Porphyromonas gingivalis LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells.

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Running title: LPS inhibits differentiation in PDLSCs

Abstract

Objective: Porphyromonas gingivalis (*P. gingivalis*) lipopolysaccharide (LPS) induces pro-inflammatory cytokines, such as interleukin-1 β (IL-1β), IL-6, and IL-8, which induce periodontal tissue destruction. Periodontal ligament stem cells (PDLSCs) play an important role in periodontal tissue regeneration and are expected to have future applications in cellular therapies for periodontitis. However, no studies have examined the effects of *P. gingivalis* LPS on PDLSCs. The aim of this study was to investigate how *P. gingivalis* LPS affects the osteoblastic differentiation and pro-inflammatory cytokine production of PDLSCs.

Design: PDLSCs were obtained from healthy adult human mandibular third molars. The identification of PDLSCs was confirmed by immunohistochemical evaluations of the mesenchymal stem cell markers STRO-1 and SSEA-4. Cell proliferation and osteoblastic differentiation were investigated by culturing the PDLSCs in a normal or osteogenic medium with *P. gingivalis* LPS (0, 1, or 10 µg/mL) and then measuring the alkaline phosphatase (ALP) activity and the production of collagen type 1 Alpha 1 (COL1A1), osteocalcin production, and mineralization. Additionally, we examined the

production of IL-1β, IL-6, and IL-8 in the PDLSCs.

Results: *P. gingivalis* LPS inhibited the ALP activity, COL1A1 and osteocalcin production, and mineralization in the PDLSCs, which are positive for STRO-1 and SSEA-4. *P. gingivalis* LPS also promoted cell proliferation and produced IL-1β, IL-6, and IL-8.

Conclusions: This study provides the first findings that *P. gingivalis* LPS inhibits osteoblastic differentiation and induces pro-inflammatory cytokines in PDLSCs. These findings will help clarify the relationship between periodontitis and periodontal tissue regeneration.

Introduction

One of the most common causes of tooth loss is periodontal disease, which is defined as the destruction of the alveolar bone and periodontal ligament by bacterial infection.[1] Many of the bacteria that cause periodontal disease are Gram-negative bacilli, such as *Porphyromonas gingivalis* (*P. gingivalis*), *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*.[2,3] *P. gingivalis* has been implicated as the causal pathogen in most cases of periodontal disease.[4-6] Lipopolysaccharide (LPS) is an outer membrane component of Gram-negative bacterial cell walls and an endotoxin with a wide range of biological activities. LPS induces the production of inflammatory cytokines, such as interleukin-1 β (IL-1β), IL-6, and IL-8.[7,8] These cytokines and LPS induce alveolar bone resorption and destroy periodontal tissue.[9]

Periodontal tissue is composed of the periodontal ligament (PDL), cementum, alveolar bone, and gingiva. The PDL is composed of fibrous connective tissue that connects the tooth root to the alveolar bone.[10] PDL cells have roles in maintaining tissue regeneration[11]. PDL cells produce bone-related proteins; they also express alkaline phosphatase (ALP) activity, collagen type 1 alpha 1, osteocalcin, and possess

mineralization capacity.[12-14] Although PDL cells are a component of connective tissues,

they also exhibit osteoblastic differentiation capacity.

Periodontal ligament stem cells (PDLSCs) exist in periodontal tissues and participate

in periodontal tissue regeneration.[15] Similar to other mesenchymal stem cells (MSCs),

PDLSCs have the potential to differentiate into osteoblasts.[16,17] PDLSCs have tissue

regeneration capacity.[18-20] PDLSCs exhibit greater potential to regenerate periodontal

tissues than other MSCs, such as bone marrow stromal cells.[21] Therefore, PDLSCs play

a particularly important role in periodontal tissue regeneration. Moreover, PDLSCs are

predicted to be an important source of cells in periodontal tissue regeneration therapy.

However, no studies have reported the effects of LPS on PDLSCs; therefore, this area of

investigation is critical.

The aim of this study was to characterize the effects of *P. gingivalis* LPS on

osteoblastic differentiation and mineralization in PDLSCs. Additionally, we examined

the production of inflammatory cytokines, such as IL-1β, IL-6, and IL-8 in the PDLSCs.

