

Original Article

Effects of 1,25-dihydroxyvitamin D₃ on Macrophage Cytokine Secretion Stimulated by *Porphyromonas gingivalis*

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SUMMARY: Vitamin D is known to be closely associated with periodontitis; however, its exact mechanisms remain to be clarified. The present study aimed to investigate the influence of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) on *Porphyromonas gingivalis* (Pg)-stimulated cytokine production and the involved signaling pathways in macrophages. The main observation was that 1,25(OH)₂D₃ inhibited Pg-induced interleukin (IL)-6 cytokine expression but up-regulated the expression of anti-inflammatory cytokine IL-10. Further analyses showed that 1,25(OH)₂D₃ decreased p38 mitogen-activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK)1/2 phosphorylation. Inhibited phosphorylation of p38 MAPK and ERK1/2 was associated with decreased level of IL-6 expression, but was not related to increased level of IL-10 expression in macrophages stimulated with Pg. These results suggest that 1,25(OH)₂D₃ might exert its anti-inflammatory effects on Pg-stimulated macrophages partly through its inhibitory effect on the p38 MAPK and ERK1/2 signaling pathway.

INTRODUCTION

Periodontitis is a highly prevalent, chronic immune inflammatory disease of the periodontium that results in progressive loss of the gingival tissue, the periodontal ligament, and the adjacent supporting alveolar bone. The disease is initiated by specific bacteria within the plaque biofilm and progresses due to abnormal immune responses to these bacteria (1,2). *Porphyromonas gingivalis* (Pg), a gram-negative anaerobe, has been strongly associated with the etiology of periodontal disease (3). Its virulence factors including lipopolysaccharides, fimbriae, hemagglutinins, and proteolytic enzymes can cause periodontal inflammation and destruction (4). Macrophages are important inflammatory and immunologic effector cells, and are more abundant in periodontitis lesions than in normal tissue. Macrophages can defend against the invasion of periodontal pathogens through phagocytosis. However, they also produce different types of cytokines upon the stimulation by pathogens. Some cytokines have proinflammatory effects, some exert anti-inflammatory effects, and both of these types affect the progression of periodontitis.

Vitamin D is obtained by the human body through diet, supplements, and exposure to sunlight. Through a series of hydroxylase enzymatic reactions, the active vitamin D metabolite 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) is formed. The biological effects of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR), also known as calcitriol receptor, which is a member of the superfamily of nuclear hormone receptors (5). Vitamin D, despite its traditional association with calcium and phosphate homeostasis, has been shown to exhibit immunological effects, influencing both innate and adaptive immune systems (6–8). In recent years, many studies have found that vitamin D can affect periodontal status. Some studies suggest that increased serum vitamin D levels might be beneficial for reducing tooth loss, attachment loss, and gingivitis susceptibility, indicating a significant inverse association between serum concentrations of vitamin D and periodontal disease (9–14). In contrast, other studies show that serum vitamin D levels in patients with aggressive periodontitis are higher than those in healthy controls and can be systemically and locally reduced after initial periodontal therapy, suggesting a positive relationship between serum vitamin D levels and the severity of periodontal inflammation (15,16). Therefore, the role of vitamin D in periodontal inflammation and its underlying mechanism need to be further investigated.

The purpose of the present study was to investigate the influence of 1,25(OH)₂D₃ on Pg-stimulated cytokine production by macrophages as well as the impact on the mitogen-activated protein kinase (MAPK) signaling pathway. Our findings will serve to enhance the understanding of the role of 1,25(OH)₂D₃ in the process of periodontitis and provide clues for the treatment of this disease.

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MATERIALS AND METHODS

Cell culture: The RAW264.7 cell line, which has strong adhesion capacity and the ability to engulf antigens, can be stably subcultured; hence, it is commonly used as a macrophage line in microbiology and immunology research. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, S. Logan, UT, USA), penicillin (50 U/mL) and streptomycin (50 µg/mL) (Hyclone), in a humidified atmosphere of 5% CO₂ at 37°C. After reaching 80% confluence, cells were subcultured at a 1:3–1:6 ratio.

