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Original Article

Inducing cyclooxygenase-2 expression, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ production of human dental pulp cells by activation of toll-like receptor-3, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase and p38 signaling

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KEYWORDS
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Abstract *Background/purpose:* Bacterial infection was the major etiology for pulpal/root canal infection. This study aimed to investigate the activation of toll-like receptor-3 (TLR) on cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ production of human dental pulp cells (HDPCs) and associated signaling.

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inflammation;
Prostaglandin;
Signal transduction;
Toll-like receptor 3

Materials and methods: HDPCs were exposed to different concentrations of Poly (I:C) (a TLR3 activator). Cell viability was determined by 3- (4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay and alkaline phosphatase (ALP) activity was evaluated by ALP staining. Activation of extracellular signal-regulated kinase (ERK) and p38 by Poly (I:C) was determined by immunofluorescent staining. The COX-2 protein expression was analyzed by Western blot. PGE_2 and $PGF_{2\alpha}$ production was measured by enzyme-linked immunosorbent assay. The mRNA expression was studied by real-time polymerase-chain reaction. Moreover, HDPCs were exposed to Poly(I:C) with/without U0126 or SB203580 treatment and analysis of COX-2 expression and prostanoid production were conducted.

Results: Poly (I:C) showed little effect on ALP activity, but decreased viability of HDPCs. It stimulated COX-2 mRNA and protein expression. Poly (I:C) induced PGE_2 and $PGF_{2\alpha}$ production of HDPCs. Poly (I:C) activated *p*-ERK, and p-p38 protein expression. Treatment by U0126 (a mitogen-activated protein kinase kinase (MEK)/ERK inhibitor) and SB203580 (a p38 inhibitor) attenuated Poly (I:C)-induced COX-2 mRNA and protein expression as well as PGE_2 and $PGF_{2\alpha}$ production.

Conclusion: TLR3 activation is involved in the infection and inflammatory responses of pulp tissues, via MEK/ERK, and p38 signaling to mediate COX-2 expression as well as PGE_2 and $PGF_{2\alpha}$ production, contributing to the pathogenesis and progression of pulpal/periapical diseases.

Introduction

Pulpitis and apical periodontitis are generally infectious diseases involving a number of gram-negative and anaerobic microorganisms. The pathogenic microorganisms may generate various toxic products such as lipopolysaccharide (LPS), metabolic products (propionic acid and butyric acid). These toxic products may induce pulpal/root canal and periapical infection via activation of various cell surface receptors (toll-like receptors [TLRs] and nucleotide binding oligomerization domain [NOD] receptors), leading to pulpal/periapical inflammation, connective tissue breakdown, pulp necrosis, and apical/periodontal bone loss. Accordingly, human dental pulp has been shown to express various TLRs and NOD receptors, including TLR3.[1] TLRs over the odontoblasts may sense the pathogenic microorganisms to induce cytokine/chemokine production, and inflammatory cell infiltration to kill the invading microorganisms[2] Microorganism are suggested to be the major etiologic factors of pulpal/periapical and periodontal diseases. Dental plaque and biofilm on the root surface or in the root canals contain a number of microorganisms.[3,4] Root canal debridement and enlargement as well as removal of dental plaque and calculus over root surface are important for treatment of periapical and periodontal diseases. Presence of microorganisms in the root canal and periodontal pocket is the reason for treatment failure or disease recurrence. Among these micro-organism, *P. gingivalis* is widely identified in periodontal pocket and infected root canals.[5,6] It contains several toxic factors, including fimbria, LPS, and short chain fatty acid. These factors may lead to tissue destruction and resorption via inducing cytokine, eliciting inflammation, and compromising the cell viability of pulpal or periodontal cells in the adjacent tissue.[7—12] Interaction of pathogenic microorganisms with pulpal/periapical and periodontal tissues may stimulate the release of inflammatory mediators such as interleukin-1β (IL-1β), IL-6, prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNFα), as well as IL-8 and leukotrienes that may induce chemotaxis of inflammatory cells.[13—16] IL-1β and TNF-α are two molecular markers relating to bone destruction in periodontitis and apical periodontitis, possibly via stimulation of IL-8, intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), lipoxygenase, with downstream PGE_2 and leukotrienes production.

An increased COX-2 expression, PGE_2 and $PGF_{2\alpha}$ production in experimental pulpitis tissues has been reported.[17—19] COX-2 and its metabolic product PGE_2 are shown to increase the vascular permeability, and mediate localized inflammatory cell infiltration especially in the dental pulp.[17] $PGF_{2\alpha}$ is shown to be involved in the diseased processes of acute and chronic inflammation, atherosclerosis, cardiovascular diseases, rheumatoid arthritis etc.[20] This event is tightly associated with infectious and inflammatory diseases of the dental pulp. However, limited information is known about the involvement of TLR3 activation in the inflammation and repair of dental pulp. Poly (I:C) binds and activates the TLR3 receptors, and stimulate downstream signals. A number of signal transduction molecules, such as focal adhesion kinase (FAK), mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), p38, and nuclear factor-kappa B (NF-κB), are shown to be responsible for the TLRs activation-mediated expression and production of various inflammatory and effector molecules.[16,21—23] We hypothesized that TLR3 activation by Poly (I:C) may induce inflammatory responses in pulp tissue and downstream signaling molecules (MEK/ERK and p38) to mediate COX-2 expression and prostaglandins' production. These results can facilitate our understanding the pathogenesis of

pulpal/periapical diseases, and further benefit their prevention and treatment.