Materials and methods

Culture and isolation of human PDLSCs

The human tissue experiments were performed in accordance with the guidelines of the

Osaka Dental University for Medical Ethics, and all experiments were approved by the

Osaka Dental University Medical Ethics Committee (approval no. 110712). All

participants provided written informed consent to participate in this study, and the study

design was approved by the appropriate ethics review boards. PDLSCs were isolated

and cultured as described in our previous study.[22] After extraction, the teeth were rinsed

in Dulbecco's Modified Eagle's Medium (DMEM) with 500 U/mL penicillin and 500

μg/mL streptomycin (all from Nacalai Tesque, Kyoto, Japan). After the teeth were

rinsed, the PDL tissues were collected with a scalpel from the middle third of the root

surface and were minced into 1-mm^3 pieces. The minced tissues were digested for 1 h at

37°C in a solution of 3 mg/mL collagenase type I (Wako Pure Chemical Industrials Ltd.,

Tokyo, Japan) and 4 mg/mL dispase (Gibco BRL, Grand Island, NY, USA). The

digested tissue samples were pooled, and single-cell suspensions were obtained by

passing the pooled tissues through a 70-μm strainer (Falcon BD, Franklin Lakes, NJ,

USA). The cells were centrifuged at 1,000 rpm for 5 min and were resuspended in

normal culture medium containing 10% fetal bovine serum (Gibco BRL), 500 U/mL

penicillin (Nacalai Tesque), and 500 µg/mL streptomycin (Nacalai Tesque). The cells

were then seeded onto T75 culture dishes (Falcon BD) and incubated at 37°C in 5%

CO_2. After 5-10 days, the presence of single-cell colonies was confirmed, PDLSCs of

passage zero (P0) were seeded, and cells at passage P3-P5 were used for further

experiments.

Characterization of human PDLSCs as MSCs

The PDLSCs were evaluated by immunocytochemistry, as previously described.[22] The

PDLSCs were plated at 2.0×10^4 cells/mL in 300 µL of normal culture medium on

Lab-Tek® Chamber Slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and

were incubated for 3 days. The cells were fixed in cold 70% ethanol for 10 min at -20°C,

blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for

30 min at room temperature, and then incubated with mouse anti-human vimentin

antibody (Dako, Glostrup, Denmark), anti-STRO-1 antibody (Santa Cruz Biotechnology,

Santa Cruz, CA, USA), or anti-SSEA-4 antibody (Santa Cruz Biotechnology) overnight

at 4°C. After being washed with PBS, the cells were incubated for 60 min at room

temperature with a fluorescently labeled secondary anti-rabbit polyclonal IgG antibody (Dako). The samples were washed with PBS and mounted in Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The negative control was treated with PBS instead of a primary antibody. Images were obtained with a BZ-II all-in-one fluorescence microscope (Keyence Corporation, Osaka, Japan). The isolated PDLSCs differentiated into osteoblasts after being treated with an osteogenic differentiation medium containing 50 µM L-ascorbic acid 2-phosphate (Nacalai Tesque), 10 mM β-glycerophosphate (Wako), and 10 nM dexamethasone (Nacalai Tesque).

Cell proliferation assay

PDLSCs were plated in 96-well microplates at 2×10^4 cells/mL in normal culture medium (100 µL/well). After a 24-h culture for cell adherence, the medium was replaced with a medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL) (InvivoGen, San Diego, CA, USA) and the cells were incubated for 1, 3, 8, 24, 72, 120, and 168 h. The number of viable cells at each time point was determined by measuring the amount of formazan generated in 6 wells per group using the Cell Count Reagent SF (Nacalai Tesque). The formazan absorbance was measured at 450 nm, and the data were analyzed with the Soft Max® Pro Microplate Data Acquisition and Analysis software (Molecular

Devices, Sunnyvale, CA, USA).

Measurement of collagen type 1 Alpha 1

PDLSCs were plated at 4×10^4 cells/well in 24-well plates and were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the incubation continued for 7 days. The culture supernatant was collected, and collagen type 1 Alpha 1 (COL1A1) was detected with a detection kit (USCN Life Science Inc., Wuhan, China).

Measurement of alkaline phosphatase (ALP) activity

PDLSCs were plated at 4×10^4 cells/well in 24-well plates and were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for 7 or 14 days and then washed with PBS and lysed with 300 µL of 0.2% Triton X-100 (Sigma, St. Louis, MO, USA). ALP was measured by using a 1-step pNPP substrate (Pierce Biotechnology, Inc., Rockford, IL, USA), and the absorbance was measured at 405 nm. The DNA content was measured using the PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). To normalize the ALP activity, the amount of ALP was normalized to the

amount of DNA in the cell lysate. The data were analyzed with the Soft Max$^®$ Pro software.

Measurement of osteocalcin

PDLSCs were plated at 4×10^4 cells/well in 24-well plates and were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for 21 days. The culture supernatant was collected, and the osteocalcin levels were measured with an osteocalcin detection kit (Takara-Shuzo Co. Otsu, Shiga, Japan).

Measurement of calcium deposition

PDLSCs were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for 21 days. The extracellular calcium deposition was measured after the calcium was dissolved with 10% formic acid. The calcium was quantified using a Calcium E-test Kit (Wako) according to the manufacturer's protocol. The reaction product absorbance was measured at 610 nm and analyzed with the Soft Max$^®$ Pro software.

