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Porphyromonas gingivalis-Derived Lipopolysaccharide-Mediated Activation of MAPK Signaling Regulates Inflammatory Response and Differentiation in Human Periodontal Ligament Fibroblasts

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***Porphyromonas gingivalis* (*P.g.*), which is a potential pathogen for periodontal diseases, contains lipopolysaccharide (LPS), and this endotoxin stimulates a variety of cellular responses. At present, *P.g.*-derived LPS-induced cellular responses in human periodontal ligament fibroblasts (PDLFs) are not well characterized. Here, we demonstrate that *P.g.*-derived LPS regulates inflammatory responses, apoptosis and differentiation in PDLFs. Interleukin-6 (IL-6) and -8 (IL-8) were effectively upregulated by treatment of *P.g.*-derived LPS, and we confirmed apoptosis markers including elevated cytochrome c levels, active caspase-3 and morphological change in the presence of *P.g.*-derived LPS. Moreover, when PDLFs were cultured with differentiation media, *P.g.*-derived LPS reduced the expression of differentiation marker genes, as well as reducing alkaline phosphatase (ALP) activity and mineralization. *P.g.*-derived LPS-mediated these cellular responses were effectively abolished by treatment of mitogen-activated protein kinase (MAPK) inhibitors. Taken together, our results suggest that *P.g.*-derived LPS regulates several cellular responses via activation of MAPK signaling pathways in PDLFs.**

***Keywords*:** apoptosis, cell differentiation, lipopolysaccharide, mitogen-activated protein kinase, periodontal ligament, *Porphyromonas gingivalis*

Introduction

The periodontal ligament (PDL) is an important structure that supports the teeth and alveolar bone. The PDL acts to restrict the movement of each tooth, while also absorbing mechanical stresses. In addition to this basic feature, the PDL has the capacity to regenerate into periodontal tissue (Beertsen *et al.*, 1997). The PDL is largely composed of cementoblasts, osteoclasts, osteoblasts, and fibroblasts (Isaka *et al.*, 2001; Seo *et al.*, 2004). Among these compartments, osteoclasts and osteoblasts have a role in bone resorption and bond formation, respectively. These functions are regulated by various factors, including hormones, growth factors, and cytokines, all acting in concert to promote proper bone-remodeling (Olney, 2003).

PDL cells have been shown to have characteristics similar to osteoblastic cells. This includes alkaline phosphatase (ALP) activity and the formation of inducible mineralized nodules (Kawase *et al.*, 1988; Basdra and Komposch, 1997; Hagewald *et al.*, 1998). ALP activity, which is often used as a marker for osteoblastic differentiation, is regulated by several factors including annexin-2 and certain cytokines (Gillette and Nielsen-Preiss, 2004). Several complex mechanisms are required for the differentiation of cells into osteoblasts, including proliferation and regulation of protein expression (Manolagas, 2000). In addition to ALP, collagen type I α 1 (ColA1), osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP) have been used as maker proteins to confirm osteoblastic differentiation (Darongsuwan and Pavasant, 2001). Since a recent study suggested that PDL cells may have potential for the treatment of periodontal diseases (Seo *et al.*, 2004), it is important to have a detailed characterization of these cells.

Previous studies have shown that stimuli such as infectious agents, inflammatory response, and various signaling pathways can induce cellular responses in PDL cells. For example, the bone forming capacity of PDL cells is affected by retinoic acid, chitosan, nitric oxide, and interleukin-1β (IL-1β) (Lee *et al.*, 2009), while proliferation is regulated by inorganic polyphosphate, the cytolethal distending toxin of *Aggregatibacter actinomycetemcomitans* (*A.a.*) (Sun *et al.*, 2007). In addition, mechanical stresses induce apoptosis, whereas cytokine responses are affected by bacterial infection and H_2O_2 exposure in PDL cells (Yamamoto *et al.*, 2006; Lee *et al.*, 2008).

Porphyromonas gingivalis (*P.g.*) is a Gram-negative bacterium typically found in oral tissue. While the exact mechanisms and connections are unclear, *P.g.* is etiologically associated with periodontal diseases. Recently, it was shown that exposure to this bacterium can regulate a number of functions, including mitochondrial dysfunction, production of cytokines and adhesion molecules, bone formation, proliferation, and apoptosis, in various cell types (Bullon *et al.*, 2011; Hashizume *et al.*, 2011).

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Lipopolysaccharide (LPS) is found in the outer membrane of Gram-negative bacteria, where it contributes to the structural integrity and stabilization of membranes. LPS also acts as an endotoxin, and as such, it is an important agent in the induction of various metabolic responses including the immune response (Aly *et al.*, 2010; Angrisano *et al.*, 2010). Although it has been reported that *P.g.*-dervied LPS induced galectin-9 expression and released the receptor activator of the nuclear factor-κB (NF-κB) ligand in human PDL cells during the inflammation process (Kasamatsu *et al.*, 2005), whether *P.g.*-derived LPS induces PDL cell responses remains a question. The objective of this study was to characterize the cellular responses of human PDL cells to *P.g.*-derived LPS.