Materials and methods

Materials

Dimethyl-sulfoxide (DMSO), fast blue 2′,5′-diethoxybenzanilide (BB) salt, naphthol-AS-phosphate, and 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) were purchased from Sigma (Sigma Chemical Company, St Louis, MO, USA). Cell culture medium and reagents are from Life Technologies (Life Technologies, Thermo Fisher Scientific Inc., New York, NY, USA). Poly (I:C) was obtained from InvivoGen (San Diego, CA, USA). SuperscriptTM III First Strand Synthesis System was from Invitrogen (Thermo Fisher Scientific Inc.). SYBR green real-time polymerase chain reaction (PCR) kits were from PCR Biosystems LTD (London, UK). By the approval of Ethics Committee, National Taiwan University Hospital, human dental pulp cells (HDPCs) were cultured by explant technique as described previously.[24,25] They were cultured in Modified Eagle's Medium alpha (αMEM) with 10 % fetal bovine serum (FBS), penicillin/streptomycin and passaged when growth of cells near confluence. The passage number of 3—8 were used for this study. Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2 and $PGF_{2\alpha}$ were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA).

Effect of Poly (I:C) on the viability and alkaline phosphatase (ALP) activity of HDPCs

In brief, 1 x 10^4 HDPCs were inoculated into 24-well culture plate. After 24 h, medium was replaced by fresh medium containing various concentration of Poly (I:C) for 3 days. Photographs of HDPCs were taken for record of morphological changes. Cell viability was estimated by MTT assay as before.[26,27]

For ALP activity assay, 1 $\times$ 10^5 HDPCs were inoculated into 24-well culture plate. After 24 h, medium was changed and cells were exposed to Poly (I:C) for further 5 days. The ALP activity of HDPCs was determined by ALP staining as described previously.[25] In short, after draw off the medium, cells were rinsed with phosphate-buffered saline (PBS), and then stained with 200 μl of the freshly-prepared stock substrate solution (10 mg fast blue 2′,5′-diethoxybenzanilide (BB) salt/50 ml ddH_2O, 3.94 g Tris-base containing 0.015 g naphthol AS phosphate and 250 μl of N,N-dimethylformamide) for 30—60 min in the dark. The pictures of ALP staining results were examined under the Olympus microscope IX71 (Olympus Corporation, Tokyo, Japan) and photographed by a camera.

Effect of Poly (I:C) on COX-2 mRNA expression of HDPCs as analyzed by real-time PCR

In brief, 5 $\times$ 10^5 HDPCs were inoculated into 6-well culture plate. After 24 h, medium was changed and Poly (I:C) with/without various inhibitors (U0126, or SB203580) was added for 30 min before the addition of Poly (I:C) (test) or solvent (control) and incubated for 24 h. RNA was isolated and subjected to reverse transcription with Invitrogen SuperscriptTM III First Strand Synthesis System. The generated cDNA was applied for real-Time PCR analysis of mRNA expression by using SYBR green real-time PCR kit.[28,29] The reaction mixture contained SYBR master mix, primers, cDNA and diethyl pyrocarbonate (DEPC)-treated water. The condition of PCR reaction was arranged at stage 1: 95 °C for 2 min, 1 cycle; then stage 2: 95 °C 5 s, 60 °C for 30 s for 40 cycles. The sequence of primers for COX-2 and beta-actin (β-actin, BAC) was described previously.[30,31] For quantitative assessment of the PCR results, the Delta/Delta Cyclic threshold values ($\Delta\Delta Ct$ = mean ΔCt [treated] - mean delta (Δ) Ct [control]) was employed to quantify the changes of gene expression. The fold of alterations in the study groups relative to the solvent control group were analyzed by using $2^{-\Delta\Delta Ct}$ method. The level of BAC gene expression was utilized as the internal control throughout the PCR tests.

Effect of Poly (I:C) on COX-2 protein expression, PGE2 and PGF2α production of HDPCs

HDPCs cells (5 $\times$ 10^5 cells/well) were inoculated into 6-well culture plates in 2 ml fresh medium containing various concentrations of Poly (I:C) (1—40 μg/ml) with/without inhibitors. Cells were incubated for 24 h. Culture medium was collected for analysis of PGE_2 and $PGF_{2\alpha}$ levels by ELISA.

Proteins in cell lysate were isolated and used to western blotting analysis as described previously.[32,33] Briefly cells were rinsed with PBS, and cells were disrupted by lysis buffer containing 10 mm Tris—HCl, pH 7; 140 mm sodium chloride; 3 mm magnesium chloride; 0.5 % NP-40; 2 mm phenylmethylsulfonyl fluoride; 1 % aprotinin; and 5 mm dithiothreitol. Protein concentrations were determined Bio-Rad protein assay kits. Same amounts of proteins of cell lysates were loaded for 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blotted with mouse (or rabbit) anti-human COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies for 2 h. This was followed by incubation in respective secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. After washing of the membrane by tris-buffered saline with Tween 20 (TBST buffer), Immobilon enhanced chemiluminescence (ECL) Ultra Western HRP substrate (EMD Millipore Corp., Burlington, MA, USA) was added and the chemiluminescence was detected by an imaging system (Image Quant LAS4000 multipurpose CCD camera system, Fujifilm, Tokyo, Japan). Multiple exposures of the membranes were done for 10 s to 5 min. The intensity of developed bands was normalized by the expression of GAPDH.

Immunofluorescent staining

Briefly 1 $\times$ 10^5 HDPCs were inoculated into 24-well culture plate with coverslips. After 24 h, HDPCs were exposed to Poly (I:C) for 0 (control), 5, 10, 30, 60, and 120 min.

