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Porphyromonas gingivalis lipopolysaccharide regulates interleukin (IL)-17 and IL-23 expression via SIRT1 modulation in human periodontal ligament cells

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ABSTRACT

Increased interleukin (IL)-17 and IL-23 levels exist in the gingival tissue of periodontitis patients, but the precise molecular mechanisms that regulate IL-17 and IL-23 production remain unknown. The aim of this study was to explore the role of SIRT1 signaling on *Porphyromonas gingivalis* lipopolysaccharide (LPS)-induced IL-17 and IL-23 production in human periodontal ligament cells (hPDLCs). IL-17 and IL-23 production was significantly increased in LPS-treated cells. LPS treatment also led to the upregulation of SIRT1 mRNA and protein expression. LPS-induced IL-17 and IL-23 upregulation was attenuated by pretreatment with inhibitors of phosphoinositide 3-kinase (PI3K), p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and NF-κB, as well as neutralizing antibodies against Toll-like receptors (TLRs) 2 and 4. Sirtinol treatment (a known SIRT1 inhibitor) or SIRT1 knockdown by small interfering RNA blocked LPS-stimulated IL-17 and IL-23 expression. Further investigation showed that LPS decreased osteoblast markers (i.e., ALP, OPN, and BSP) and concomitantly increased osteoclast markers (i.e., RANKL and M-CSF). This response was attenuated by inhibitors of the PI3K, p38, ERK, JNK, NF-κB, and SIRT1 pathways. These findings, for the first time, suggest that human periodontopathogen *P. gingivalis* LPS is implicated in periodontal disease bone destruction and may mediate IL-17 and IL-23 release from hPDLCs. This process is dependent, at least in part, on SIRT1-Akt/PI3K-MAPK-NF-κB signaling.

1. Introduction

Periodontitis is a chronic destructive inflammatory disease of teeth-supporting tissues [i.e., connective tissue of the periodontal ligament and alveolar bone]. This inflammatory condition is initiated by a complex bacterial biofilm localized in the subgingival environment, where *Porphyromonas gingivalis* is a major causative pathogen [1]. Elevated *P. gingivalis* levels are present in periodontal lesions and are significantly reduced by successful therapy [2]. *P. gingivalis* modulates the expression of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and TNF-α by gingival epithelial cells [3,4]. Several reports indicate that specific components of *P. gingivalis*, including fimbriae and lipopolysaccharide (LPS), induce pro-inflammatory cytokine production (i.e., IL-1β, IL-6, IL-8, and TNF-α) in human and murine monocytes and macrophages [5,6]. In addition, *P. gingivalis* LPS leads to periodontal tissue destruction and alveolar bone resorption through IL-6 and IL-8 release from human periodontal ligament cells (hPDLCs; [7]), and these inflammatory cytokines play a role in the destruction and disintegration of the extracellular matrix [8]. We previously reported that hPDLCs may play an important role, as these cells produce inflammatory cytokines, including receptor activator of nuclear factor-κB ligand (RANKL; [9–14]), which is related to bone metabolism in periodontal diseases. However, the mechanisms by which local immune responses against bacterial virulence factors result in the destruction of tooth-supporting tissues remains to be established.

Among the inflammatory cytokines implicated in inflammatory disease, much interest is focused on two recently identified cytokines: IL-23 and IL-17. IL-17 (also known as IL-17A), a pro-inflammatory cytokine that is regulated by IL-23, is crucial for the development of a novel $CD4^+$ T-cell subset called T-helper 17 (Th17) cells, which promote tissue inflammation in host-defense responses against infection [15]. Enhanced IL-17 expression is reported in various inflammation models, including rheumatoid arthritis, periodontitis, asthma, and organ rejection [16–18]. In

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addition, IL-17 may affect osteoclast resorption indirectly through osteoblasts, like other pro-inflammatory cytokines [19,20]. IL-17 levels in inflamed gingival tissues of periodontitis are higher than those in healthy control tissues [16,21]. Similarly, the amount of IL-17 in gingival crevicular fluid samples is significantly higher in periodontitis patients [22,23]. Furthermore, IL-17RA-deficient mice have enhanced periodontal bone destruction following *P. gingivalis* infection, suggesting a bone-protective role of IL-17 [24]. IL-23, a heterodimeric protein that is composed of the IL-12p40 subunit and a specific IL-23p19 subunit, is primarily produced by activated dendritic cells, monocytes, and macrophages [25]. IL-23, in conjunction with IL-1, then contributes to the expansion and maintenance of Th17 cells that, once activated, release the cytokines IL-17A, IL-17F, IL-22, TNF-α, and IL-6, all of which can induce inflammatory responses [26]. Furthermore, high numbers of IL-17- and IL-23-expressing cells are present in the ileum of Crohn's disease [27], active uveitis of Bechet's disease [28], lupus nephritis [29], and gingival tissue of periodontitis patients [16].

SIRT1 is a prominent member of the NAD^+-dependent enzyme family and affects a variety of cellular functions ranging from gene silencing, cell cycle regulation, apoptosis, and differentiation to energy homeostasis [30–32]. Several recent studies implicated SIRT1 in the regulation of inflammatory responses [33,34]. SIRT1 can also inhibit nuclear transcription factor-κB (NF-κB), a master transcription factor involved in the regulation of pro-inflammatory cytokines, which enhances cell death in response to the inflammatory cytokine TNF-α [35]. However, very little is known regarding whether SIRT1 regulates LPS-induced IL-23 or IL-23 axis signaling, particularly in periodontitis bone destruction.

This study was designed to gain further knowledge on the impact of *P. gingivalis* LPS on the regulation of IL-17 and IL-23 production, and homeostasis between osteoblastic and osteoclastic genes in hPDLCs. We also investigated potential mechanisms involved in this process, including the role of SIRT1.

2. Materials and methods

2.1. Reagents

Sirtinol and LPS (from *P. gingivalis*) were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO). Human IL-17 ELISA Kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Affinity purified polyclonal antibodies against mouse TLR2, TLR4, IκB-α, NF-κB p65 and β-actin monoclonal antibodies were obtained from Santa Cruz Biotechnology (Delaware Avenue, CA). Anti-human TLR2 and TLR4 monoclonal antibodies (Abcam, Cambridge, UK) were used for in vitro neutralization studies in hPDL cells. Antibodies (Abs) against phospho-ERK (p-ERK), ERK, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK), and JNK were purchased from Cell Signaling. Wortmannin was purchased from Calbiochem (San Diego, CA).