Materials and Methods

Cell culture

Human PDLFs (ScienCell, USA) were cultured in α-MEM (Hyclone, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, UK). Cells were maintained at 37°C in a 5% CO_2 incubator. Indicated concentration of *P.g.*-derived LPS was used in each experiment. For differentiation conditions, the cells were cultured in the above media with 10 mM β-glycerophosphate (Invivogen, USA), 50 mg/ml L-ascorbic acid (Sigma, USA), and 100 nM dexamethasone (Sigma). For the MAPK inhibitor experiment, PDLFs were treated with indicated inhibitors including p38 (SB203580, Sigma), ERK (PD98059, Sigma), and JNK (SP600125, Sigma) for 1 h before treatment of LPS. p38 and ERK inhibitors were used at concentrations of 20 mM, and JNK inhibitor was used at a concentration of 10 mM.

Proteome profiling

Human PDLFs were washed with phosphate buffered saline (PBS) and collected in 1.5-ml tubes. Cells were lysed using lysis buffer 6 from the Proteome Profiler Antibody Array kit (R&D Systems, USA). Next, 40 μg of protein extracts were bound on part A and part B membranes, both of which had been pre-blocked overnight prior to their use. Each membrane was then incubated with the appropriate detection antibody cocktail for 2 h. After washing, the membranes were incubated with streptavidin-conjugated HRP for 30 min. Proteins were detected by using an enhanced chemiluminescent substrate (Thermo Scientific, USA).

Western blot analysis

Human PDLFs were washed with PBS and lysed using RIPA buffer (50 mM Tris; pH 8.0, 150 mM NaCl, 5 mM EDTA; pH 8.0, 0.8% Triton X-100) with an added protease inhibitor cocktail (Roche, Switzerland). Equal amounts of the extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked using 3% BSA and incubated overnight with a 1:1,000 dilution of primary antibody (Cell Signaling Technology, USA). Membranes were then incubated for 1 h with 1:5,000 dilution of anti-rabbit secondary antibody (Cell Signaling Technology). The membrane was developed using an ECL reagent and the signals were detected using a fluorescence imaging system (Fuji Film, Japan).

Annexin-V staining assay

The Annexin V-FITC Apoptosis Detection Kit (Komabiotech, Korea) allows detection of phosphatidylserine on the cell surface. Assays were performed according to the manufacturer's instructions. Briefly, the cells were trypsinized, washed with cold PBS, and resuspended with 0.5 ml of 1× binding buffer. The cells were incubated with 1.25 μl FITC Annexin-V, washed with cold 1× binding buffer, and incubated with 10 μl propidium iodide (PI, 50 μg/ml) for 15 min in darkness. The cells were immediately analyzed by flow cytometry (Becton-Dickinson, USA).

Real time PCR

The total RNA was extracted from human PDLFs using an RNA extraction kit (INTRON, Korea). First-strand cDNA was synthesized from 1 μg RNA by using a reverse transcriptase PCR kit (Applied Biosystems, USA). The cytokines and differentiation marker genes are amplified using following primer sets: IL-8 (F: 5′-TCTGCTAGCCAGGAT CCACAA-3′ and R: 5′-GTGCTTCCACATGTCCTCACA-3′), IL-6 (F: 5′-ATGAACTCCTTCTCCACAAGCGC-3′ and R: 5′-GAAGAGCCCTCAGGCTGGACTG-3′), ColA1 (F: 5′-ATGCCGTGACTTGAGACTCAGC-3′ and R: 5′-CCGGA TACAGGTTTCGCCAGTA-3′), OCN (F: 5′-AAAGGTGC AGCCTTTGTGTCC-3′ and R: 5′-TCACAGTCCGGATT GAGCTCA-3′), OPN (F: 5′-TTCACTCCAGTTGTCCCC ACA-3′ and R: 5′-TGGATGTCAGGTCTGCGAAAC-3′), BSP (F: 5′-GAACAAGGCATAAACGGCACC-3′ and R: 5′-TTCTGCATTGGCTCCAGTGAC-3′). cDNA templates were assayed by using the real-time quantitative PCR kit (Applied Biosystems) containing each primer in a final reaction volume of 15 μl. The real-time PCR device (Applied Biosystems) conditions were as follows: i) serial heating at 95°C for 10 min; ii) amplification for 45 cycles consisting of 15 sec denaturation at 95°C, 15 sec annealing at 56°C, and 33 sec extension at 72°C. Quantification of each value was normalized by using the housekeeping gene *GAPDH*.

Measurement of ALP activity

ALP activity was analyzed using an ALP assay kit (BioAssay Systems, USA). PDLFs were washed with PBS and resuspended with alkaline buffer followed by 4 min of incubation with the ALP assay working solution. After incubation, the absorbance of p-nitrophenol was measured at 405 nm using a microplate reader (Molecular Devices, USA).

Mineralization assay

For the mineralization assays, PDLFs were washed with PBS and fixed in ice-cold 70% ethanol for 10 min. Cells were washed twice with distilled water followed by staining with Alizarin red S (Sigma) for 45 min. The stained cells were photographed using a phase-contrast microscope, and the mineralized areas were quantified using an image-processing system.

Statistical analysis

Means and standard deviations were calculated for quantitative data. The collected data for each group was compared by Repeated Measures Analysis of Variance using a statistical software package. $P<0.05$ was considered to indicate statistical significance.

Results

P.g.-derived LPS regulates inflammatory responses in PDLFs

To determine whether *P.g.*-derived LPS induces expression of cytokines as several reported previously, we performed real-time PCR using LPS-treated or -untreated PDLFs. First, we test to set the proper concentration of LPS and time condition in PDLFs. Using primer sets for IL-6 and IL-8, we confirm that mRNA levels of these cytokines are effectively increased at 24 h post-treatment and 200 to 500 ng/ml concentration of *P.g.*-derived LPS (Figs. 1A and 1B). After confirming proper time and concentration condition, we performed equal experiment at 24 h post-treatment of LPS in the presence of different concentration of LPS. As shown in Fig. 1C and 1D, IL-6 and IL-8 were upregulated about 4-fold in the presence of 500 ng/ml concentration of LPS. These data suggest that *P.g.*-derived LPS can regulate the mRNA levels of inflammatory cytokines in PDLFs.