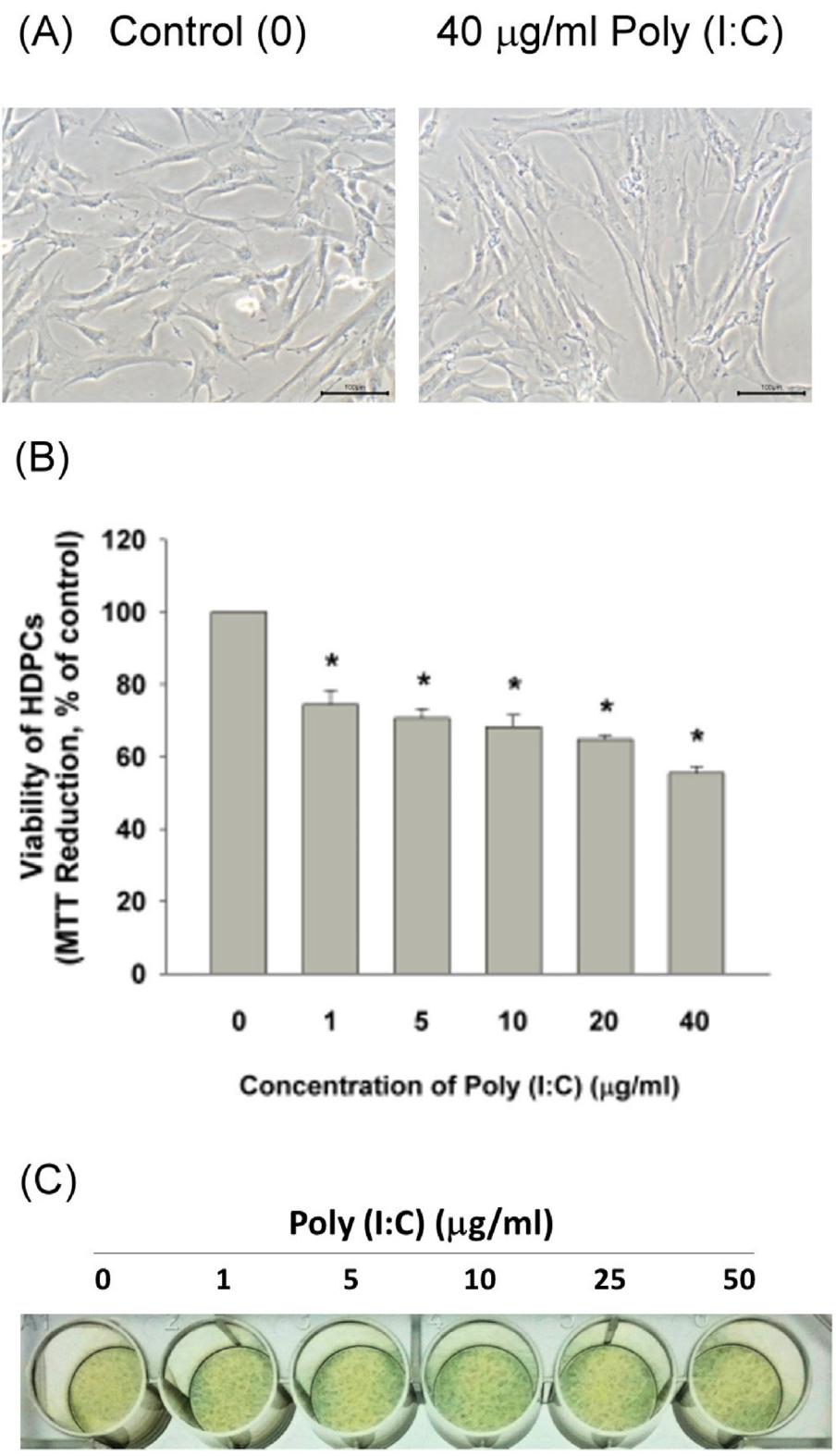
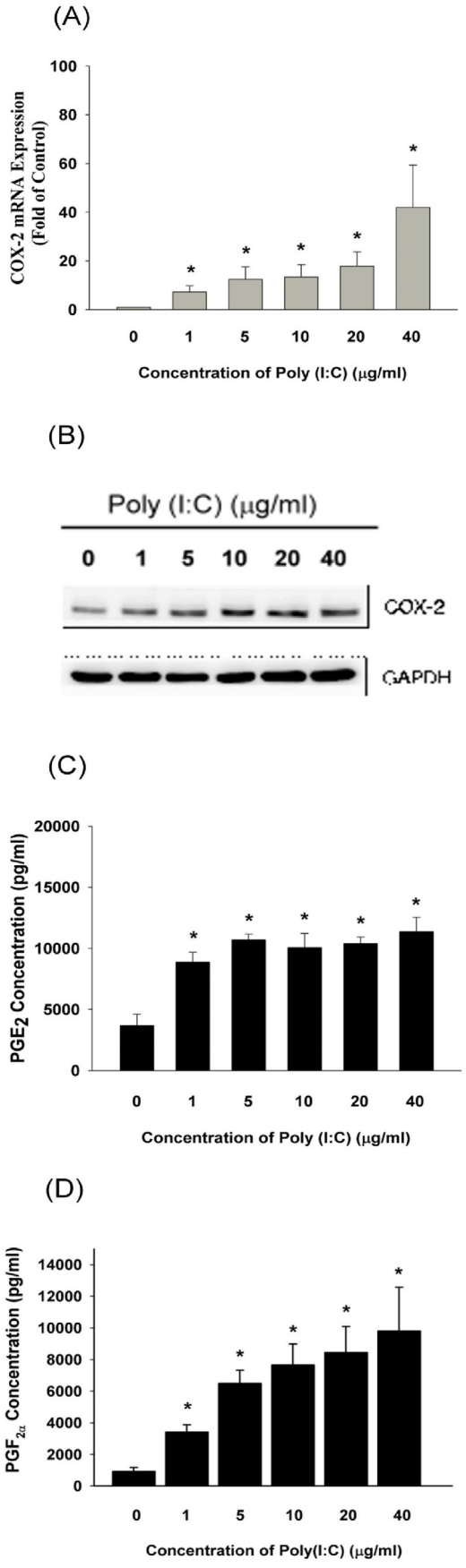


Fig. 1 Effect of Poly (I:C) on morphology and alkaline phosphatase (ALP) activity of human dental pulp cells (HDPCs). HDPCs were exposed to various concentrations of Poly (I:C) for indicated time points. **(A)** Morphology of HDPCs treated by solvent control (0 mg/ml) and HDPCs after exposed to Poly (I:C) (40 μg/ml) for 3 days. One representative morphology picture was shown. **(B)** Effect of Poly (I:C) on the viability of HDPCs. Results were expressed as percentage (%) of control (as 100 %). *denotes statistically significant difference when compared with control (0) group. **(C)** HDPCs were exposed to Poly(I:C) (1—50 μg/ml) for 5 days. ALP staining was performed. One representative ALP staining result was shown.