2.2. Cell culture

Immortalized hPDLCs, transfected with human telomerase catalytic component (hTERT), were kindly provided by professor Takashi Takata (Hiroshima University, Japan) [37]. Cells were cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Western blot analysis

Cells (1×10^6) from each set of experiments were harvested and washed twice in cold tris-buffered saline. Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Proteins (20 μg) were resolved by SDS–PAGE (10% acrylamide), transferred to nitrocellulose membranes, and probed with specific Abs (diluted 1/1000), followed by incubation with secondary HRP-conjugated Ab (1/5000). Proteins were detected by enhanced chemiluminescence system according to the manufacturer's instructions and exposed to X-ray film. Densitometricanalysis of each blot was performed with a computerized image processing system (Quantity One; Bio-Rad, Hercules, CA).

2.4. RNA isolation and Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells (5×10^5) were cultured in 60-mm culture dishes until 70% confluency, and incubated for 24 h in a serum free medium containing stimuli as indicated. The total RNA of pulp cells was extracted using the Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction. Then 1 μg RNA was reverse-transcribed for first strand cDNA synthesis (Gibco BRL, Rockville, MD). The cDNA was amplified in a final volume of 20 μL containing 2.5 mM magnesium dicholoride, 1.25 units Ex Taq polymerase (Bioneer, Daejeon, Korea) and 1 μM specific primers. Amplification was carried out for 30 cycles in a DNA thermal cycler. Primer sequences for differentiation markers are detailed in Table 1. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. The intensity of each band after normalization with beta-actin mRNA was quantified on the photographed gels with a densitometer (Quantity One; Bio-Rad, Hercules, CA).

2.5. Measurement by enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-17 in the culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommended procedure. The plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA).

2.6. SIRT1 siRNA transfection

siRNA-annealed oligonucleotide duplexes for SIRT1 (Sequence 5′ → 3′ sense: GAUGAAGUUGACCUCCUCAtt; antisense: UGAGGAG GUCAACUUCAUCtt) and negative control (Catalog No. SN-1003)

Table 1
Reverse transcriptase-polymerase chain reaction (RT-PCR) primers and conditions.

Gene	Sequence (5′–3′)	Size (bp)	T_m (°C)
IL-17	Forward: CGATGACTCCTGGGAAGACCTC Reverse: GTGTGGGCTCCCCAGAGCTCTTA	490	60
IL-23	Forward: GCAGATTCCAAGCCTCAGTC Reverse: TTCAACATATGCAGGTCCCA	524	60
ALP	Forward: ACGTGGCTAAGAATGTCATC Reverse: CTGGTAGGCGATGTCCTTA	475	55
OPN	Forward: AATGAAAACGAAGAAAGCGAAG Reverse: ATCATAGCCATCGTAGCCTTGT	347	55
BSP	Forward: TGGAGATGACAGTTCAGAAG Reverse: GTACTGGTGCCGTTTATGC	333	52
OPG	Forward: TGCAGTACGTCAAGCAGGAG Reverse: TGACCTCTGTGAAAACAGC	575	56
RANKL	Forward: GCCAGTGGGAGATGTTAG Reverse: TTAGCTGCAAGTTTTCCC	486	55
M-CSF	Forward: ATGACAGACAGGTGGAACTGCCAGTGTAGAGG Reverse: TCACACAACTTCAGTAGGTTCAGGTGA TGGGC	437	60
SIRT-1	Forward: GCAACATCTTATGATTGGCACA Reverse: AAATACCATCCCTTGACCTGAA	820	60
β-Actin	Forward: CATGGATGATGATATCGCCGCG Reverse: ACATGATCTGGGTCATCTTCTCG	371	55

