyromonas gingivalis; PVDF, polyvinylidene fluoride; p-p65, phospho-p65; p-IκBα, phospho-IκBα; R, root; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; RUNX2, Runt-associated transcription factor 2; Sal, Salidroside; siRNA, Small interfering RNA; SD, standard deviation; TNF-α, tumor necrosis factor-α; α-MEM, α-minimum essential medium.

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Disclosure

The authors report no conflicts of interest in this work.

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