Immunofluorescent staining of *p*-ERK1/2 and p-p38 protein expression in HDPCs was performed as described before.[29,33]

Effect of U0126 and SB203580 on Poly (I:C)-induced COX-2 mRNA and protein expression as well as PGE_2 and $PGF_{2\alpha}$ production in HDPCs.

HDPCs were plated as above. Cells were then pretreated with U0126, SB203580 or solvent control (control group, 0) for 30 min and then Poly(I:C) (final 20 μg/ml) was added and further incubated for 24 h. Culture medium was collected for PGE_2 and $PGF_{2\alpha}$ ELISA. Cellular RNA and protein were isolated and analyzed by real-time PCR and western blotting analysis as described above.

Statistical analysis

All experiments were repeated more than three times and the means were used as the results. Difference between control and experimental groups was evaluated by paired student's *t*-test. A P value < 0.05 was regarded to indicate the presence of a statistically significant difference between groups.

Results

Effect of Poly (I:C) on morphology, viability and ALP activity of HDPCs

HDPCs were generally spindle shaped in appearance (Fig. 1A, left). Poly(I:C) showed no remarkable effect on cell morphology of HDPCs even at a concentration of 40 μg/ml (Fig. 1A, right). Unexpectedly, Poly (I:C) decreased the viability of HDPCs at concentrations higher than 1 μg/ml, as analyzed by MTT assay (Fig. 1B). The exposure to Poly (I:C) (<50 μg/ml) for 5 days showed no obvious effect on the ALP of HDPCs (Fig. 1C).

Effect of Poly (I:C) on COX-2 expression, PGE2 and PGF2α production of HDPCs

Poly (I:C) (a TLR3 agonist) was shown to stimulate COX-2 mRNA expression of HDPCs at concentrations ranging from 1 to 40 μg/ml of Poly(I:C) (Fig. 2A). In addition, Poly(I:C) also induced the COX-2 protein expression of HDPCs as analyzed by western blotting (Fig. 2B). Therefore, Poly (I:C) (1—40 μg/ml) also markedly stimulated PGE_2 and $PGF_{2\alpha}$

Fig. 2 **(A)** Effect of Poly(I:C) on cyclooxygenase-2 (COX-2) mRNA expression as analyzed by real-time polymerase chain reaction (PCR). Results were expressed as fold of control (Mean ± SE). **(B)** Effect of Poly (I:C) on COX-2 protein expression human dental pulp cells (HDPCs). One representative western blotting result was shown. **(C)** Effect of Poly (I:C) on prostaglandin E_2 (PGE_2) production of HDPCs. **(D)** Effect of Poly (I:C) on $PGF_{2\alpha}$ production of HDPCs. Results were expressed as pg/ml (Mean ± SE). *denotes statistically significant difference when compared with control group (0, solvent treated group). #indicates statistically significant difference when compared with Poly (I:C)-treated group.

production of HDPCs as measured by ELISA (Fig. 2C and D), suggesting the activation of TLR3 may mediate COX-2 expression and prostaglandins' production in HDPCs.

Effect of Poly(I:C) on *p*-ERK and p-p38 expression of HDPCs

To examine the expression of *p*-ERK and p-p38 expression of HDPCs after exposure to Poly (I:C), immunofluorescent (IF) staining was performed. As shown in Fig. 3, Exposure to Poly (I:C) for 30—120 min stimulated the *p*-ERK1/2 and p-p38 protein expression, as indicated by increased red fluorescence of HDPCs in immunofluorescent staining results (Fig. 3). These results reveal that Poly (I:C) may potentially activate TLR3, and their downstream cellular signaling molecules, to regulate cell behavior and inflammatory/repair responses.

Role of MEK/ERK, and p38 in the poly (I:C)-induced COX-2 mRNA and protein expression of HDPCs

Intriguingly, Poly (I:C)-induced COX-2 mRNA expression of HDPCs was prevented by U0126 (10 & 20 μM), and SB203580 (10 and 20 μM) as shown by real-time PCR results (Fig. 4A