Alizarin Red staining

Alizarin Red is used to ascertain the presence of mineralised nodules formed by cells of osteogenic lineages; these nodules indicate the calcification of the bone matrix. We qualitatively analyzed the calcium deposition in the cell cultures using Alizarin Red staining as a biochemical mineralisation assay. PDLSCs were plated at 4×10^4 cells/well in 24-well plates and were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for 21 days. The medium was removed, the cells were washed with PBS, and the PDLSCs were fixed in 70% ethanol for 10 min at -20°C. The PDLSCs were stained with a solution of 1% Alizarin Red S (Wako) for 3 min at room temperature and were washed 3 times with distilled water.

Measurements of IL-1β, IL-6, and IL-8

The PDLSCs were plated at 4×10^4 cells/well in 24-well plates and were cultured to confluence in normal medium. The medium was replaced with osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/ml), and the cells were cultured for 21 days. The production of IL-1β, IL-6, and IL-8 in the supernatant was measured with a

detection kit (USCN Life Science Inc.)

Statistical analysis

In this study, 6 wells were prepared for the cell proliferation assay, and 3 wells were prepared for the remaining experiments; each experiment was repeated 3 times. The statistical analysis was performed with IBM SPSS Statistics. Significant differences were determined using Student's *t*-test. The control (0 µg/mL *P. gingivalis* LPS) and experimental groups (1 or 10 µg/mL *P. gingivalis* LPS) were compared to identify significant differences (*$p < 0.05$).

Results

Isolation and characterization of PDLSCs

The PDL-derived cells formed clonogenic clusters of fibroblast-like cells (Fig. 1A). No staining was detected in control (Fig. 1B). The PDLSCs derived from the PDL cells were positive for vimentin (Fig. 1C). The PDLSCs were also positive for STRO-1 (Fig. 1D) and SSEA-4 (Fig. 1E), which are MSC markers. The isolated PDLSCs were capable of differentiating into osteogenic cells (Fig. 1F).

Cell proliferation

The proliferation of the PDLSCs in the normal culture medium containing *P. gingivalis* LPS was significantly enhanced compared with the cells cultured without LPS (Fig. 2, 1 h, 3 h, 8 h, 120 h, and 168 h *$p < 0.05$). The proliferation of PDLSCs was significantly enhanced only in culture medium at a concentration of 10 μg/mL *P. gingivalis* LPS (Fig. 2, 24 h and 72 h *$p < 0.05$).

ALP activity

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The ALP activity of PDLSCs was significantly enhanced only in osteogenic medium at a concentration of 10 μg/mL *P. gingivalis* LPS (Fig. 3A, day 7, *p < 0.05; Fig. 3B, day 14, *p < 0.05).

COL1A1 and osteocalcin production

The COL1A1 production was significantly lower in the osteogenic medium containing *P. gingivalis* LPS than in the control (Fig. 4A, day 7, *p < 0.05). The osteocalcin production was significantly lower in the osteogenic medium containing *P. gingivalis* LPS than in the control (Fig. 4B, day 21, *p < 0.05).

Extracellular calcium deposition and Alizarin Red staining

The calcium deposition was significantly lower in the osteogenic medium containing *P. gingivalis* LPS (Fig. 5A, *p < 0.05).

The number of calcified nodules stained with Alizarin Red was lower in the osteogenic medium containing *P. gingivalis* LPS than in the control medium (Fig. 5B, day 21, *p < 0.05).

IL-1β, IL-6, and IL-8 production

The production of IL-1β and IL-8 was significantly enhanced in the osteogenic medium

containing *P. gingivalis* LPS compared with the control medium (Fig. 6A and C, day 21,

*p < 0.05). The production of IL-6 was significantly enhanced only in the osteogenic

medium at a concentration of 10 μg/mL *P. gingivalis* LPS (Fig. 6B, day 21, *p < 0.05).

Discussion

The results of this study indicated that *P. gingivalis* LPS enhanced the proliferation of

PDLSC cells. The osteoblastic differentiation assay revealed that the ALP activity,

COL1A1 production, osteocalcin production, and mineralization were significantly

suppressed by *P. gingivalis* LPS. *P. gingivalis* LPS also enhanced the production of

IL-1β, IL-6, and IL-8.

PDLSCs express STRO-1, a marker of bone marrow mesenchymal stem cells

(BMSCs).[16] SSEA-4 is also a marker of MSCs[23] and PDLSCs.[24] In this study, the

PDLSCs that originated from the PDL were positive for STRO-1 and SSEA-4.

Therefore, we investigated whether *P. gingivalis* LPS would affect cell proliferation and

the differentiation of PDLSCs into hard tissue-forming osteoblasts.