Effect of *P.g.*-derived LPS treatment on apoptosis

To determine whether *P.g.*-derived LPS affects apoptosis in PDLFs, we examined the expression levels of cytochrome-c and the mitochondrial apoptosis-associated protein. In addition, we measured the levels of procaspase-3 and cleaved caspase-3, which is the active form of this protein. As shown in Fig. 2A, *P.g.*-derived LPS increased the expression of cytochrome-c, while expression of Bax was not affected. In addition, LPS treatments increased both procaspase-3 and cleaved caspase-3 levels in a dose-dependent manner. Next, we utilized light microscopy to screen for morphological changes in PDLFs following treatment with various concentrations of *P.g.*-derived LPS. Following 48 h of treatment with 0 μg/ml, 5 μg/ml, 10 μg/ml, and 20 μg/ml of LPS, we observed a significant reduction in spindle-shaped fibroblasts in treated cells, as compared to untreated cells. In addition, there was an increase in cell death for PDLFs that were exposed to *P.g.*-derived LPS (Fig. 2B). Then, we examined cellular apoptosis in PDLFs by using FITC-conjugated Annexin V staining. Annexin V has a high affinity for phosphatidylserine (PS), a plasma membrane phospholipid. In apoptotic cells, PS is exposed to the extracellular environment, and then Annexin V binds to PS. Therefore, cells that stain positive for Annexin V-FITC and negative propidium iodide (PI) are undergoing apoptosis. Cells that stain positive for Annexin V-FITC and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive. H_2O_2 treatment induced apoptotic cell death (Fig. 2C-b). *P.g.*-derived LPS treatment showed an increased level of Annexin V staining compared to untreated cells (Fig. 2C-a), indicating that *P.g.*-derived LPS induces apoptosis in PDLFs (Figs. 2C-c and d). Together, these results suggest that apoptotic marker proteins are in-

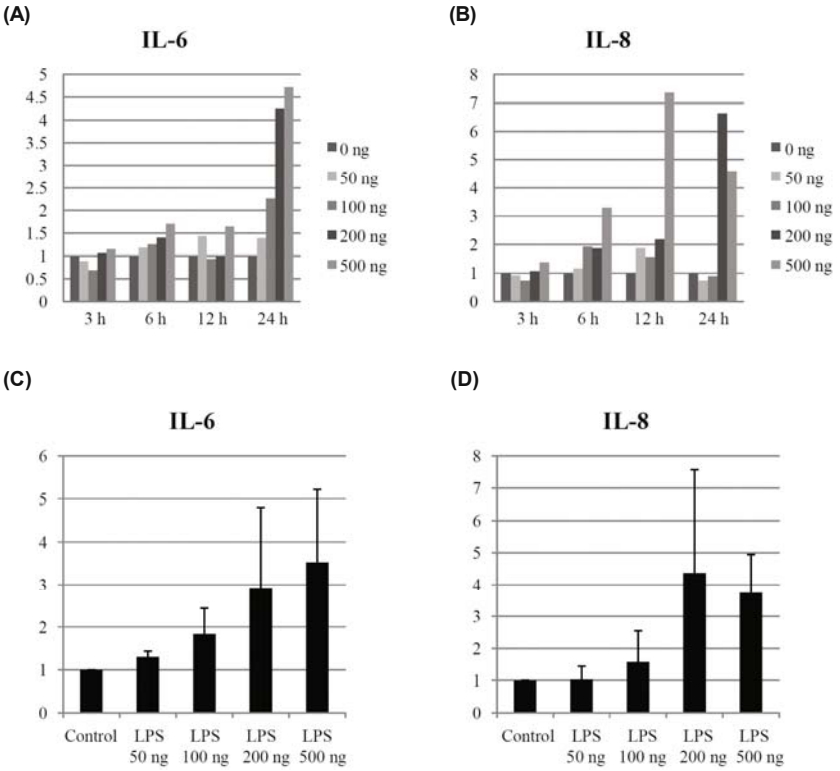

Fig. 1. *P.g.*-derived LPS regulates inflammatory responses in PDLFs.(A, B) PDLFs were treated with indicated concentrations of *P.g.*-derived LPS for different time points. After post-treatment of LPS at indicated time points, mRNA levels of IL-6 and IL-8 were measured by real-time PCR. (C, D) PDLFs were treated with indicated concentration of LPS at 24 h post-treatment. mRNA levels were measured by real-time PCR. *GAPDH* was used for normalization of each result.