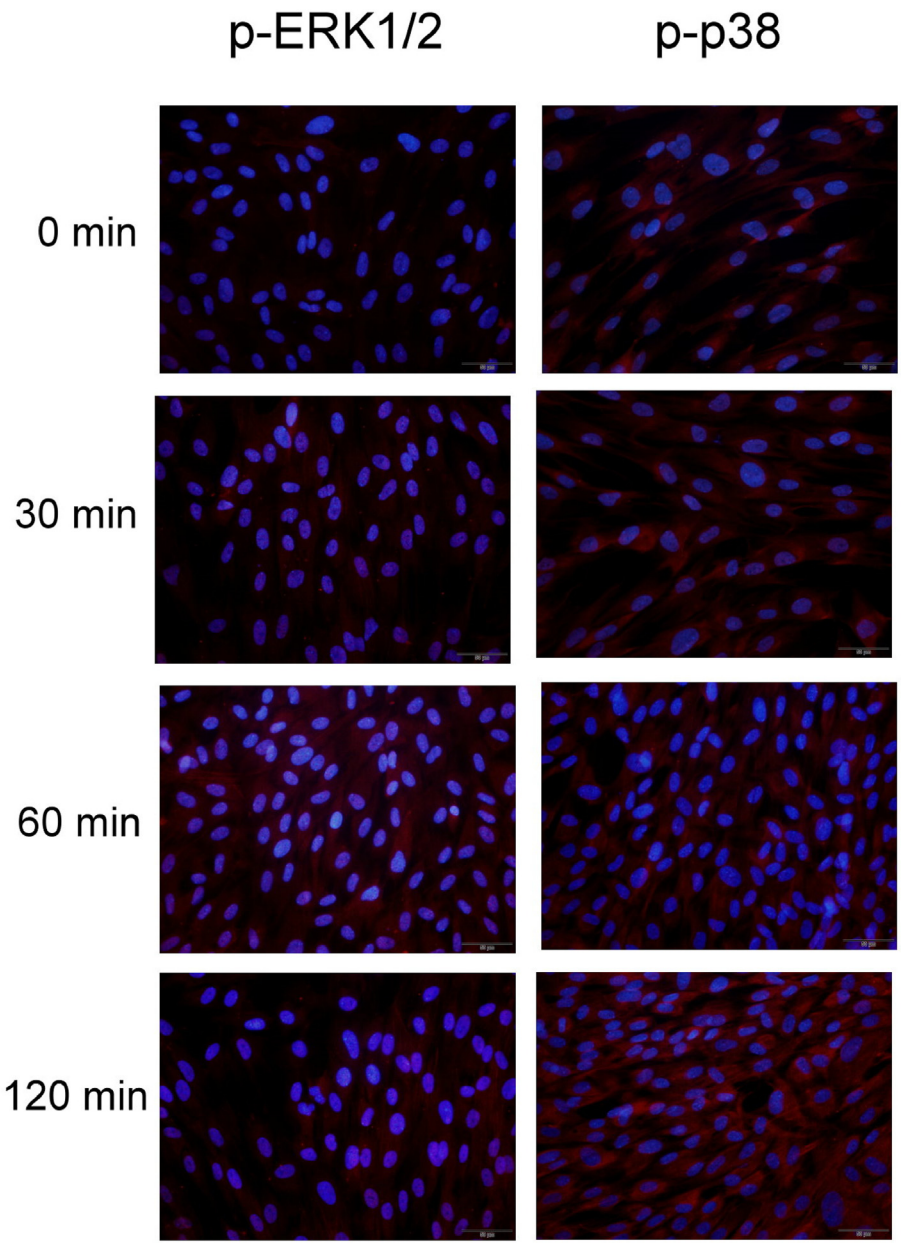


Fig. 3 Expression of phospho-extracellular signal-regulated kinase (*p*-ERK) (left) and phospho-p38 (p-p38) (right) protein after exposure of HDPCs to Poly (I:C) for 0 (control), 30 min, 60 min and 120 min. One representative immunofluorescent staining picture was shown with other similar results. Increased red fluorescence (*p*-ERK, p-p38) after exposure to Poly(I:C) for 30, 60, and 120 min was noted. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) (as Blue fluorescence). 400x of original magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and B). U0126 and SB203580 also prevented the Poly (I:C)-induced COX-2 protein expression as shown by decreased COX-2 protein bands' density of western blotting pictures (Fig. 4C and D).

Role of MEK/ERK, and p38 in the poly (I:C)-induced PGE_2 and $PGF_{2\alpha}$ production of HDPCs

To know whether inhibition of COX-2 by MEK/ERK and p38 may lead to decreased production of prostaglandins, we analyzed and found that Poly (I:C)-induced PGE_2 production of HDPCs was effectively prevented by both U0126 and SB203580 (Fig. 5A and B). Similarly, the Poly(I:C)-stimulated $PGF_{2\alpha}$ production of HDPCs was also attenuated by U0126 and SB203580 (Fig. 5C and D), suggesting the involvement of MEK/ERK and p38 signaling.

Discussion

During infection of periodontal, pulpal and periapical tissues, various bacterial products may affect the neighboring oral cells via the activation of TLRs and NODs receptors. The expression of TLRs and NODs receptors in periodontal tissues and dental pulp tissues have been reported.[1,34] Activation of TLRs has been shown to affect the osteoclast differentiation and bone loss.[35] In stromal osteoblasts, activation of TLR3 leads to PGE_2-mediated periodontal bony destruction.[36] TLRs on the surface of odontoblasts may detect the invaded pathogenic microorganisms and stimulate inflammatory cytokine/chemokine releases, as well as inflammatory cell infiltration to kill the pathogenic bacteria.[2] However, limited is known about the activation of TLR3 on the dental pulp inflammation and repair. Using Poly (I:C) (a specific TLR3 agonist) may clarify the effects of TLR3 activation on downstream signaling molecules and effective cellular responses. This is especially important because periodontal/pulpal infection and TLRs receptors' activation has been linked to several systemic diseases (Rheumatoid arthritis, cardiovascular diseases, etc.), and even cancer.[37–40] In this study, Poly(I:C), a TLR3 agonist, decreased the cell viability of HDPCs, but showed little effect on the ALP activity of confluent HDPCs, suggesting that Poly (I:C) may potentially affect the repair of human dental pulp. The inhibitory effect of 8 μg/ml Poly(I:C) on the proliferation of HDPCs at non-confluent status was reported.[41] Activation of TLR3 also stimulated ALP activity in aortic valve interstitial cells.[42] Corresponding well our current results, poly(I:C)-conditioned medium of peripheral blood mononuclear cells showed little effect on ALP activity of mesenchymal stem cells.[43] More studies are needed to clarify these events in different experimental conditions and cell types.

P. gingivalis LPS and other bacterial toxins were found to differentially stimulate the mRNA and protein expression as well as protein production/secretion of COX-2/PGE2, IL-6, TNF-α, IL-1β, vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1 of HDPCs and periodontal cells.[15,44–46] PGE_2, $PGF_{2\alpha}$ and the above mediators may be involved in tissue inflammatory responses (e.g., inflammatory cell infiltration, increase vascular permeability, chemotaxis of inflammatory cells etc.) of the