P. gingivalis LPS and *Escherichia coli* (*E. coli*) LPS inhibit the proliferation of dental

follicle cells (DFCs).[25] *E. coli* LPS inhibits the proliferation of PDL fibroblasts,[26]

whereas *P. gingivalis* LPS promotes PDL fibroblast proliferation.[27] *E. coli* LPS

promotes mouse MSC proliferation.[28] In this study, PDLSC proliferation was promoted

by *P. gingivalis* LPS. PDL cells may activate immune cells in response to bacterial

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LPS.[29,30] *E. coli* LPS promotes the proliferation of peripheral blood mononuclear cells (PBMCs), which are important immune system components.[31] Thus, we determined that the proliferation of PDLSCs was activated by a reaction to LPS.

The formation of new bone requires the production of collagen type 1.[32] Collagen type 1 is produced in the early stages of osteoblastic differentiation. *P. gingivalis* LPS inhibits COL1A1 mRNA expression in human PDL fibroblasts[33] and in mouse calvarial osteoblasts.[34] In this study, *P. gingivalis* LPS inhibited COL1A1 production in PDLSCs, suggesting that *P. gingivalis* LPS can inhibit the osteoblastic differentiation of PDLSCs at an early stage of differentiation.

ALP is a marker of the osteoblastic phenotype and is secreted during mid-stage differentiation,[35] which is when mineralization is initiated.[36] *P. gingivalis* LPS has been found to inhibit ALP activity in PDL fibroblasts, dental pulp cells (DPCs),[37] and BMSCs.[38] In contrast, *E. coli* LPS promotes ALP activity in DFCs.[25] In this study, *P. gingivalis* LPS inhibited the ALP activity in PDLSCs, suggesting that *P. gingivalis* LPS can inhibit the osteoblastic differentiation of PDLSCs during mid-stage differentiation.

Osteocalcin is a non-collagenous protein component of bone matrix and a late marker of osteoblastic differentiation.[39] *P. gingivalis* LPS suppresses osteocalcin mRNA expression in PDL fibroblasts[33] and mouse BMSCs.[38] In this study, *P. gingivalis* LPS

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inhibited osteocalcin production, thus inhibiting the differentiation of PDLSCs into mature osteoblasts.

We quantified the calcium deposition in cultured PDLSCs using a biochemical mineralisation assay based on Alizarin Red staining and extracellular calcium deposition. Alizarin Red is used to ascertain the presence of mineralised nodules formed by cells of osteogenic lineages; these nodules are indicative of bone matrix calcification. In general, Alizarin Red staining is used as a biochemical mineralisation assay to qualitatively analyze the calcium deposition in cell cultures. This technique has also been used to assess the mineralisation of PDLSCs.[16,17] *E. coli* LPS inhibits the mineralisation of PDL fibroblasts[33] and mouse osteoblasts.[40] In another study, *P. gingivalis* LPS inhibited the mineralisation of rat calvarial cells.[41] In the present work, *P. gingivalis* LPS reduced the number of mineralized nodules and the amount of extracellular calcium deposition in PDLSCs. The presence of mineralisation during osteoblastic differentiation indicates that differentiation into mature osteoblasts was inhibited by *P. gingivalis* LPS. This result is consistent with the observation that the production of osteocalcin, a marker of mature osteoblastic differentiation, was inhibited.

LPS induces pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-8, which are strongly related to the destruction of periodontal tissue.[42,43] *P. gingivalis* LPS induces

IL-1β and IL-6 in human monocytic cell.[44] Another study suggested that *P. gingivalis* LPS induces IL-6 in mouse alveolar macrophage cell[45] and human gingival fibroblasts.[46] *E. coli* LPS induces IL-1β, IL-6, and IL-8 in DFCs.[25] *P. gingivalis* LPS induces IL-1β, IL-6, and IL-8 in PDL cells.[47] On the other hands, *P. gingivalis* LPS but not *E. coli* LPS induces IL-6 in PDL cells.[48] In the present work, *P. gingivalis* LPS induced the production of IL-1β, IL-6, and IL-8 in PDLSCs. These results suggest that the production of IL-1β, IL-6, and IL-8 associated with periodontal tissue destruction is enhanced by *P. gingivalis* LPS. Thus, PDLSCs may perform a function similar to that of the active immune cells that produce pro-inflammatory cytokines in response to stimulation by *P. gingivalis* LPS.

Seo et al. suggested that the inhibitory effect of *P. gingivalis* LPS on osteoblastic differentiation in PDL fibroblasts was activated by mitogen-activated protein kinase signaling pathways.[33] Uehara et al. suggested that the inhibitory effect of *P. gingivalis* LPS on osteoblastic differentiation in PDL fibroblasts was regulated by DNA hypermethylation.[49] Robert et al. reported that *P. gingivalis* LPS inhibits osteoblastic differentiation of rat alveolar bone osteoblasts through alteration of decorin and biglycan, which involve alveolar bone destruction.[50] However, these molecular mechanisms of the present study are not yet clear. Therefore, additional studies on this

issue would be needed to further clarify the mechanisms of *P. gingivalis* LPS-inhibited osteoblastic differentiation in PDLSCs.