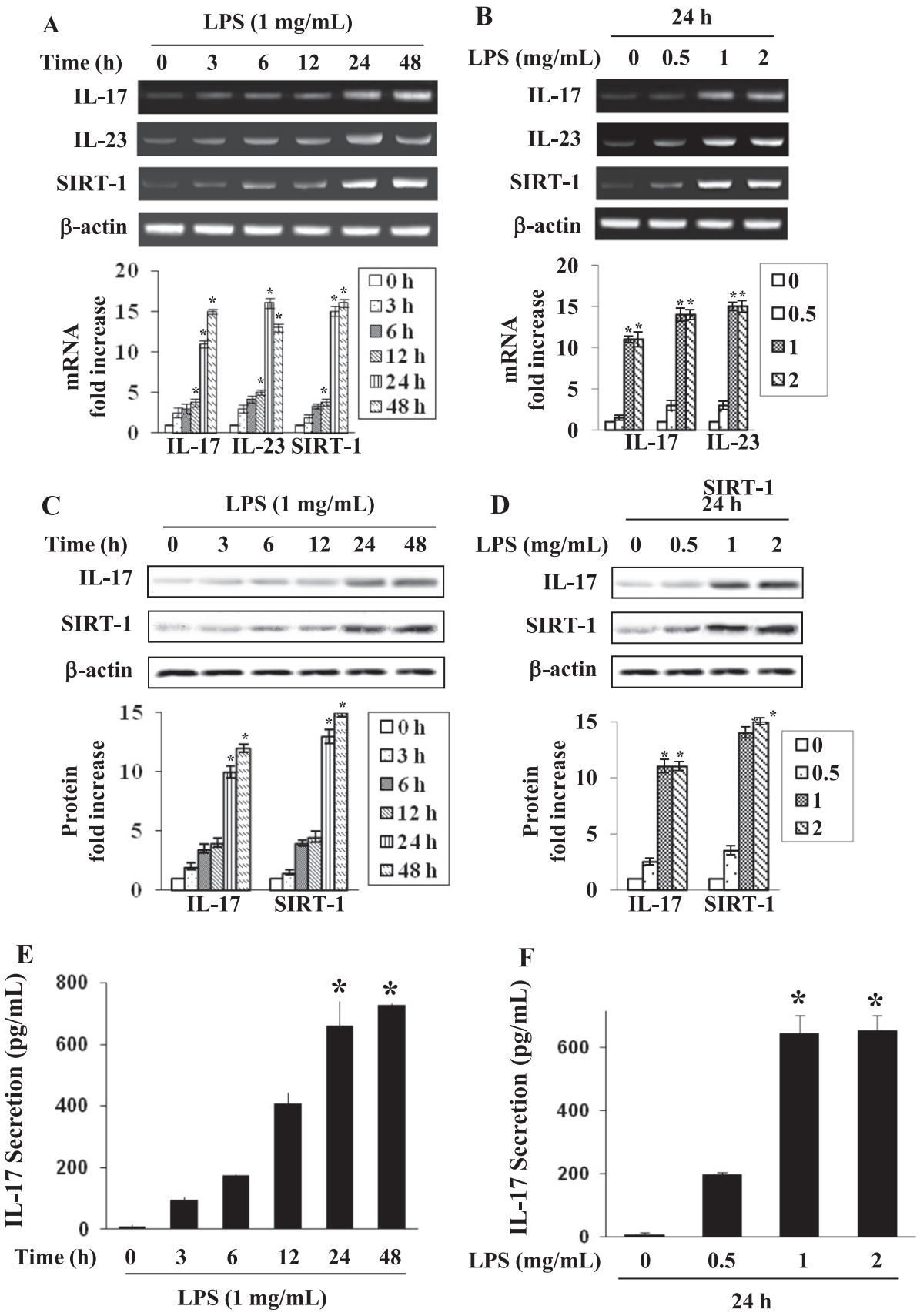

Fig. 1. Effects of LPS on IL-17, IL-23, and SIRT1 mRNA and protein and IL-17 production n hPDLCs. mRNA and protein expression and production levels were determined by semiquantitative RT-PCR (A and B), Western blotting (C and D), and ELISA (E and F). Western blotting, PCR, and ELISA results are representative of three independent experiments. The histogram shows the quantification of mRNA and protein expression by densitometry, and is presented as fold increases compared to non-stimulated control cells. *Statistically significant difference versus control, $p < 0.05$.

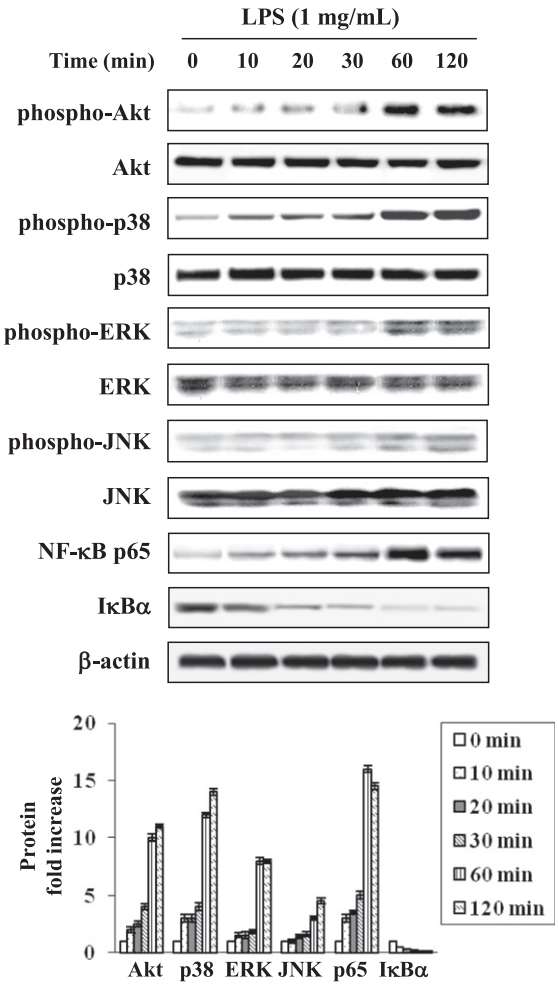

Fig. 2. Effects of *P. gingivalis* LPS on activation of the Akt, MAPK, and NF-kB pathways. Activation was determined by Western blot analysis. The graph shows the quantification of protein expression by densitometry, and is presented as fold increases compared to control cells. Similar results were obtained in three independent experiments. *Statistically significant difference versus control, $p < 0.05$.

were purchased from Bioneer (Bioneer Corporation, Daejeon, South Korea) and hPDL cells were transfected using lipofectamine 2000 (Gibcoen ltd, paisley, UK) following the manufacturer's instructions.

2.7. Statistical analysis

Differences among the groups were analyzed using one-way analysis of variance combined with the Duncan's multiple range tests.

3. Results

3.1. Effects of LPS on IL-17, IL-23, and SIRT1 mRNA and protein expression

To investigate whether LPS could induce IL-17 and IL-23 cytokines in hPDLCs, cells were stimulated with various LPS concentrations, and cytokine production was measured by reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and ELISA (Fig. 1A–F). IL-17 and IL-23 mRNA and protein expression were upregulated by LPS in a time- and concentration-dependent fashion, with cytokine accumulation peaking at 1 μg/ml 24 h LPS posttreatment (Fig. 1A–D). After 24 h LPS stimulation, hPDLCs secreted significant amounts of IL-17; a LPS concentration of 1 μg/ml induced the largest production (Fig. 1E and F).

To understand the role of SIRT1 in LPS-induced cell signaling processes, we first assessed the expression of SIRT1 mRNA and protein by RT-PCR and Western blotting. As shown in Fig. 1A–D, LPS treatment significantly increased SIRT1 protein and mRNA levels in a concentration- and time-dependent manner, with a maximal effect at 1 μg/ml for 24 h. LPS (1 μg/ml) did not adversely affect cell viability (data not shown) and was thus used in subsequent experiments.

3.2. Signaling pathways of LPS-induced IL-17 and IL-23 expression

To determine the signaling pathways involved in the upregulation of IL-17 and IL-23 expression by LPS in hPDLCs, we examined the activation states of Akt, MAPK, and NF-κB. LPS treatment induced phosphorylation of Akt, p38, ERK, and JNK without affecting the total levels of these kinases. In addition, LPS treatment increased the NF-κB p65 nuclear translocation and IκB-α degradation in hPDLCs (Fig. 2).

To further examine the roles of Akt, MAPK, and NF-κB in LPS-induced IL-17 and IL-23, pharmacological inhibitors of signaling intermediates were used. Pretreatment with inhibitors of phosphatidylinositol 3-kinase (PI3K) (LY294002 and wortmannin), p38 (SB203580), ERK (PD98059), JNK (SP600125), and NF-κB (pyrrolidine dithiocarbamate, PDTC) blocked LPS-induced SIRT1, IL-17, and IL-23 expression, and IL-17 secretion in hPDLCs (Fig. 3A–C).

To determine whether the increased SIRT1 level was associated with LPS-induced IL-17 and IL-23 expression, hPDLCs were pretreated with the pharmacological SIRT1 inhibitor sirtinol. After 1 h sirtinol pretreatment, cells were treated with LPS for 24 h and subjected to immunoblot analysis. The data showed that LPS-induced SIRT1, IL-17, and IL-23 expression and that IL-17 secretion was attenuated by sirtinol pretreatment (Fig. 3A–C).