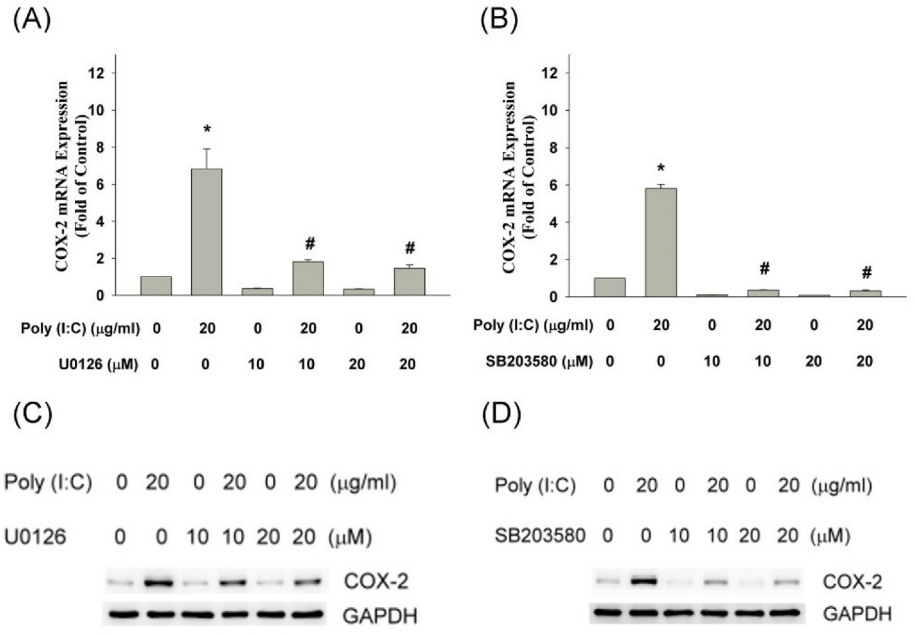


Fig. 4 **(A)** Effect of U0126, on Poly (I:C)-induced cyclooxygenase-2 (COX-2) mRNA expression of human dental pulp cells (HDPCs). **(B)** Effect of SB203580 on Poly (I:C)-induced COX-2 mRNA expression of HDPCs as measured by real-time polymerase chain reaction (PCR). Results were expressed as fold of control (as 1) (Mean ± SE). *denotes statistically significant difference when compared with control group. #indicates statistically significant difference when compared with Poly (I:C)-treated group. **(C)** Effect of U0126, on Poly (I:C)-induced COX-2 protein expression of HDPCs. **(D)** Effect of SB203580 on Poly (I:C)-induced COX-2 protein expression of HDPCs as analyzed by western blotting. One representative western blotting picture was shown.

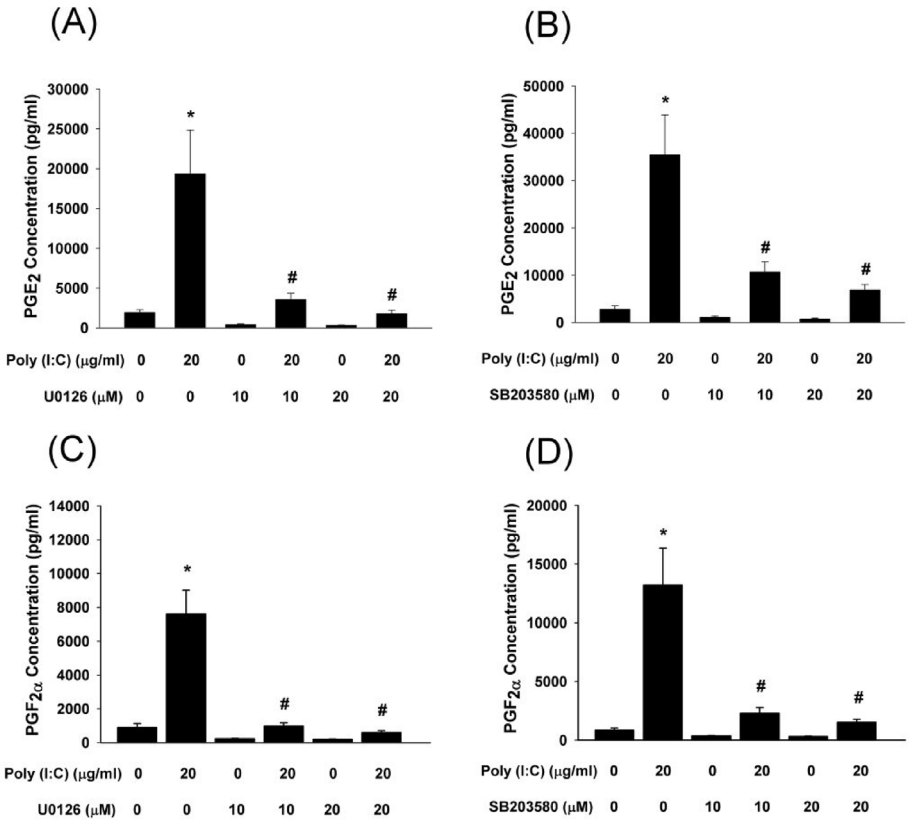


Fig. 5 **(A)** Effect of U0126, on Poly (I:C)-induced prostaglandin E2 (PGE_2) production of human dental pulp cells (HDPCs). **(B)** Effect of SB203580 on Poly (I:C)-induced PGE_2 production of HDPCs as measured by enzyme-linked immunosorbent assay. **(C)** Effect of U0126, on Poly (I:C)-induced $PGF_{2\alpha}$ production of HDPCs. **(D)** Effect of SB203580 on Poly (I:C)-induced $PGF_{2\alpha}$ production of HDPCs. Results were expressed as pg/ml (Mean ± SE). *denotes statistically significant difference when compared with control group. #indicates statistically significant difference when compared with Poly (I:C)-treated group.