Bacterial strains, culture, and counting: *Pg* ATCC33277 was cultured and maintained on Columbia blood agar plates (Land bridge, Beijing, China) in an anaerobic atmosphere of 10% H₂, 5% CO₂, and 85% N₂ at 37°C. For the preparation of *Pg* for cell stimulation, bacteria were harvested, centrifuged, and washed with PBS. The number of bacteria was determined using a spectrophotometer with 1 mL cuvettes. One optical density (OD) unit equals approximately 10⁹ cells/mL at a wavelength of 600 nm (17).

Cell treatment: RAW264.7 cells were seeded in 24-well plates at a concentration of 1 × 10⁶ cells/well in DMEM with 10% FBS without antibiotics. Twenty-four hours later, cells were pretreated with 5 × 10⁻¹ µM 1,25(OH)₂D₃ (Sigma-Aldrich, St. Louis, MO, USA) or the corresponding vehicle control (control group) for 2 h; subsequently, *Pg* suspensions were added to cells at a multiplicity of infection (MOI) of 100. At 2, 6, 12, and 24 h after *Pg* addition, 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to each well to obtain the cell lysate for mRNA analysis. At 24 h after *Pg* stimulation, cell culture supernatants were collected and stored at -70°C for further analysis of interleukin (IL)-6, IL-10, IL-1β, and IL-12p40 protein levels. In some experiments, RAW264.7 cells were pre-incubated with SB203580 and/or U0126 (Cayman, Ann Arbor, MI, USA) at 10 µM for 2 h before exposure to *Pg*.

Real-time PCR: Total RNA was prepared with Trizol and reverse transcribed to cDNA using Oligo dT and ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR amplification was then performed, probing for IL-6, IL-10, IL-1β, and IL-12p40, with the SYBR Green real-time PCR kit (Invitrogen), using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. β-actin served as an internal reference for normalization of RNA expression. The primer sequences for target genes are listed in

Table 1.

Enzyme-linked immunosorbent assay (ELISA): The protein levels of IL-6, IL-10, IL-1β, and IL-12p40 in the cell culture supernatants were assessed by an enzyme-linked immunosorbent assay (ELISA), using an ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

Western blot analysis: To assess MAPK activation following stimulation of RAW264.7 with 1,25(OH)₂D₃ and *Pg*, RAW264.7 cells were cultured in 6-well plates (2 × 10⁶ cells/well). Cells were pretreated with 5 × 10⁻¹ µM 1,25(OH)₂D₃ for 2 h followed by *Pg* (MOI = 100) treatment for 0, 10, 30, 60, and 120 min or were pre-incubated with SB203580 and/or U0126 at 10 µM for 2 h before exposure to *Pg* for 30 min. To prepare cytoplasmic extracts, an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo, Waltham, MA, USA) was used according to the manufacturer's protocol. Briefly, cells were washed twice with ice-cold PBS and were lysed in ice-cold CER I and CER II buffers; cells were vortexed and centrifuged at 16,000 × g for 5 min at 4°C. The supernatants (cytoplasmic extracts) were collected and evaluated by Western blotting. The protein content was measured using a BCA assay (Pierce, Waltham, MA, USA). Equivalent amounts of protein samples from cell extracts were subjected to SDS-PAGE, and electrotransferred to polyvinylidene difluoride membranes (Roche, Basel, Switzerland), and subsequently probed using specific antibodies against the phosphorylated forms of p38 (Thr180/Tyr182), extracellular signal regulated kinase (ERK)1/2 (P44/42, Thr202/Tyr204), or stress-activated protein kinase /c-Jun N terminal kinase (JNK) (Thr183/Tyr185) (Cell Signaling Technology, Danvers, MA, USA). Detection of bands was performed using a horseradish peroxidase-linked rabbit IgG antibody and ECL Western blotting detection reagents (Thermo), according to the manufacturer's instructions. Equal protein loading was monitored by the assessing total p38. Densitometric analysis of distinct bands was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). Relative band intensities were compared by determining the ratio of the area densities of the target protein to total p38 bands.

Statistical analysis: Results are expressed as the means ± standard deviation (SD). All statistical analyses were performed using a Student's *t*-test for 2 independent groups or a one-way ANOVA for more than 2 groups, using SPSS 10.0 software (SPSS, Chicago, IL, USA). The differences were considered significant at *p* < 0.05 and highly significant at *p* < 0.01.