3.3. Effects of signaling pathway inhibitors on LPS-induced osteogenic and osteoclastogenic gene expression

To investigate the putative mechanism linking IL-17 and IL-23 to alveolar bone homeostasis, we analyzed the expression pattern of osteogenic and osteoclastogenic genes associated with bone-loss conditions. As shown in Fig. 4, LPS treatment concomitantly downregulated the expression of alkaline phosphatase (ALP), osteopontin (OPN), and bone sialoprotein (BSP) mRNA expression, and upregulated the expression of nuclear factor κ B-ligands (RANKL) and macrophage colony-stimulating factors (M-CSF), but not osteoprotegerin (OPG). Treatment with sirtinol, LY294002 and wortmannin (PI3K inhibitor), SB203580 (p38 inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor), and PDTC (NF-κB inhibitor) blocked the effects of LPS on the upregulation of RANKL and M-CSF, and downregulation of ALP, OPN, and BSP mRNA expression (Fig. 4). The vehicle control was 0.1% dimethyl sulfoxide (DMSO) because sirtinol was dissolved in DMSO.

3.4. Functional role of TLR2 and TLR4 on LPS-induced response

Since TLR2 or TLR4 serves as the main mediator of responses to LPS, we initially examined whether LPS influenced the expression of TLR2 and TLR4 in hPDLCs. Following LPS exposure, TLR2 and TRL4 protein levels increased in a time- and concentration-dependent manner (Fig. 5A and B). To assess the functional involvement of TLR2 and TLR4 in LPS-induced cytokine response of hPDLCs, cells were incubated with anti-TLR2 or anti-TLR4 monoclonal antibody (10 μg/ml) before LPS stimulation. Antibody specificity was assessed by incubating with control IgG from non-immunized

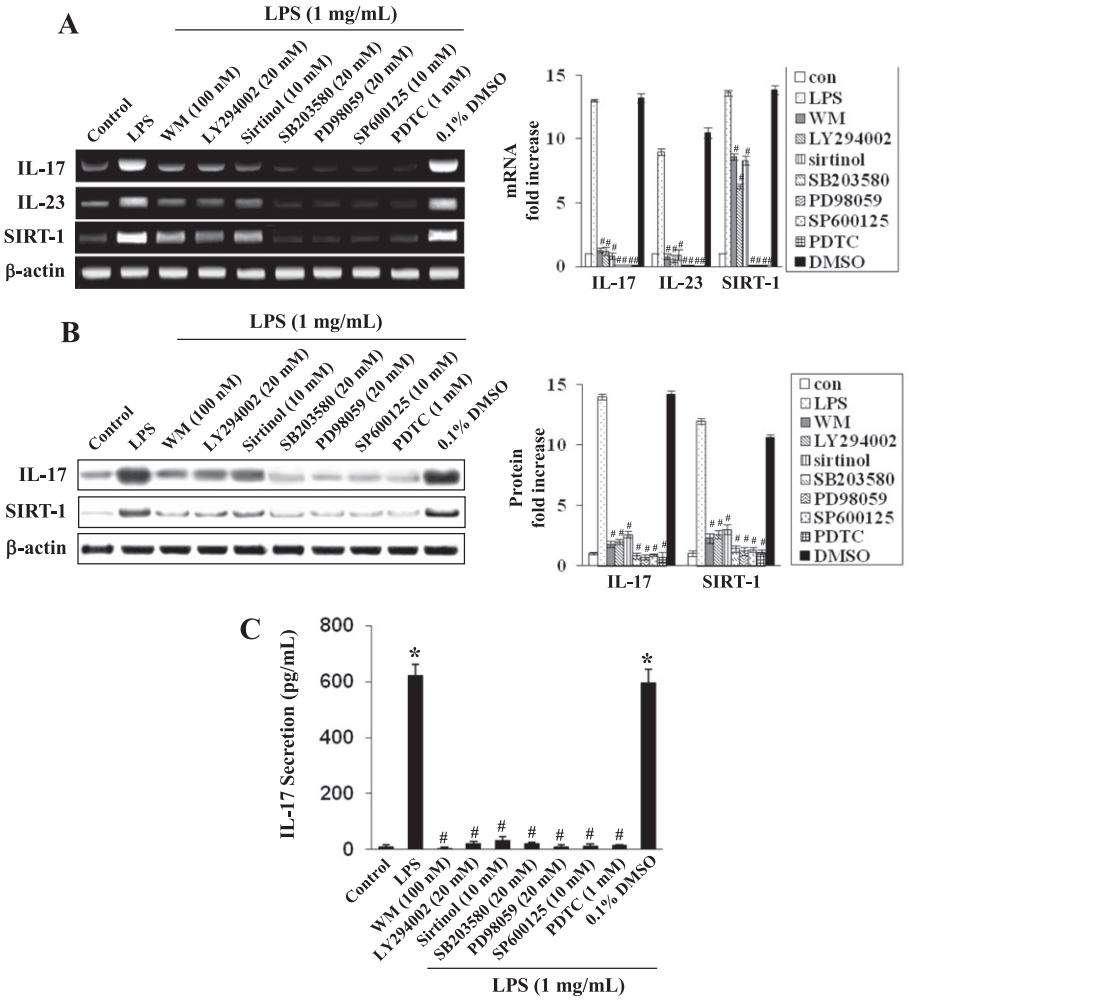

Fig. 3. Effects of various signaling pathway inhibitors on *P. gingivalis*-induced IL-17, IL-23, and SIRT1 expression. Cells were preincubated with (A) medium only or with wortmannin (WM; PI3K inhibitor), LY294002 (PI3K inhibitor), sirtinol (SIRT1 inhibitor), SB203580 (p38 kinase inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), PDTC (NF-kB inhibitor), or vehicle (0.1% DMSO) for 60 min before stimulation with 1 μg/ml *P. gingivalis* LPS for 1 h. RT-PCR (A), Western blot (B) and ELISA (C) results are representative of three independent experiments. The histogram shows the quantification of mRNA and protein expression by densitometry, and is presented as fold increases compared to control cells. *Statistically significant difference compared to the control group, $p < 0.05$. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