infected dental pulp tissues. A recent study showed that *in vivo* administration of Poly(I:C) into mice every two days may induce systemic inflammation with associated changes in the dental pulp including increased multipotent progenitor cells, and influx of B cells.[47] Activation of TLR3 by Poly(I:C) *in vitro* was found to stimulate the expression of various factors such as brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), monocyte chemotactic factor-1 (MCP-1), stroma cells-derived factor-1 (SDF-1), IL-6, IL-11, metalloproteinase-9 (MMP-9), and tissue inhibitor metalloproteinase-1 (TIMP-1) in HDPCs.[41] Poly(I:C) was also shown to induce IL-8, IL-6, C—C motif chemokine ligand 2 (CCL2), and C-X-C motif chemokine ligand 10 (CXCL10) RNA expression in HDPCs.[1] In this study, it is interestingly that we found the induction of COX-2 mRNA and protein expression of HDPCs by Poly(I:C). Poly(I:C) further stimulated PGE_2 and $PGF_{2\alpha}$ production of HDPCs. In the dental pulp, PGE_2 and $PGF_{2\alpha}$ are found to stimulate IL-8 expression/production via prostaglandin EP and FP receptors, adenylate cyclase, phospholipase C, calcium mobilization and MEK/ERK signaling, but affect the ALP activity of human dental pulp cells (HDPCs).[48—50] PGE_2 was also shown to stimulate the mineralization-related genes' expression (bone morphogenetic protein-2 [BMP-2], Runx-2) in mouse dental pulp stem cells,[51] and provoke the gene expression of various angiogenesis and differentiation markers (vascular endothelial growth factor [VEGF], fibroblast growth factor 2 [FGF2], dentin sialophosphoprotein [DSPP], type I collagen) of HDPCs via EP2/EP4 receptors[52] These results indicate that TLR3 activation may be involved in the infection, inflammation and repair of dental pulp via stimulation of PGE_2/$PGF_{2\alpha}$ and differential activation of EP and FP receptors.

Activation of TLR3 and downstream signaling molecules may possibly mediate the above induced events. TLR3-mediated activation of NF-κB and mitogen-activated protein kinases (MAPKs) is reported to associate with TRAF6/TAK1/TAB2/PKR signaling.[21] However, the downstream signaling after TLR3 activation in the HDPCs is not fully understood. The induction of IL-8 expression of HDPCs by Poly(I:C) is correlate to TLR3/MAPKs/NF-κB activation.[1] In this study, Poly (I:C) as a TLR3 agonist was found to activate ERK and p38 signaling in HDPCs within 60 min of exposure. Activation of these signaling molecules may potentially affect the downstream effector molecules, such as inflammatory mediators (Prostaglandins, IL-6, IL-8, IL-1β, TNF-α etc.) to regulate the infection, inflammation, and wound healing/repair responses. Accordingly, we further found that Poly(I:C)-induced COX-2 expression, PGE_2 and $PGF_{2\alpha}$ production of HDPCs can be prevented by U0126 and

SB203580, suggesting the important contribution of TLR3-MEK/ERK and -p38 signaling in these events.

In conclusion, these results indicate that pulpal pathogens may contribute to the pathogenesis of pulpal infection, inflammation, healing, and repair processes via activation of TLR3 and downstream MEK/ERK and p38 signaling in HDPCs. TLR3 activation may decrease cell viability, stimulate COX-2 mRNA and protein expression with increased production of PGE_2 and $PGF_{2\alpha}$. Further studies are necessary to clarify the mechanisms of the up-stream signaling molecules and the down-stream effector molecules to further identify more diseased markers. The results may facilitate our future prevention and treatment of pathogen-associated pulpal/periapical diseases.

Declaration of competing interest

All authors declare there are no conflict of interest for this submission.

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References

1. Ha YE, So YJ, Im J, Yun CH, Park JC, Han SH. TLR3 recognition of viral double-stranded RNA in human dental pulp cells is important for the innate immunity. *Int Immunopharm* 2023; 119:110161.
2. Yumoto H, Hirao K, Hosokawa Y, et al. The roles of odontoblasts in dental pulp innate immunity. *Jpn Dent Sci Rev* 2018; 54:105—17.
3. Tanner A, Maiden MF, Macuch PJ, Murray LL, Kent Jr RL. Microbiota of health, gingivitis, and initial periodontitis. *J Clin Periodontol* 1998;25:85—98.
4. Nishihara T, Koseki T. Microbial etiology of periodontitis. *Periodontol* 2004;36:14—26. 2000.
5. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. *Microbiol Mol Biol Rev* 1998;62:1244—63.
6. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of Porphyromonas gingivalis. *Periodontol* 1999;20:168—238. 2000.
7. Isogai H, Isogai E, Yoshimura F, Suzuki T, Kagota W, Takano K. Specific inhibition of adherence of an oral strain of Bacteroides gingivalis 381 to epithelial cells by monoclonal antibodies against the bacterial fimbriae. *Arch Oral Biol* 1998;33:479—85.
8. Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA. A role for fimbriae in Porphyromonas gingivalis invasion of oral epithelial cells. *Infect Immun* 1997;65:1980—4.
9. Sojar HT, Han Y, Hamada N, Sharma A, Genco RJ. Role of the amino-terminal region of Porphyromonas gingivalis fimbriae in adherence to epithelial cells. *Infect Immun* 1999;67:6173—6.
10. Wilson M, Reddi K, Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. *J Periodontal Res* 1996;31:393—407.
11. Chang MC, Tsai YL, Chen YW, et al. Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts. *J Periodontal Res* 2013;48: 66—73.
12. Tsai YL, Chang MC, Lin LD, et al. Stimulation of prostanoids and IL-8 production in human gingival fibroblasts by Porphoromonas gingivalis LPS is associated with MEK/ERK signaling. *J Dent Sci* 2014;9:78—84.
13. Kabashima H, Yoneda M, Nagata K, Hirofuji T, Maeda K. The presence of chemokine (MCP-1, MIP-1alpha, MIP-1beta, IP-10, RANTES)-positive cells and chemokine receptor (CCR5, CXCR3)-positive cells in inflamed human gingival tissues. *Cytokine* 2002;20:70—7.
14. Zhang F, Engebretson SP, Morton RS, Cavanaugh Jr PF, Subbaramaiah K, Dannenberg AJ. The overexpression of cyclooxygenase-2 in chronic periodontitis. *J Am Dent Assoc* 2003; 134:861—7.
15. Paula-Silva FW, Petean IB, da Silva LA, Faccioli LH. Dual role of 5-lipoxygenase in osteoclastogenesis in bacterial-induced apical periodontitis. *J Endod* 2016;42:447—54.
16. Tsai YL, Wang CY, Chuang FH, et al. Stimulation of phosphatidylinositol-3- kinase/protein kinase B signaling by Porphyromonas gingivalis lipopolysaccharide mediates interleukin-6 and interleukin-8 mRNA/protein expression in pulp inflammation. *J Formos Med Assoc* 2023;122:47—57.
17. Okiji T, Morita I, Suda H, Murota S. Pathophysiological roles of arachidonic acid metabolites in rat dental pulp. *Proc Finn Dent Soc* 1992;88(Suppl 1):433—8.
18. Miyauchi M, Takata T, Ito H, et al. Immunohistochemical demonstration of prostaglandin E2, F2 alpha and 6-keto-prostaglandin F1 alpha in rat dental pulp with experimentally induced inflammation. *J Endod* 1996;22:600—2.
19. Nakanishi T, Shimizu H, Hosokawa Y, Matsuo T. An immunohistological study on cyclooxygenase-2 in human dental pulp. *J Endod* 2001;27:385—8.
20. Basu S. Novel cyclooxygenase-catalyzed bioactive prostaglandin F2alpha from physiology to new principles in inflammation. *Med Res Rev* 2007;27:435—68.
21. Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BR, Li X. Poly(I-C)-induced toll-like receptor 3 (TLR3)-mediated activation of NFkappaB and MAP kinase is through an interleukin-1 receptor associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. *J Biol Chem* 2003;278:16713—9.
22. Ok CY, Park S, Jang HO, Bae MK, Bae SK. Involvement of the visfatin/toll-like receptor 4 signaling axis in human dental pulp cells senescence: protection via toll-like receptor 4 blockade. *J Dent Sci* 2023;18:1177—88.
23. Fernandez A, Herrera D, Hoare A, Hernandez M, Torres VA. Lipopolysaccharides from Porphyromonas endodontalis and Prophyromonas gingivalis promote angiogenesis via Toll-like receptor 2 and 4 pathways in vitro. *Int Endod J* 2023;56: 1270—83.
24. Chang MC, Chen LI, Chan CP, et al. The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. Biomaterials 2010;31:8164—71.