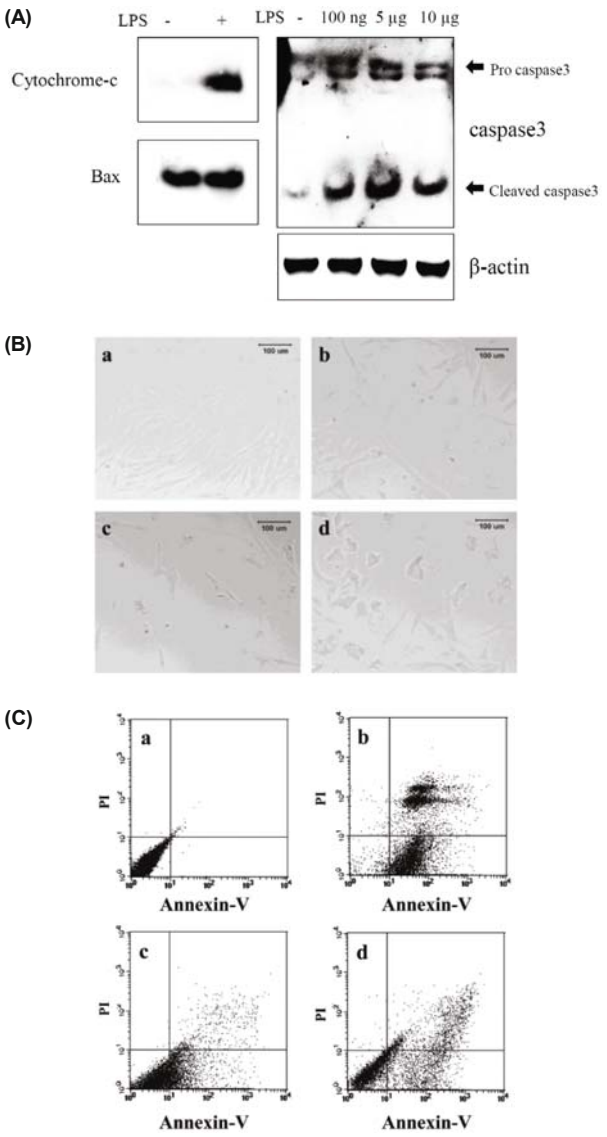

Fig. 2. Effects of *P.g.*-derived LPS on apoptotic responses in PDLFs. PDLFs were treated with 100 ng/ml of *P.g.*-derived LPS unless otherwise indicated. Apoptosis-associated responses were confirmed by western blot analysis and phase-contrast microscopy. (A) Total cell lysates from *P.g.*-derived LPS-treated and untreated PDLFs were subjected to SDS-PAGE and western blot analysis of cytochrome-c, Bax, and caspase-3. (B) Apoptotic effects of treating PDLFs with high concentrations of *P.g.*-derived LPS were observed using phase-contrast microscopy. (a) control, (b) 5 μg/ml of LPS, (c) 10 μg/ml of LPS, and (d) 20 μg/ml of LPS (bar=100 μm). (C) Annexin V-FITC staining of LPS-treated PDLFs. PDLFs were incubated with H_2O_2 (C-b) or 5 μg/ml (C-c) or 10 μg/ml (C-d) of *P.g.*-derived LPS for 20 h (C-a) control.

creased in the presence of *P.g.*-derived LPS, whereas high concentrations of LPS can cause visible morphological changes and apoptosis in PDLFs.

P.g.-derived LPS-induced activation of signal transduction proteins

Next, we performed proteome profiling to find some factors which affected LPS-mediated dysregulated cellular responses in PDLFs. For this study, we confirmed different phosphorylation levels of various signal transduction proteins in the presence or absence of *P.g.*-derived LPS. As shown in Fig. 3A, the presence of LPS affected the phosphorylation levels of various signaling proteins, including p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), signal transducer and activator of transcription (STAT) proteins, p90 ribosomal S6 kinase (RSK), and p53. Since the p38, ERK, and JNK MAPK family members are associated with inflammation and various cellular responses in many types of cells, we focused our subsequent studies on these proteins. As such, phosphorylation of these proteins was quantified via western blot analysis of lysates from LPS-treated or untreated PDLFs (Fig. 3B). Interestingly, the phospho-forms of ERK and p38 were significantly increased in the presence of LPS (Fig. 3A), whereas the phosphorylation level of JNK was not affected.

Inhibition of MAPK signaling reduces LPS-mediated upregulation of inflammatory responses

To confirm that inhibition of MAPK signaling pathways rescue from upregulated mRNA levels of cytokines in PDLFs, we determined cellular responses in the presence or absence of each MAPK inhibitor. First, we validated inhibition of activated MAPKs via treatment of each inhibitor including p38 (SB203580), ERK (PD98059), and JNK (SP600125). For this assay, we used common concentration of each inhibitor; SB203580 and PD98059 were used for concentration of 20 mM, and SP600125 was used for concentration of 10 mM. As shown in Fig. 4A, LPS-mediated active form of p38 and ERK, but not JNK, are effectively inhibited by treatment of respective inhibitors. Also, native forms of each protein were not affected by treatment of LPS or respective inhibitors. To determine whether these effects regulate LPS-mediated inflammatory responses in PDLFs, we determined mRNA levels of cytokines in the presence or absence of each inhibitor. In the case of SB203580 treatment, both mRNA levels of IL-6 and IL-8 were effectively downregulated compared with only LPS-treated PDLFs as well as non-treated control PDLFs (Fig. 4B). These effects were considered that spontaneous activated p38 pathway in PDLFs was also affected by its inhibitor. In addition, ERK inhibitor, PD98059, reduced mRNA levels of both IL-6 and IL-8, but lower than in the case of p38 inhibitor. However, JNK inhibitor did not rescue from LPS-mediated upregulated mRNA levels of cytokines in PDLFs. As shown in western blot analysis, it seems that JNK pathway rarely affects LPS-mediated these cytokines regulation in PDLFs. Taken together, we suggest that *P.g.*-derived LPS-mediated activation of some MAPKs pathways can induce inflammatory responses in PDLFs.