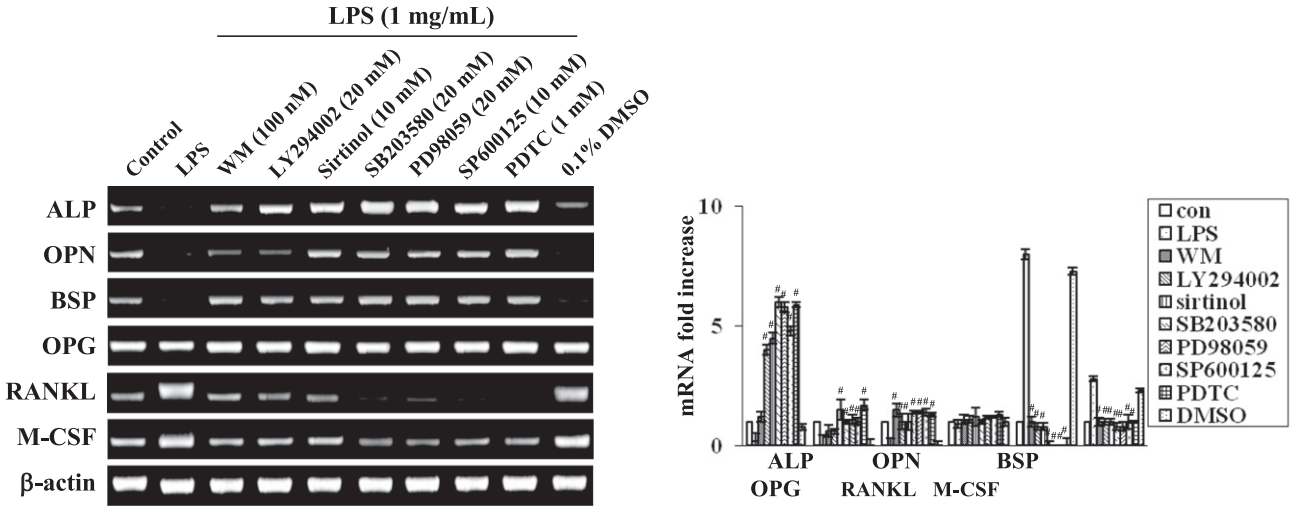

Fig. 4. Effects of various signaling pathway inhibitors, including SIRT1, on *P. gingivalis* LPS-induced osteoblastic and osteoclastic genes. Cells were preincubated with wortmannin (WM; PI3K inhibitor), LY294002 (PI3K inhibitor), sirtinol (SIRT1 inhibitor), SB203580 (p38 kinase inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), PDTC (NF-kB inhibitor), or vehicle (0.1% DMSO) for 60 min before stimulation with 1 mg/ml *P. gingivalis* LPS for 24 h. mRNA expression level was determined by semiquantitative RT-PCR. Histogram is densitometric quantitation of mRNA expression. Similar data were obtained from three independent experiments. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

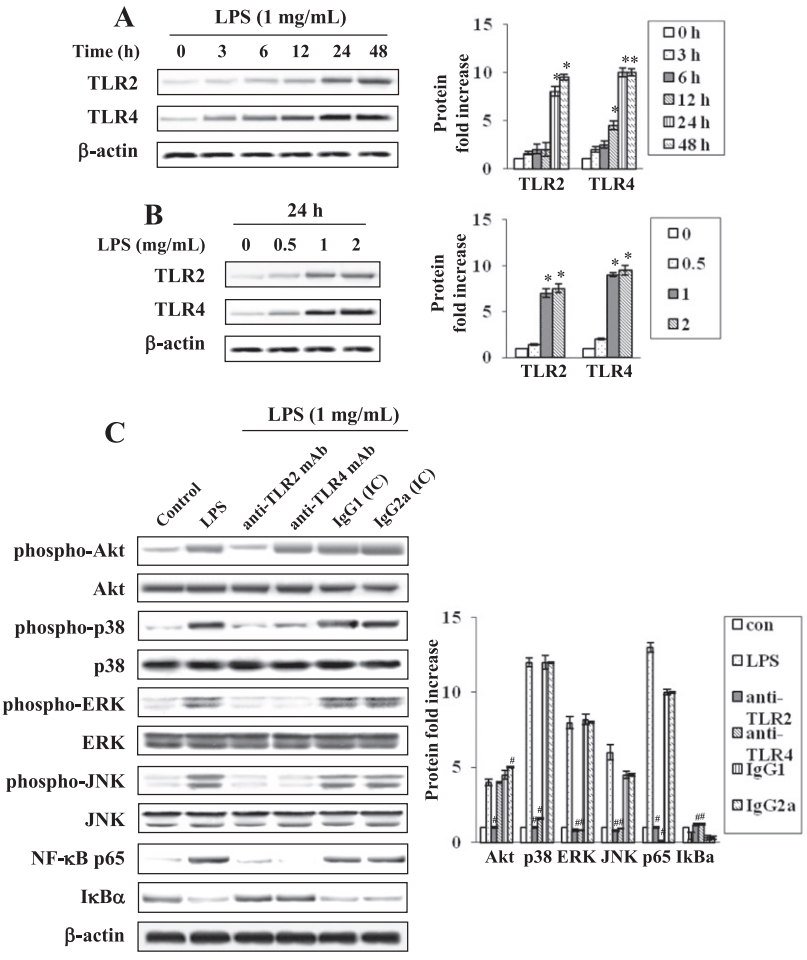

Fig. 5. Effects of *P. gingivalis* LPS on TLR and TLR4 expression (A and B). TLR2 and TLR4 block LPS-induced Akt, MAPK, and NF-kB activation (C). Cells were preincubated with or without TLR2 or TLR4 mAbs, or isotype control (IC) abs for 60 min before stimulation with 1 μg/ml *P. gingivalis* LPS for 1 h. Expression was determined by Western blot analysis. Histogram is densitometric quantitation of protein expression. Similar results were obtained in three independent experiments. *Statistically significant difference compared to the control group, $p < 0.05$. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

animals. LPS-induced Akt, p38, ERK, JNK, and NF-κB activation was inhibited by anti-TLR2 and anti-TLR4 antibodies (Fig. 5C). In addition, treatment of cells with anti-TLR2 and anti-TLR4 antibodies blocked LPS-induced SIRT1, IL-17, and IL-23 upregulation, but IgG1 and IgG2a did not (Fig. 6A–C). The anti-TLR4 and anti-TLR2 antibodies also attenuated LPS-induced osteogenic and osteoclastogenic gene mRNA expression (Fig. 6D).

3.5. Effect of SIRT1 siRNA on LPS-induced responses

To further confirm the role of SIRT1 in LPS-induced IL-17, IL-23, and osteoblastic and osteoclastogenic gene expression, we knocked down SIRT1 with a specific siRNA. As shown in Fig. 7A, SIRT1 siRNA successfully knocked down SIRT1 expression in hPDLCss and blocked LPS-induced IL-17 secretion, expression of the mRNAs encoding IL-17 and IL-23, osteoblastic genes (ALP, OPN, BSP), and osteoclastogenic genes (RANKL and M-CSF), whereas transfection of cells with an equivalent amount of nonspecific siRNA had no effect (Fig. 7B–E).