Table 1. Primer sequences in quantitative real-time PCR

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
IL-6	GAGGATACCACTCCCAACAGAC	GTGCATCATCGTTGTTTCATAC
IL-10	GGTTGCCAAGCCTTATCGGA	AATCGATGACAGCGCCTCAG
IL-1β	AGCCCATCCTCTGTGACTCAT	CATTGAGGTGGAGAGCTTTC
IL-12p40	AGATTCAGACTCCAGGGGACA	GGGAGTCCAGTCCACCTCTA
β-actin	TCCTGTGGCATCCATGAACTA	CCAGGGCAGTAATCTCCTCTG

RESULTS

Cytokine mRNA levels in RAW264.7 cells treated with 1,25(OH)₂D₃ combined with *Pg*: To understand the effects of 1,25(OH)₂D₃ on cytokine mRNA expression levels in RAW264.7 cells in response to *Pg* stimulation, cells were pretreated with 1,25(OH)₂D₃ for 2 h followed by *Pg* treatment. We used real-time PCR to determine the mRNA levels of cytokines. Normalized to the 2 h control group, the mRNA levels of IL-6 in the control group gradually increased, reaching a peak at 12 h. IL-6 mRNA in the experimental group was lower than that in the control group and these difference were statistically significant at 2 and 12 h ($p < 0.01$) (Fig. 1A). The mRNA level of IL-10 also gradually increased, and the level in the experimental group was significantly higher than that in the control group at 24 h ($p < 0.01$) (Fig. 1B). However, the mRNA expression levels of IL-1 β and IL-12p40 were not significantly different between the 2 groups ($p > 0.05$) (Fig. 1C and D).

Cytokine protein levels in RAW264.7 cells treated with 1,25(OH)₂D₃ combined with *Pg*: ELISA was used to determine the protein expression levels of cytokines in RAW264.7 cells. The results showed that the expression of IL-6 in the experimental group decreased significantly compared to that in the control group ($p < 0.01$) (Fig. 2A). In addition, the expression of the anti-inflammatory cytokine IL-10 in the cell culture supernatant of the experimental group was higher than that in the control group ($p < 0.05$) (Fig. 2B). There were no significant differences in IL-1 β and IL-12p40 expression between the 2 groups ($p > 0.05$) (Fig. 2C and D).

Activation of MAPK signaling pathway in RAW264.7 cells treated with 1,25(OH)₂D₃ combined

with *Pg*: Western blotting was performed to analyze the activation of inflammation-related MAPK signaling pathways, which include p38 MAPK, ERK1/2, and JNK pathways (Fig. 3A). The results showed that with *Pg* stimulation, p38 MAPK phosphorylation levels gradually increased over time. p38 phosphorylation levels in the experimental group was lower than that in the control group and was statistically significant at 0, 10, 30, 60, and 120 min ($p < 0.01$) (Fig. 3B). ERK1/2 phosphorylation levels in the experimental group was lower than that in the control group and was statistically significant at 0, 10, 30, and 60 min ($p < 0.05$) (Fig. 3C). With *Pg* stimulation, JNK phosphorylation levels gradually increased over time, reaching a peak after approximately 10–30 min, following which time, they gradually declined. However, JNK phosphorylation levels were not significantly different between the 2 groups ($p > 0.05$) (Fig. 3D).

Next, MAPK inhibitors were used to further investigate the regulatory roles of p38 MAPK and ERK1/2 in the production of proinflammatory and anti-inflammatory cytokines by *Pg*-stimulated RAW264.7 cells. Initially, we evaluated the specific inhibitory effect of each inhibitor (p38 MAPK: SB203580; ERK1/2: U0126) on MAPK activation. SB203580 inhibited the phosphorylation of p38 MAPK, without affecting the activation of ERK. Similarly, U0126 inhibited the phosphorylation of ERK, whereas no inhibition of p38 phosphorylation was observed. Combined pretreatment with SB203580 and U0126 significantly inhibited the phosphorylation of both p38 MAPK and ERK (Fig. 4A). IL-6 mRNA levels were suppressed by SB203580