Effect of *P.g.*-derived LPS on differentiation regulatory proteins

PDLFs can differentiate into osteoblasts when they are maintained under the proper growth conditions. Therefore, we determined whether *P.g.*-derived LPS would affect the ex-

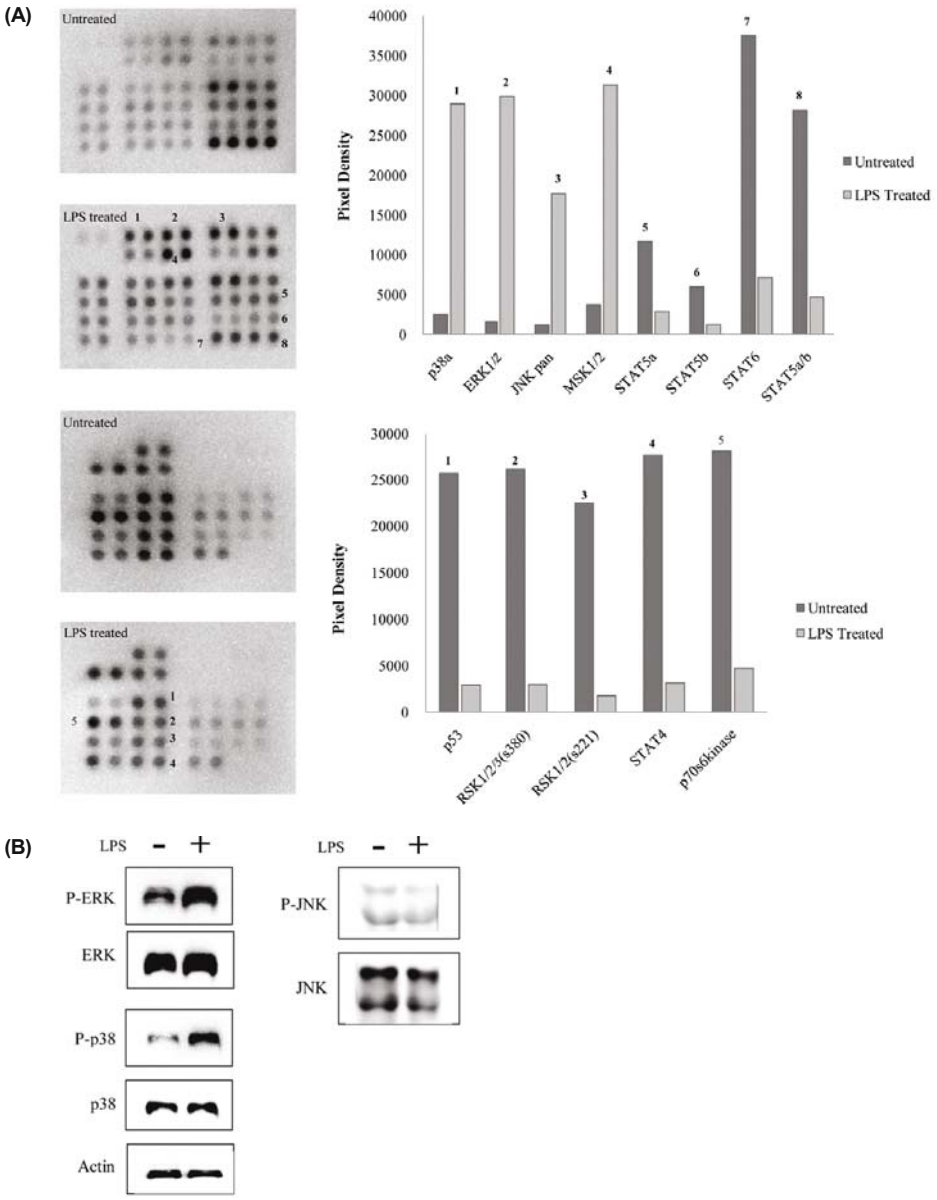

Fig. 3. Proteome profiling and *P.g.*-derived LPS-mediated activation of MAPK pathways. PDLFs were treated with 100 ng/ml of *P.g.*-derived LPS, and expressed factors were compared with untreated cells. (A) Proteome profiling was performed using a human phospho-kinase array kit. Changes in protein levels are represented graphically. (B) ERK, p38, JNK, and their active forms were analyzed by western blot analysis. Total cell lysates were normalized with β-actin.

pression of differentiation marker genes such as ColA1, OCN, OPN, and BSP (Figs. 5A–5D). Real time PCR analysis revealed that the expression of all four of these genes increased over time in untreated control cells. All of these genes expression was higher at the fourth week as compared to the first week. In contrast, gene expression decreased in *P.g.*-derived LPS-treated PDLFs when compared to the controls. OCN and BSP, in particular,exhibited a significant increase in expression by the fourth week for controls, whereas each had decreased expression levels following treatment with *P.g.*-derived LPS (Figs. 5B and 5D). ColA1 and OPN were also reduced by *P.g.*-derived LPS at the second and fourth weeks, respectively (Figs. 5A and 5C). Therefore, these results suggest that *P.g.*-derived LPS can decrease the ability of PDLFs to differentiate into osteoblasts, most likely via the regulation of gene expression.