To elucidate the molecular basis of the responses to SIRT1 inhibition, we examined the effects of SIRT1 siRNA on LPS-induced Akt, MAPK, and NF-κB signaling pathways in hPDLCs. As shown in Fig. 8, SIRT1 siRNA pretreatment decreased LPS-induced phosphorylation of Akt, p38, ERK, and JNK, and the degradation of IκBα and the nuclear translocation of p65 (NF-κB).

4. Discussion

Periodontal disease is triggered by potentially hazardous microorganisms, which induce consequent immune-inflammatory responses. IL-17 treatment of human gingival fibroblasts (HGFs) leads to IL-6 production, and IL-8 and intercellular adhesion molecule (ICAM)-1 expression [37], which may contribute to local tissue inflammation. Furthermore, IL-17 induces RANKL expression via induction of prostaglandin E2 production in osteoblasts and is released by IL-23 stimulation [38,39]. Moreover, both IL-23 and IL-27 inhibit bone marrow cell osteoclastogenesis induced by M-CSF/soluble RANKL [40]. Whether the IL-23/IL-17 pathway is involved in periodontal bone destruction is unclear, although increased IL-17 and IL-23 levels are found in gingival tissue and crevicular fluid of patients with periodontitis [16,21–23]. Therefore, our study was designed to investigate the association between periodontal bone loss and production of IL-23 and IL-17 by the periodontopathogen *P. gingivalis* LPS in hPDLCs.

As primary hPDLCs are heterogeneous cell populations with varied proliferative potential, long-term culture of hPDLCs is apt to reduce the number of cells with low proliferative potential, so that their primary phenotype is lost [41]. Consequently, reproducible results are difficult to obtain and the biology and regenerative mechanism of hPDLCs is difficult to clarify by using primary cells [42]. Therefore, to address this issue, we used immortalized hPDLCs, which retain the features of their original primary cells,

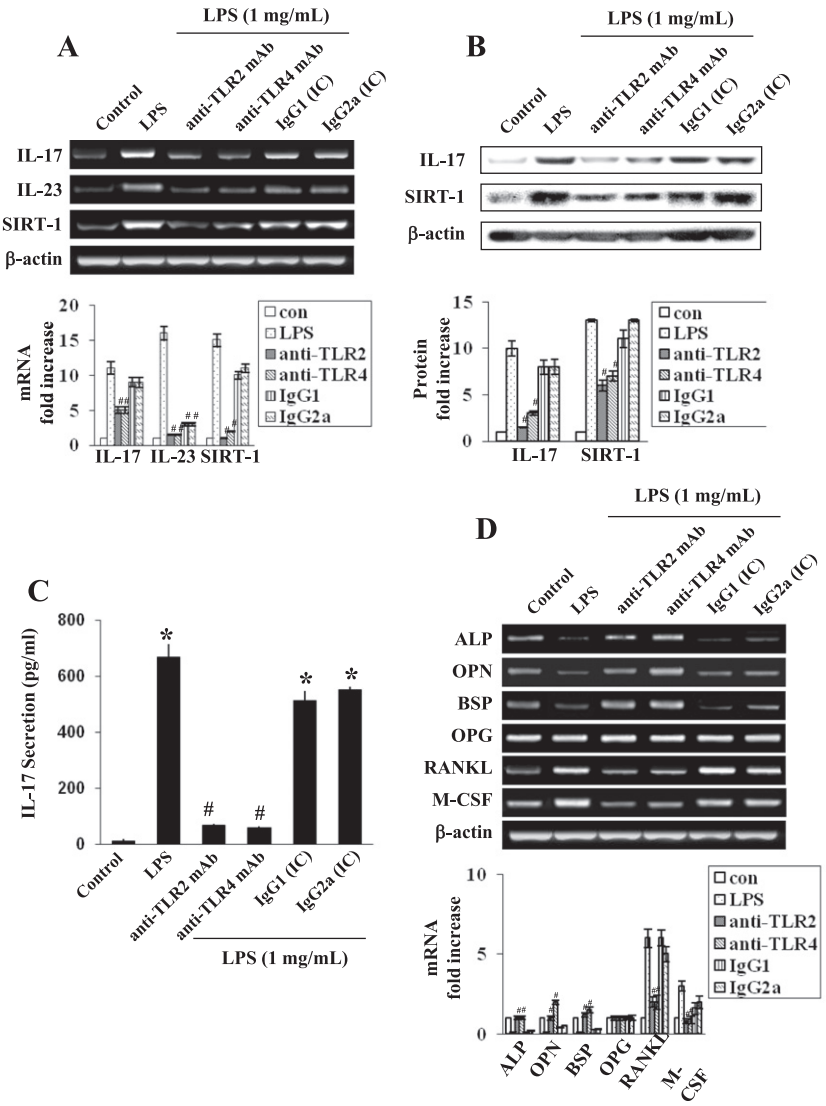

Fig. 6. Effect of blocking TLR2 and TLR4 on LPS-induced IL-17 and IL-23 production (A–C), SIRT1 expression (A and B), and osteoblastic and osteoclastic genes (D). Cells were preincubated with or without TLR2 or TLR4 mAbs or isotype control (IC) abs for 60 min before stimulation with 1 μg/ml *P. gingivalis* LPS for 24 h. Histogram is densitometric quantitation of mRNA or protein expression. Similar results were obtained in three independent experiments. *Statistically significant difference compared to the control group, $p < 0.05$. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

viz., hPDLCs [36]. In addition, Kitagawa et al. [36] demonstrated that F-spondin increased the expressions of ALP, OCN, and BSP mRNA, and ALP activity in immortalized hPDLCs. Thus, immortalized this cell line is helpful tools for studying the biology and regenerative mechanisms of hPDLCs.