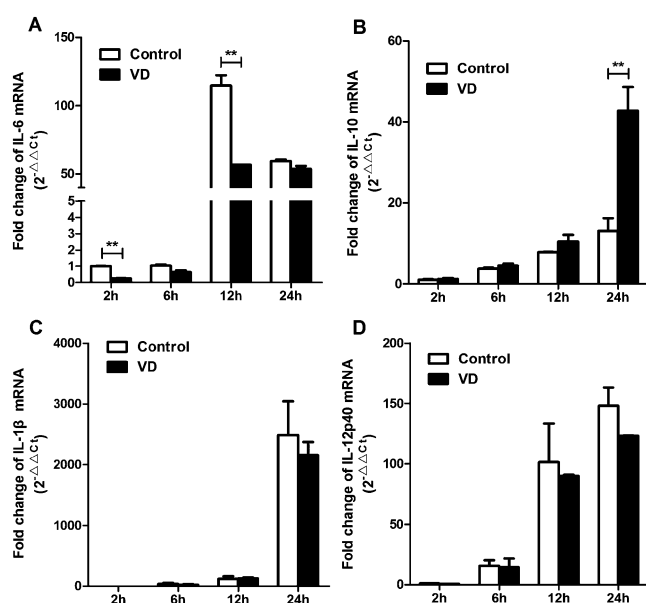


Fig. 1. The mRNA levels of cytokines in RAW264.7 cells treated with 1,25(OH)₂D₃ (VD) and *Pg*. Total RNA was prepared, and the mRNA levels of IL-6 (A), IL-10 (B), IL-1 β (C), and IL-12p40 (D) were determined by quantitative real-time PCR. Data were expressed as the means \pm standard deviation of 3 independent experiments. Symbols for statistical significance: **, significantly different from the control group ($p < 0.01$).

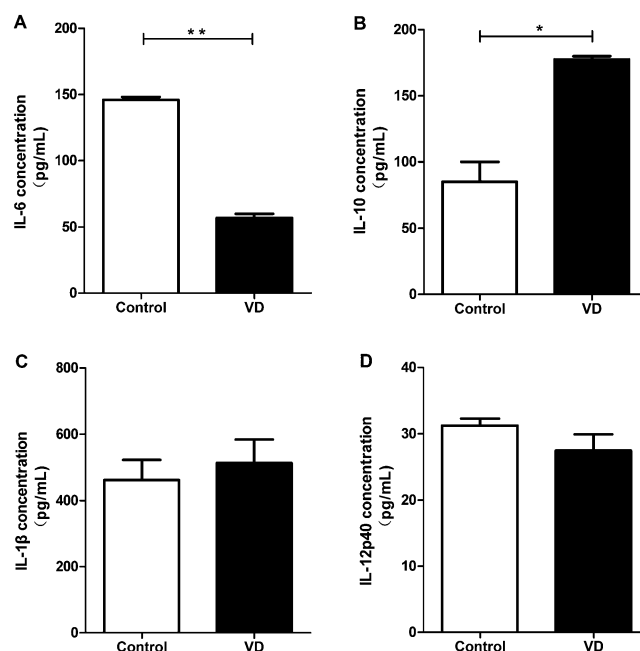


Fig. 2. The protein expression levels of cytokines in RAW264.7 cells treated with 1,25(OH)₂D₃ (VD) and *Pg*. Cell culture supernatants were collected, and the protein expression levels of IL-6 (A), IL-10 (B), IL-1 β (C), and IL-12p40 (D) were determined by ELISA. Data were expressed as the means \pm standard deviation of 3 independent experiments. Symbols for statistical significance: **, significantly different from the control group ($p < 0.01$); *, significantly different from the control group ($p < 0.05$).