Effect of *P.g.*-derived LPS treatment on ALP activity and mineralization

Next, we sought to determine the effect of *P.g.*-derived LPS on the differentiation of PDLFs. For these experiments, we measured ALP activity, which is a specific marker of early differentiation of PDLFs. We also screened cells for mineralization, which is their final differentiation status. For the measurement of ALP activity, PDLFs were cultured in differentiation media for two weeks in the presence or absence of LPS. ALP activity was indicated by the generation of p-nitrophenol, which was measured at an absorbance of 405 nm. As shown in Fig. 6A, ALP activity was significantly reduced (~4-fold) in *P.g.*-derived LPS treated PDLFs when compared to untreated cells. For the estimation of mineralization levels, we used Alizarin red S staining, which is routinely used to determine the mineralization of bone

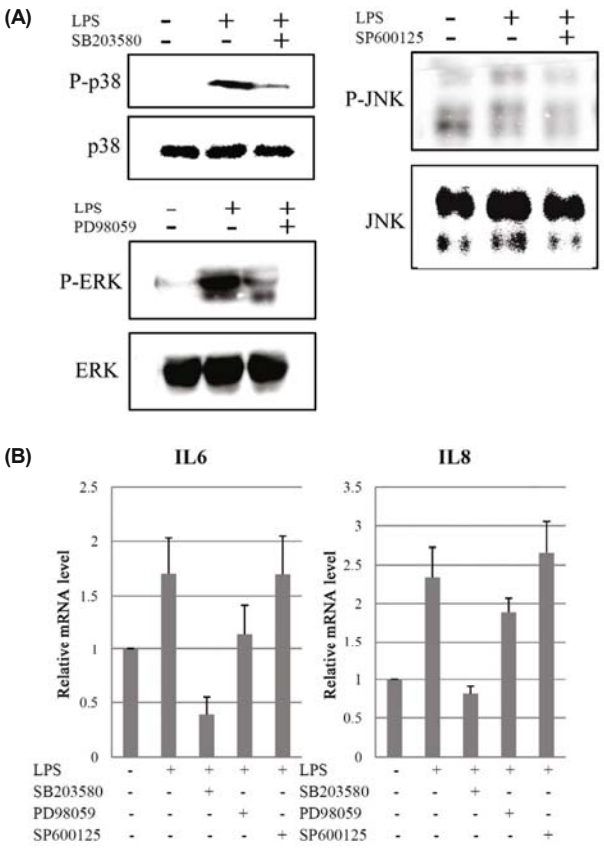

Fig. 4. Inhibition of MAPK signaling reduces LPS-mediated upregulation of inflammatory responses. (A) PDLFs were treated with *P.g.*-derived LPS in the presence or absence of each MAPK inhibitor. After 24 h post-treatment, expressed MAPKs and phosphorylation forms were analyzed by western blot analysis. (B) PDLFs were treated with LPS in the presence of each MAPKs inhibitor. After 24 h post-treatment, mRNA levels of IL-6 and IL-8 were measured by real-time PCR. The results were normalized with *GAPDH*, and were represented by relative fold value compared with control PDLFs.

matrix. In *P.g.*-derived LPS treated PDLFs, the levels of staining were lower than for untreated cells (Fig. 6B). Together, these results suggest that *P.g.*-derived LPS affects both the differentiation and mineralization of PDLFs.

Inhibition of MAPK signaling increases expression of differentiation regulatory proteins

Finally, we determine whether LPS-mediated reduction of differentiation marker genes is affected by inhibition of MAPK pathways. For this assay, PDLFs were cultured with differentiation media in the presence of *P.g.*-derived LPS with or without respective MAPK inhibitor. JNK inhibitor was not used in this experiment, because *P.g.*-derived LPS did not affects activation of JNK as well as JNK inhibitor did not rescues from LPS-mediated inflammatory responses in PDLFs. After four weeks post-differentiation, we confirmed mRNA levels of each differentiation regulatory genes using real-time PCR. As shown in Fig. 7, ColA1 and OCN are rarely affected by treatment of p38 and ERK inhibitors. However, p38 inhibitor increased mRNA levels of OPN and BSP about 3-fold and 2.7-fold, respectively (Figs. 7C and 7D). ERK inhibitor also induced expression of those genes about 3.5-fold and 4-fold, respectively. Compared to above results (Fig. 5), these increased ratios in the presence of MAPK inhibitors indicate that inhibition of MAPKs abolishes the *P.g.*-derived LPS-mediated downregulation of some differentiation marker genes. This result support that *P.g.*-derived LPS-mediated dysregulation of differentiation in PDLFs is partly associated with MAPKs pathway.

Discussion

Recent studies indicate that LPS causes cellular dysfunction via a variety of mechanisms. For example, LPS can induce nitric oxide (NO) synthesis and leads to inflammatory responses. However, this effect is suppressed by 3-(2-hydroxy-