PDL tissue is composed of osteoblasts, fibroblasts, and MSCs. In the present study, we used PDLSCs which particularly contribute periodontal tissue generation among PDL tissue. Then, we have made a discussion used the previous reported studies which examined the effect of LPS on PDL fibroblasts, osteoblasts, and MSCs. As these cells were designed from PDLSCs, we might be suggested that *P gingivalis* LPS inhibits osteoblastic differentiation and induces pro-inflammatory cytokines from PDLSCs as well as previous reported study.[33,40,47]

In conclusion, the results of this study suggest that *P. gingivalis* LPS inhibits osteoblastic differentiation and mineralisation and induces pro-inflammatory cytokines in PDLSCs, which play an important role in periodontal tissue regeneration. These findings will help clarify the relationship between periodontal disease and periodontal tissue regeneration. This study provides the first important findings concerning the effects of *P. gingivalis* LPS on PDLSCs.

Acknowledgments

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Figure legends
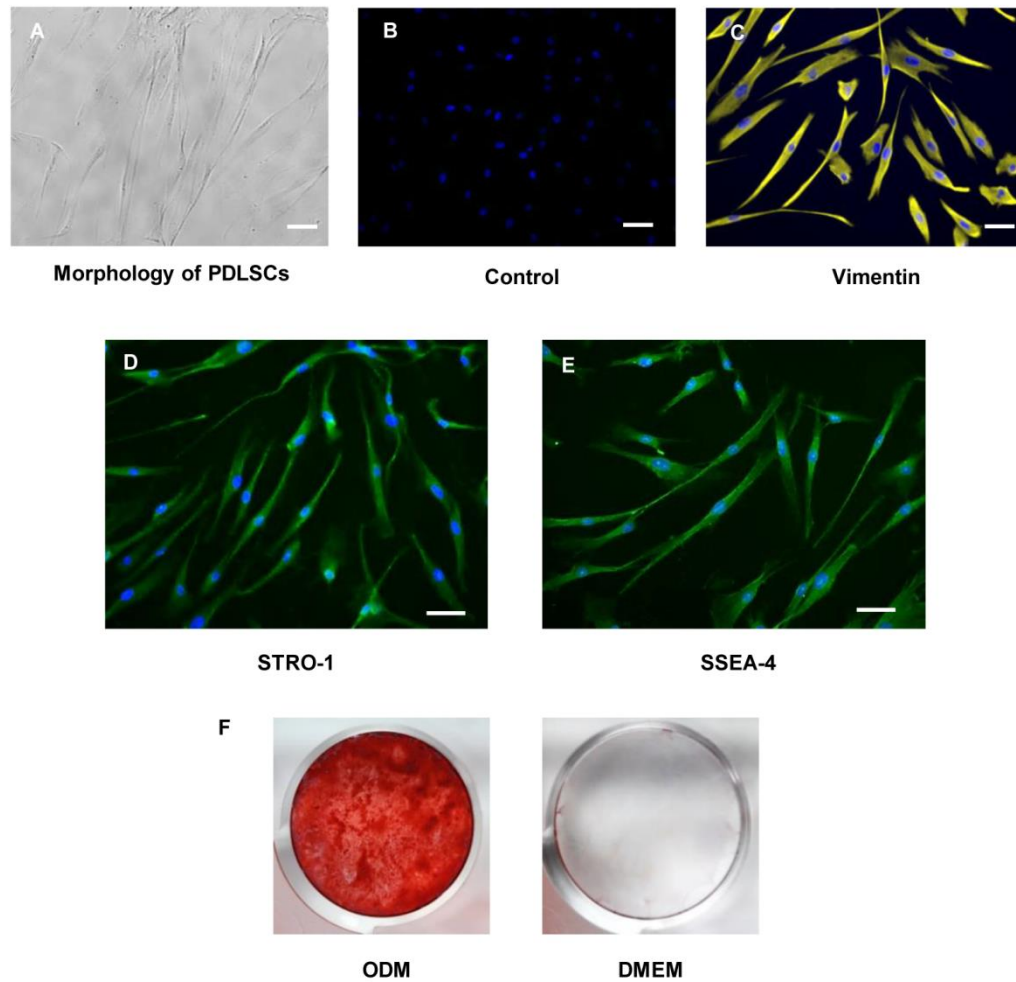
Fig. 1 Characterization of human PDLSCs as MSCs. A) Morphology of PDLSCs.

The cultured PDLSCs showed a fibroblast-like cellular morphology, including the

presence of dendrites, under phase-contrast microscopy. B) Immunocytochemical

staining of the negative control. C) Immunocytochemical staining for vimentin.

Vimentin was observed in the cytoplasm of the PDLSCs (yellow) but not in the nuclei.