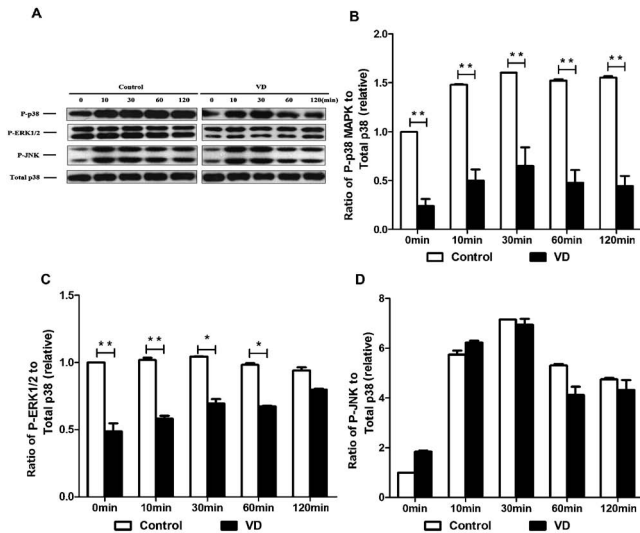


Fig. 3. The activation levels of MAPKs signaling pathway in RAW264.7 cells treated with 1,25(OH)₂D₃ (VD) and *Pg*. Cytoplasmic lysates were collected at 0, 10, 30, 60, and 120 min and analyzed by Western blotting using the indicated antibodies. Total p38 was examined as the loading control (A). Quantification of band density represents fold induction over control samples (B, C, and D). The results were shown as the means \pm standard deviation of 3 independent experiments. Symbols for statistical significance: **, significantly different from the control group ($p < 0.01$); *, significantly different from the control group ($p < 0.05$).

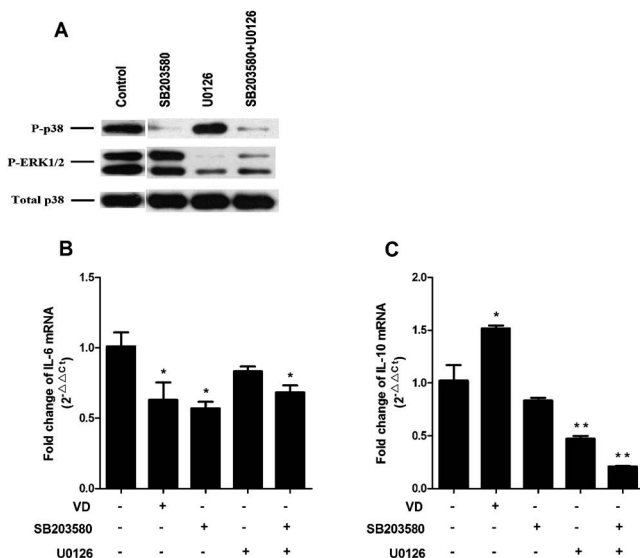


Fig. 4. The effects of p38 and ERK inhibitors on *Pg*-induced cytokine production. Cells were pre-incubated with SB203580 and/or U0126 at 10 μ M 2 h before exposure to *Pg* for 30 min. Cytoplasmic lysates were collected and analyzed by Western blotting. Total p38 was examined as the loading control (A). Cells were pre-treated with 1,25(OH)₂D₃ (VD), SB203580, or U0126 for 2 h and then stimulated with *Pg* for 2 h. The mRNA levels of IL-6 and IL-10 were determined by quantitative real-time PCR (B and C). The results were shown as the means \pm standard deviation of 3 independent experiments. **, significantly different from the control group ($p < 0.01$); *, significantly different from the control group ($p < 0.05$).

($p < 0.05$). U0126 had an inhibitory effect on IL-6 production, but the effect was not significant (Fig. 4B). U0126 resulted in a significant reduction in IL-10 levels

($p < 0.01$), whereas the inhibitory effect of SB203580 on IL-10 production was not significant. In addition, combined pre-treatment with SB203580 and U0126 significantly reduced IL-10 production (Fig. 4C).

DISCUSSION

Vitamin D is not only a nutrient, but also a type of endocrine hormone produced by the skin, liver, and kidney. Through an association with VDR, it regulates target gene transcription and translation, thus exerting a variety of biological effects. Vitamin D, in addition to its classical role in regulating calcium and phosphorus metabolism, promoting cell growth and differentiation, and modulating bone reconstruction, plays an important role in immune regulation. It can influence dendritic cell maturation and function, enhance the phagocytic and chemotactic function of monocytes and macrophages, and regulate T- and B-lymphocyte proliferation, differentiation, and cytokine secretion (18–24). In recent years, several studies have demonstrated that vitamin D can modulate inflammation via the regulation of cytokine production (21,25). Tang et al. reported that vitamin D might potentially inhibit periodontal inflammation induced by *Pg*, partly by decreasing the cytokine expression in human periodontal ligament cells (26). The periodontium is a complex tissue consisting of different cell types, and macrophages play an important role in the host response to periodontal pathogens.