LPS is a well-known endotoxin which elicits a variety of inflammatory responses. In humans, LPS binds the CD14/TLR4/MD2 receptor complex, which triggers the signaling cascade for many cell types to secrete pro-inflammatory cytokines and nitric oxide [43]. LPS-treated hPDLCs were found to respond to the endotoxin by activation of TLR4 and TLR2, promotion of NF-κB subunit p65 translocation to the nucleus and enhanced expression of pro-inflammatory cytokines [13,44]. To examine the role of IL-17 and IL-23 signaling in the LPS response, we first analyzed IL-17 and IL-23 protein and mRNA levels. Our results indicate that LPS induces IL-17 and IL-23 expression and IL-17 secretion in a time- and concentration-dependent manner, which is consistent with the previous finding that LPS stimulates pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 IL-10, TNF-α, and IL-12 in hPDLCs [13,44,45]. Following LPS exposure, parallel to IL-17 and IL-23 upregulation, we detected increased protein and mRNA levels of SIRT1. These results are consistent with our previous study, which showed that SIRT1 mRNA and protein is upregulated by LPS and heat stress in dental pulp cells [33].

Akt/PI3K, MAPK, and NF-κB pathways play a pivotal role in pro-inflammatory cytokine and chemokine regulation [18,32–34]. Our results demonstrated that LPS activated Akt/PI3K, p38, JNK, ERKK, and NF-κB pathways in hPDLCs. Based on the results of a cytotoxicity assay, we used optimal inhibitor concentrations of PI3K (20 μM LY294002 and 100 nM wortmannin), ERK (20 μM PD98059), p38 MAPK (20 μM SB203580), JNK (10 μM SP600125), and NF-κB (1 mM PDTC) that elicited inhibitory effects without toxicity. Our results showed that SB203580, PD98058, SP600125, wortmannin, LY294002, and PDTC downregulated LPS-induced IL-17 and IL-23, which suggests that Akt/PI3K, ERK, p38, JNK, and NF-κB mediate LPS-induced IL-17 and IL-23 expression in HDP cells. Consistent with our data on hPDLCs, IL-17 produced by activated rheumatoid arthritis peripheral blood mononuclear cells is completely or partly blocked in the presence of the PDTC, wortmannin, and LY294002 [18].

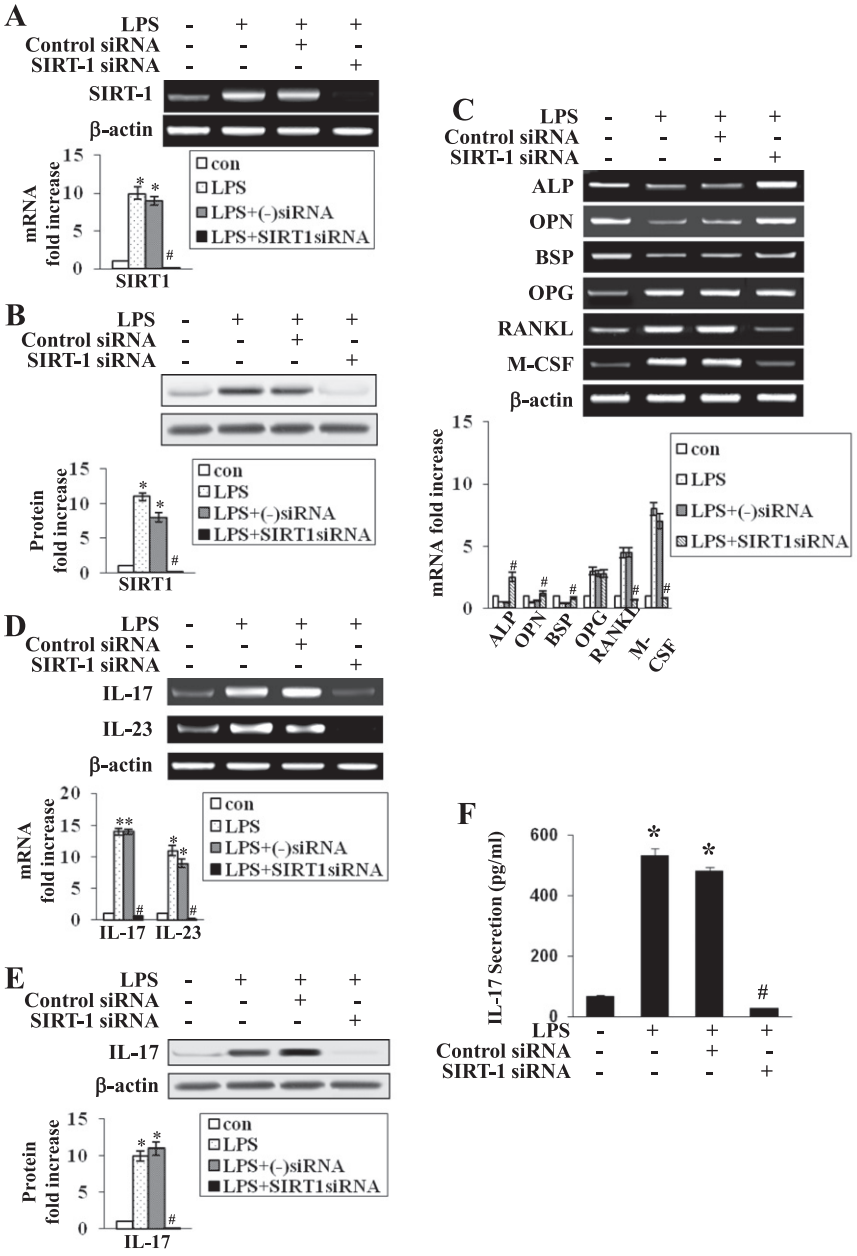

Fig. 7. Effects of SIRT1 siRNA on *P. gingivalis* LPS-induced SIRT1 mRNA and protein levels (A and B), osteoblastic and osteoclastic genes (C), and IL-17 and IL-23 production (D–F). Cells were transiently transfected with a control vector or SIRT1 siRNA, followed by 24 h LPS treatment. Similar results were obtained in three independent experiments. Histogram is densitometric quantitation of mRNA or protein expression. *Statistically significant difference compared to the control group, $p < 0.05$. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

TLR2 and TLR4, transmembrane receptors that transmit the LPS signal to intracellular components in signal transduction pathways, play important roles in the immune system [46]. TLR4 is associated with the recognition of Gram-negative bacterial LPS, and TLR2 is regarded as the receptor for Gram-positive bacteria [47]. Tabeta et al. [48] reported that *P. gingivalis* LPS stimulates human gingival fibroblasts to secrete IL-1 and IL-6 via TLR4. In contrast, Zhang et al. [49] found that *P. gingivalis* LPS could utilize both TLR2 and TLR4 to induce the production of cytokines such as TNF-α, IL-1β, and IL-6 in THP-1 cells. In the present study, we observed that TLR4 and TLR2 expression increased in hPDLCs exposed to *P. gingivalis* LPS and that neutralizing anti-TLR2 and anti-TLR4 antibodies specifically inhibited *P. gingivalis* LPS-induced expression and secretion of IL-17 and IL-23, as well as Akt/PI3K activation and its downstream pathways, including MAPK and NF-κB. These results suggest that TLR2 and TRL4 play important roles in the defense mechanisms of microorganisms by stimulating a wide repertoire of signal transduction pathways that promote inflammatory processes.