D) Immunocytochemical staining for STRO-1. The PDLSC were positive for STRO-1

(green). E) Immunocytochemical staining for SSEA-4. The PDLSC were positive for

SSEA-4 (green). Scale bar = 50 µm. (F) Osteoblastic differentiation of PDLSCs. The

isolated PDLSCs were stained with Alizarin Red after 21 days of cultivation in

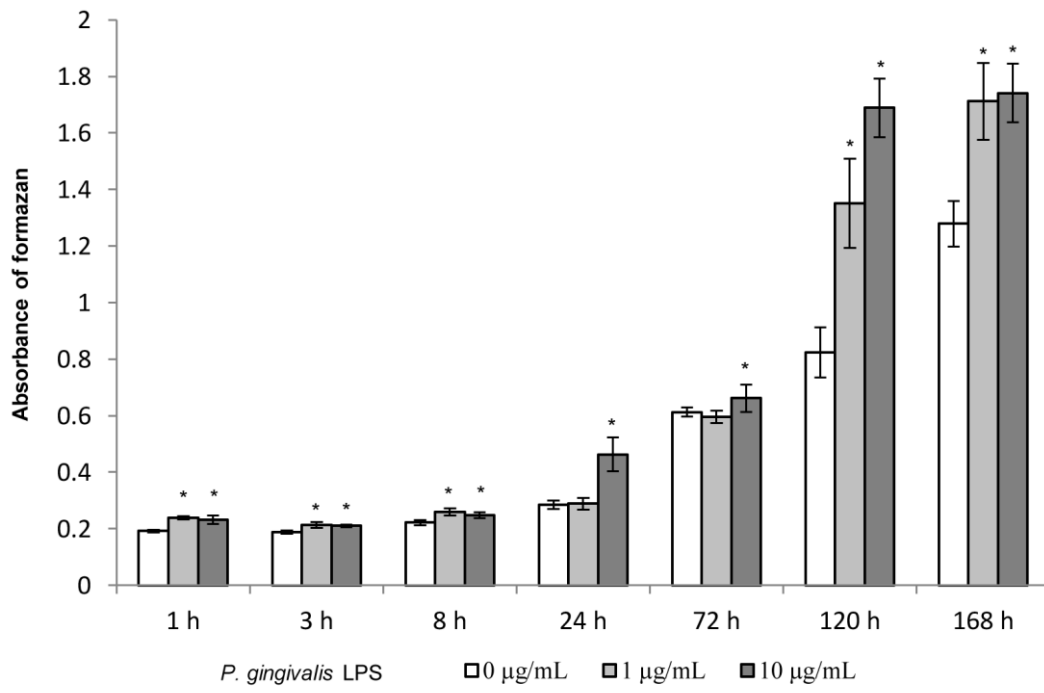osteogenic medium (ODM) and in normal culture medium (DMEM).