Therefore, we investigated the effect of 1,25(OH)₂D₃ on cytokine production by macrophages in response to *Pg* stimulation, and the underlying mechanism. The main observation was that the production of cytokines (IL-6 and IL-10) by macrophages was affected by 1,25(OH)₂D₃. IL-6, a multifunctional cytokine, is closely related to the development of periodontitis. Many studies have shown that IL-6 levels in the gingival crevicular fluid of patients with periodontitis were significantly higher than those of healthy people, and these levels were positively correlated with gingival bleeding index, periodontal pocket depth, and degree of alveolar bone absorption (27). In the present study, it was found that 1,25(OH)₂D₃ decreased *Pg*-induced IL-6 expression, signifying that vitamin D exerts an inhibitory effect on pro-inflammatory cytokine IL-6 production, thereby attenuating *Pg*-induced inflammation.

IL-10, the most important endogenous inhibitor of inflammation, plays an important role in the balance of proinflammatory response and inflammation suppression. IL-10, a critical cytokine characterized by its anti-inflammatory properties, performs a vital role in controlling infection and the progression of periodontal disease (28,29). Studies by Goutoudi et al. showed that one of the pathological features of periodontitis quiescence was the increased expression of IL-10. In periodontitis patients, after scaling and root planing treatment, local periodontal inflammation decreased and expression of IL-10 increased, suggesting that IL-10 might be involved in the repair of periodontal tissue (30). The present study demonstrated that 1,25(OH)₂D₃ improved the expression of the anti-inflammatory cytokine IL-10. We have reason to infer that increased

IL-10 might suppress the inflammatory response, promote the repair of periodontal tissue, and reduce immune-associated injury to periodontal tissue. However, the exact effect of IL-10 in vivo requires further analysis.

A better understanding of the cell signaling processes is critical for the development of new therapeutic approaches to prevent or attenuate periodontal disease. Signal transduction from inflammatory mediators might involve the activation of the MAPK signaling. MAPKs are key components of several important intracellular signaling pathways, including 3 classic pathways, namely p38 MAPK, ERK1/2, and JNK pathways. Ample evidence exists suggesting that MAPK pathways are vital for the synthesis and amplification of inflammatory cytokines (31). In particular, p38 MAPK activation was closely related to cytokine transcription and translation, making it the target of anti-inflammatory treatment (32). Studies have shown that *Pg* stimulates macrophages by activating the MAPK signaling pathway and regulating gene expression, thus inducing the release of inflammatory cytokines, leading to the destruction of periodontal connective tissue and alveolar bone absorption (33). Travan et al. also clearly showed that MAPK signaling played a significant role in the ontogeny of inflammation in periodontal disease (34). In the present study, we investigated whether the effect of vitamin D on *Pg*-induced cytokine production was through MAPK signaling pathways. The results demonstrated that 1,25(OH)₂D₃ effectively decreased *Pg*-induced phosphorylation of p38 MAPK and ERK1/2.

Furthermore, inhibition of p38 and ERK using specific inhibitors reduced *Pg*-induced IL-6 expression. These findings suggested that vitamin D might act as a potent negative regulator of p38 MAPK and ERK1/2, thereby affecting the transcriptional induction of IL-6. The observation that the p38 MAPK and ERK are involved in the regulation of IL-6 expression is in accordance with that by Morimoto et al. (35), who reported that treatment of RAW264.7 cells with a p38 or ERK inhibitor reduced *Pg* LPS-induced IL-6 production. The result that p38 and ERK inhibitors reduced IL-10 production appears to be in conflict with the finding that 1,25(OH)₂D₃ enhanced the expression of the anti-inflammatory cytokine IL-10. It is suggested that other signaling pathways could be involved in the regulation of IL-10 expression by 1,25(OH)₂D₃. It is worth noting that vitamin D seemed to reduce baseline phosphorylation levels of p38 MAPK and ERK1/2 directly, since their phosphorylation levels were lower than those of the control group at 0 min before *Pg* stimulation. The detailed molecular mechanisms through vitamin D exerts its effects on these signaling pathways remain to be elucidated.

In conclusion, the findings of our study indicate that vitamin D can inhibit *Pg*-induced IL-6 proinflammatory cytokine expression, while improving the expression of the anti-inflammatory cytokine, IL-10 in RAW264.7 cells, partly through the down-regulation of MAPK signaling. However, the exact mechanism should be explored in further studies; animal and pre-clinical experiments are also needed to further confirm the exact role of vitamin D in the regulation of periodontitis, in order

to find a more effective and nontoxic drug for prophylaxis or treatment of periodontal disease.

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Conflict of interest None to declare.

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