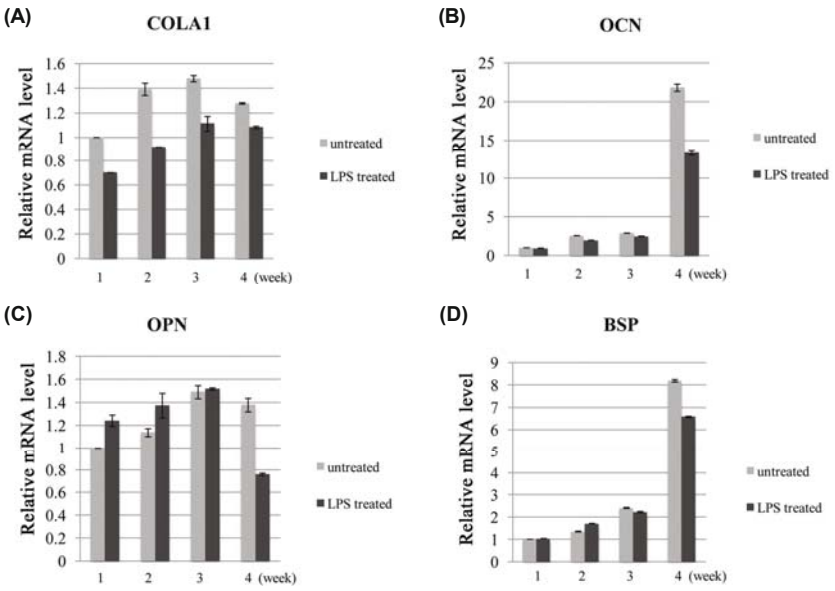

Fig. 5. *P.g.*-derived LPS reduces differentiation-associated proteins in PDLFs. (A–D) PDLFs were incubated with differentiation media in the presence or absence of *P.g.*-derived LPS. After four weeks, expression levels of ColA1, OCN, OPN, and BSP were analyzed using real-time PCR. The results were normalized with *GAPDH*.

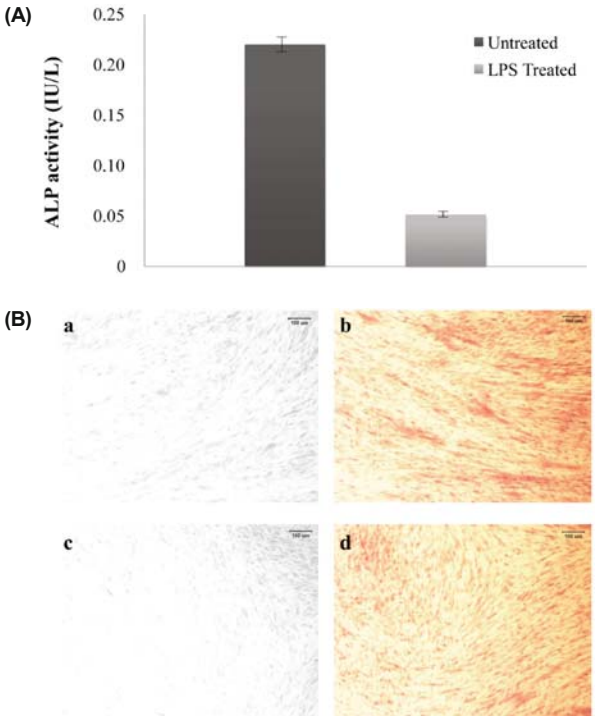

Fig. 6. *P.g.*-derived LPS affects the differentiation of PDLFs into osteoblasts. PDLFs were incubated with differentiation media for two weeks in the presence or absence of *P.g.*-derived LPS. (A) PDLF lysates were incubated with ALP working solution for 4 min, after which the level of p-nitrophenol formation was determined at an absorbance of 405 nm. The results presented are from three independent experiments and are represented graphically. (B) After incubation for two weeks, *P.g.*-derived LPS-treated and untreated PDLFs were stained with Alizarin red S. Stained cells were observed using a phase-contrast microscope. Unstained and stained *P.g.*-derived LPS-untreated PDLFs are (a) and (b), respectively. (c) and (d) indicate unstained and stained *P.g.*-derived LPS-treated PDLFs, respectively (bar=100 μm).

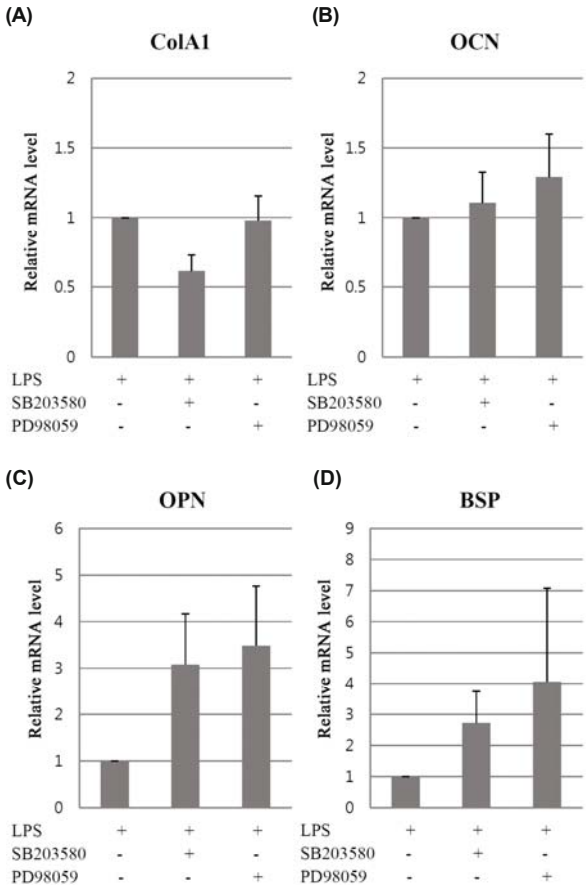

Fig. 7. Inhibition of MAPK signaling increases expression of differentiation regulatory proteins. (A–D) PDLFs were incubated with differentiation media in the presence of *P.g.*-derived LPS with or without indicated MAPK inhibitor. After 4 weeks, mRNA levels of indicated genes were measured by real-time PCR, and were represented by relative fold value compared with LPS-treated PDLFs. The results were normalized with *GAPDH*.

phenyl)-1-(5-methyl-furan-2-y-l) propenone, which inhibits p38 activation, thereby affecting the binding affinity of activating protein-1 in murine monocytic-macrophage cells (Liew *et al.*, 2011).