Fig. 2 *P. gingivalis* LPS and cell proliferation. PDLSCs were cultured in 100 μL of normal culture medium containing *P. gingivalis* LPS (0, 1, or 10 μg/mL). The proliferation of the PDLSCs was significantly enhanced compared with the cells cultured without LPS (*p < 0.05 at 1 h, 3 h, 8 h, 120 h, and 168 h) and only in culture medium at a concentration of 10 μg/mL *P. gingivalis* LPS (*p < 0.05 at 24 h and 72 h).
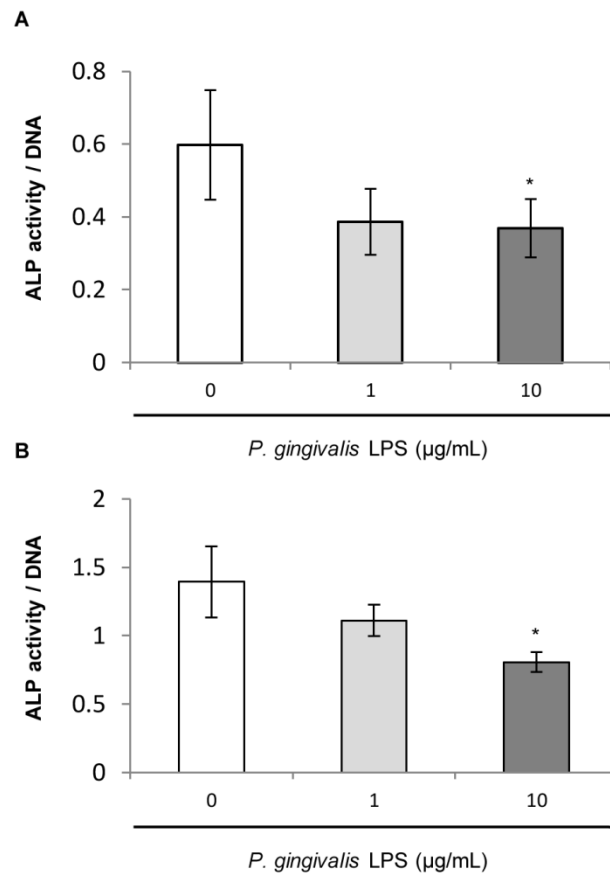
Fig. 3 *P. gingivalis* LPS and ALP activity. After the PDLSCs reached confluence, the normal culture medium was replaced with an osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for an additional 7 and 14 days. The ALP activity of PDLSCs was significantly enhanced only in osteogenic medium with *P. gingivalis* LPS (10 µg/mL) (A, day 7, *p < 0.05; B, day 14, *p < 0.05).
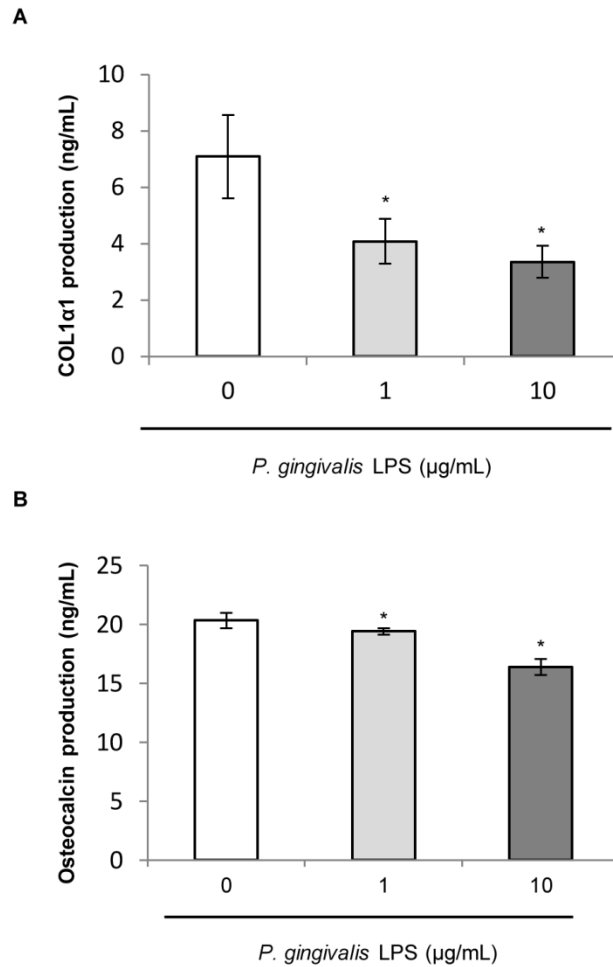
Fig. 4 *P. gingivalis* LPS and the production of collagen type 1 Alpha 1 (COL1A1) and osteocalcin. After the PDLSCs reached confluence, the culture medium was replaced with an osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL), and the cells were cultured for 7 or 21 days. The COL1A1 production (A; day 7: *p < 0.05) and the osteocalcin production were significantly lower in the osteogenic medium containing *P. gingivalis* LPS than in the control medium (B, day 21, *p < 0.05).
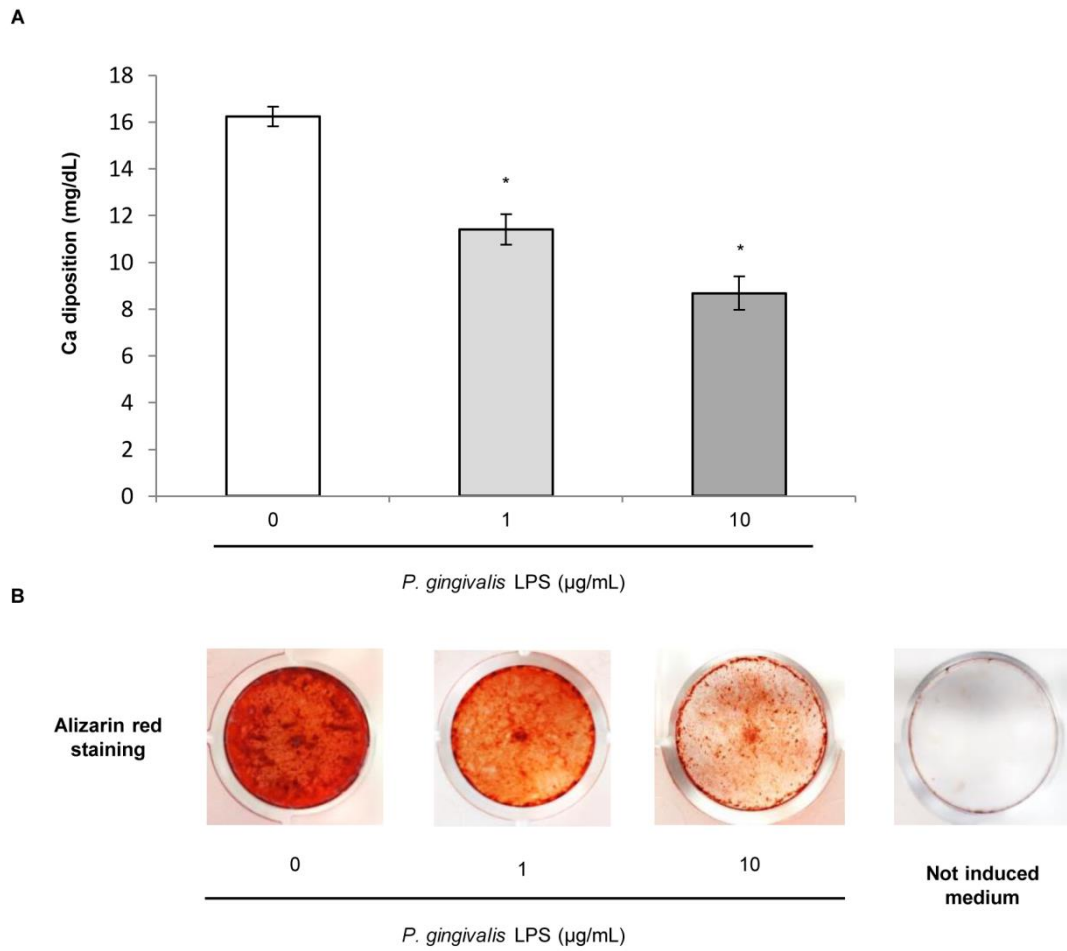
A