Recent studies implicated SIRT1 in the regulation of inflammatory responses [32–34]. Resveratrol-induced SIRT1 activation or adenoviral-mediated SIRT1 overexpression blocks the expression and release of pro-inflammatory cytokines in response to environmental stress [50,51]. To assess the role of SIRT1 in IL-17 and IL-23 expression in hPDLCs, we tested the effects of SIRT1 inhibition and gene silencing. In the present study, we showed that downregulating SIRT1 expression via SIRT1 siRNA or blocking SIRT1 activity via sirtinol prevented LPS-mediated induction of IL-17 and IL-23 expression and production. This result is consistent with the previous finding that inhibition of SIRT1 by sirtinol reduces cytokine

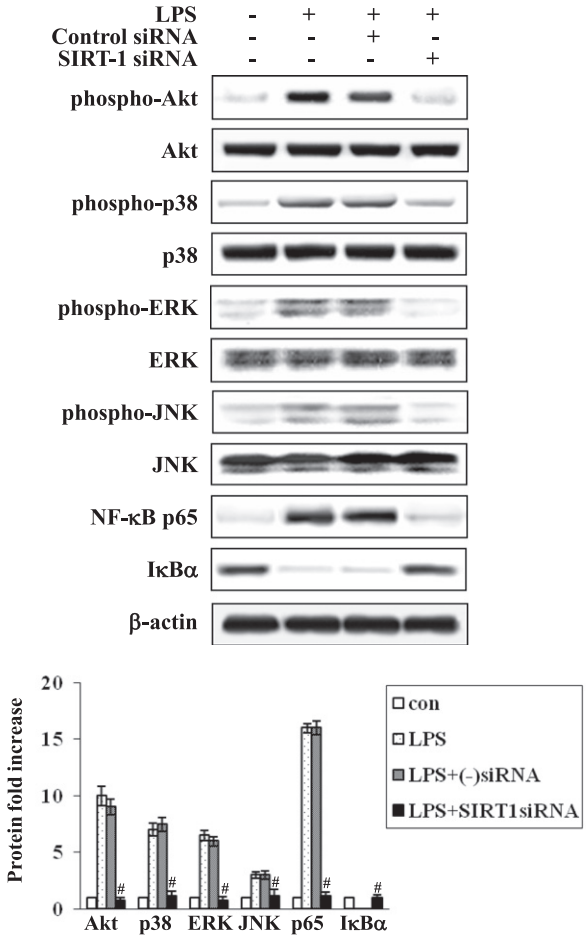

Fig. 8. Effects of SIRT1 siRNA on *P. gingivalis* LPS-induced Akt, MAPK, and NF-kB activation. Cells were transiently transfected with control vector or SIRT1 siRNA, followed by 1 h LPS treatment. Histogram is densitometric quantitation of protein expression. Similar results were obtained in three independent experiments. #Statistically significant difference compared to the LPS-treated group, $p < 0.05$.

production in a rodent model of smoke-induced airway inflammation [52], attenuates hepatic injury and pro-inflammatory cytokine production in rats [53], diminishes human dermal microvascular endothelial cell inflammatory responses to TNF-α and IL-1β [54], and blocks HMGB1-stimulated osteoclastic cytokines, including RANKL in hPDLCs [34]. These results suggested that SIRT1 inhibition may modulate the inflammatory response to periodontal pathogens in hPDLCs.

In the present study, downregulation of SIRT1 expression levels by siRNA diminished LPS-stimulated Akt, MAPK, and NF-κB activation. This may indicate that SIRT1 plays a modulatory role within the LPS signaling cascade. Reciprocally, we observed inhibitors for Akt, MAPK, and NF-κB blocked LPS-induced SIRT1 mRNA and protein expression. These results suggested that SIRT1 activation may be an important upstream target of Akt, MAPK, and NF-κB in the regulation of hPDLCs. Consistent with these findings, we previously reported that inhibitors for PI3K, MAPK and NF-κB blocked the mechanical stress-induced SIRT1 mRNA expression in hPDLCs [32]. Overall, the above findings suggest that direct functional interaction between SIRT1 and LPS signaling does exist in hPDLCs culture models.

Periodontal inflammation affects the net loss of bone by enhanced resorption and diminished bone formation. hPDLCs have osteoblastic properties and express ALP, OPN, and BSP [55]. Moreover, hPDLCs express RANKL, OPG, and M-CSF [11,14,34], which contribute to bone metabolism regulation, such as osteoclastogenesis. To evaluate hDPLCs differentiation status, we analyzed the mRNA expression of different genes involved in osteoblast (ALP, OPN, and BSP) and osteoclast differentiation (RANKL, OPG, and M-CSF). Our data demonstrated that LPS downregulated osteoblastic differentiation marker mRNA (ALP, OPN, and BSP) and concomitantly upregulated the expression of osteoclast differentiation markers (RANKL and M-CSF). These results are consistent with a previous study on the osteoclastic-inducing effects [56] and osteoblastic-inhibiting effects of *P. gingivalis* LPS [57]. In the present study, hPDLCs pretreatment with inhibitors of PI3K, p38 MAPK, ERK, JNK, NF-κB, anti-TLR4 and anti-TLR2 antibodies, sirtinol, and SIRT1 siRNA blocked LPS-induced upregulation of osteoclast markers (RANKL and M-CSF) and prevented downregulation of osteoblast markers (ALP, OPN, and BSP). Furthermore, SIRT1 inhibition by sirtinol and SIRT1 siRNA blocked LPS-induced activation of Akt/PI3K, p38, ERK, JNK, and NF-κB. These results suggest that SIRT1, AKt/PI3K, p38, ERK, JNK, and NF-κB, TLR2, and TLR4 pathways are involved in these responses. Further studies are needed to elucidate the detailed mechanism of action of LPS on SIRT1 signaling.

To our knowledge, this study is the first to demonstrate that SIRT1, Akt/PI3K, MAPK, NF-κB, TLR2, and TLR4 signaling pathways are involved in regulating the Th17-related molecules IL-17 and IL-23, which are stimulated by *P. gingivalis* LPS treatment in hPDLCs. Pharmacological modulation of SIRT1 may be a novel therapeutic approach for modulating the inflammatory response and bone destruction associated with periodontal disease.

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