In our previous study, we showed that constitutive induction of NO leads to activation of MAPK signaling factors, thereby causing cell death in PDLFs, the effects of which are regulated by N-methyl-D-aspartate (NMDA) receptor (Seo *et al.*, 2011). Previous studies have shown that MAPK pathways can be regulated differently on certain stimuli and cell types. Therefore, it is important to understand the types of responses in specific cell type against certain stimulus. At present, however, LPS-mediated activation of MAPK pathways has not been elucidated for PDLFs. In our experiments, we focused on a series of cellular responses induced by *P.g.*-derived LPS, and confirmed that those cellular responses were partly regulated via MAPK pathways. Since LPS is a stress inducer, it mediates the activation of MAPKs following the inflammatory response, while also regulating apoptosis, proliferation, and differentiation.

The cellular responses that are induced by LPS depend on the origin of this endotoxin, the cell type, and various other conditions. For example, *E. coli* (*E.c.*)-derived LPS induces the activation of JNK and delayed activation of ERK and JNK in microglial cells and astrocytes, respectively (Wang *et al.*, 2010; Gorina *et al.*, 2011). In addition, *A.a.*-derived LPS increases the activation of JNK in murine PDL cells (Patil *et al.*, 2006), while it also activates ERK1 and ERK2 in human gingival fibroblasts (Gutierrez-Venegas *et al.*, 2006). In our study with PDLFs, we showed that *P.g.*-derived LPS increases the activation of p38 and ERK, but not JNK. These results indicate that *P.g.*-derived LPS can induce MAPK signaling pathways similar to the effect of other forms of LPS in various cell types, while a different response, such as inactivation of JNK in PDLFs, may result in different cellular responses. Moreover, we confirmed that *P.g.*-derived LPS-mediated activation of MAPK pathways induced the increment of cytokines such as IL-6 and IL-8 consistent with previous study (Yamamoto *et al.*, 2006). Therefore, the study of these factors may help us gain a better understanding of the characteristics of PDLFs treated with *P.g*-derived LPS. Likewise, this could lead to better

methods for treating periodontal inflammation.

Our results additionally show that *P.g.*-derived LPS upregulates cytochrome-c without alteration of expression level of Bax. In addition, there is an increase in the active-form of caspase-3, which is usually followed by mitochondrial apoptosis. Previous studies have shown that *E.c.*-derived LPS can induce Bax-mediated apoptosis and may affect apoptosis via the expression of Bax in endothelial cells and dental pulp cells, respectively (Munshi *et al.*, 2002; Yang *et al.*, 2010). It was also reported that *A.a.*-derived LPS cannot induce expression of Bax as well as cytochrome-c in differentiated macrophage-like cells (Suzuki *et al.*, 2004). In our results, however, we showed that *P.g*-derived LPS significantly increased expression of cytochrome-c, but not Bax, compared with an LPS-untreated control. At present, the mechanisms of LPS-mediated Bax-independent apoptosis in PDLFs have not been elucidated. Thus, additional research is required in order to find the exact mechanism of *P.g*-derived LPS-induced apoptosis.

Finally, we show that *P.g.*-derived LPS affects differentiation in PDLFs. A previous study showed that *A.a.*- and *E.c.*-derived LPS can induce osteoclastogenesis via the expression of MAPK signaling factors and receptor activator of NF-κB ligand (RANKL) in murine PDLFs (Rossa *et al.*, 2008). In the case of human PDLFs, *E.c.*-derived LPS increases an mRNA level of osteoprotegerin and RANKL, while *P.g.*-derived LPS does not dramatically change the mRNA level of RANKL (Wada *et al.*, 2004; Krajewski *et al.*, 2009). Moreover, several studies showed that *P.g.*-derived LPS can reduce bone nodule formation and ALP activity in rat calvaria cells. It also decreases the level of OCN, but not BSP, OPN, or Col1 in murine cementoblast cells (Kadono *et al.*, 1999; Nociti *et al.*, 2004). Besides these factors, our previous report revealed that NMDA receptors regulate the differentiation, but not the proliferation, of PDLFs (Yu *et al.*, 2009). Dysregulation of PDL and alveolar bone formation can cause periodontal diseases and bone remodeling is regulated by a proper balance between bone formation and resorption. Therefore, the above factors must be exactly regulated by each process in PDLF. However, *P.g.*-derived LPS-mediated differentiation of PDLFs into osteoblasts has not yet been characterized. In this study, we observed that *P.g.*-derived LPS decreases the level of ColA1, OCN, OPN and BSP which are synthesized in osteoblastic cells, and inhibition of MAPK pathways partly rescues from dysregulated expression of those genes. Our results indicate that *P.g.*-derived LPS induces MAPK pathway-mediated dysfunction of bone remodeling in PDLFs, which can be a cause of periodontitis.

In conclusion, our findings show that *P.g.*-derived LPS results in the induction of apoptosis and activation of MAPK pathways leading to the expression of cytokines, as well as the reduction of differentiation into osteoblastic cells in PDLFs. These findings are important for understanding cellular responses by *P.g.*-derived LPS and finding therapeutic targets of *P.g.*-mediated periodontitis in PDLFs. We have further studies to elucidate other periodontal changes by *P.g.*-derived LPS underway.

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