B

Fig. 5 *P. gingivalis* LPS and mineralisation. A) Confluent PDLSCs were stained with Alizarin Red after 21 days of cultivation in an osteogenic medium containing *P. gingivalis* LPS (0, 1, or 10 µg/mL). B) The extracellular calcium deposition was measured on day 21 (*$p < 0.05$).
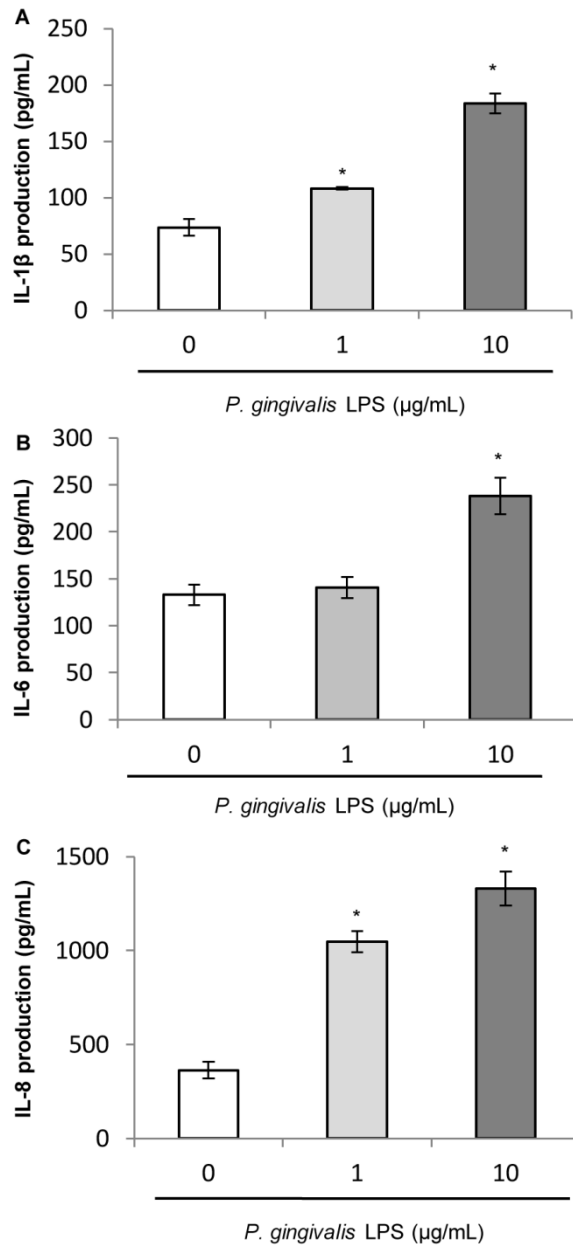
Fig. 6 *P. gingivalis* **LPS and the levels of IL-1β, IL-6, and IL-8.** After the PDLSCs

reached confluence, the culture medium was replaced with an osteogenic medium

containing *P. gingivalis* LPS (0, 1, or 10 μg/mL), and the cells were cultured for 21 days.

The production of IL-1β and IL-8 was significantly enhanced in the medium compared

with the control medium (A and C, day 21, *p < 0.05). The production of IL-6 was

significantly enhanced only in the medium with *P. gingivalis* LPS (10 µg/mL) (B, day

21, *p < 0.05).