25. Chang HH, Chen IL, Wang YL, et al. Regulation of the regenerative activity of dental pulp stem cells from exfoliated deciduous teeth (SHED) of children by TGF-β1 is associated with ALK5/Smad2. *TAK1, p38 and MEK/ERK signaling. Aging* 2020; 12:21253—72.
26. Jeng JH, Hsieh CC, Lan WH, et al. Cytotoxicity of sodium fluoride on human oral mucosal fibroblasts and its mechanisms. *Cell Biol Toxicol* 1998;14:383—9.
27. Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC. Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. *J Oral Pathol Med* 1999;28: 64—71.
28. Chang MC, Chen NY, Chen JH, et al. bFGF stimulated plasminogen activation factors, but inhibited alkaline phosphatase and SPARC in stem cells from apical papilla: involvement of MEK/ERK, TAK1 and p38 signaling. *J Adv Res* 2022;40:95—107.
29. Chang MC, Chen JH, Lee HN, et al. Inducing cathepsin L expression/production, lysosomal activation, and autophagy of human dental pulp cells by dentin bonding agents, camphorquinone and BisGMA and the related mechanisms. *Biomater Adv* 2023;145:213253.
30. Chang MC, Chen YJ, Tai TF, et al. Cytokine-induced prostaglandin E2 production and cyclooxygenase-2 expression in dental pulp cells: downstream calcium signaling via activation of prostaglandin EP receptor. *Int Endod J* 2006;39:819—26.
31. Chang MC, Hung HP, Lin LD, et al. Effect of interleukin-1β on ICAM-1 expression of dental pulp cells: role of PI3K/Akt, MEK/ERK and cyclooxygenase. *Clin Oral Invest* 2015;19: 117—26.
32. Chang MC, Chang HH, Hsieh WC, et al. Effects of transforming growth factor-β1 on plasminogen activation in stem cells from the apical papilla: role of activating receptor-like kinase 5/Smad2 and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signalling. *Int Endod J* 2020;53:647—59.
33. Chang MC, Tang CM, Lin YH, et al. Toxic mechanisms of Roth801, canals, microparticles and nanoparticles of ZnO on MG-63 osteoblasts. Mater sci eng. *C Mater Biol Appl* 2021;119: 11635.
34. Gu Y, Han X. Toll-like receptor signaling and immune regulatory lymphocytes in periodontal disease. *Int J Mol Sci* 2020;21:3329.
35. Kwon Y, Park C, Lee J, et al. Regulation of bone cell differentiation and activation by microbe-associated molecular patterns. *Int J Mol Sci* 2021;22:5805.
36. Tominari T, Akita M, Matsumoto C, et al. Endosomal TLR3 signaling in stromal osteoblasts induces prostaglandin E2-mediated inflammatory periodontal bone resorption. *J Biol Chem* 2022;298:101603.
37. Ettinger G, MacDonald K, Reid G, Burton JP. The influence of the human microbiome and probiotics on cardiovascular health. *Gut Microb* 2014;5:719—28.
38. Wallet SM, Puri V, Gibson FC. Linkage of infection to adverse systemic complications: periodontal disease, toll-like receptors and other pattern recognition systems. *Vaccines (Basel)* 2018;6:21.
39. Vitkov L, Hannig M, Minnich B, Herrmann M. Periodontal sources of citrullinated antigens and TLR agonists related to RA. *Autoimmunity* 2018;51:304—9.
40. Kamarajan P, Ateia I, Shin JM, et al. Periodontal pathogens promote cancer aggressivity via TLR/MyD88 triggered activation of integrin/FAK signaling that is therapeutically reversible by probiotic bacteriocin. *PLoS Pathog* 2020;16:e1008881.
41. Sabatini C, Ayenew L, Khan T, Hall R, Lee T. Dental pulp cell conditioning through polyinosinic-polycytidylic acid activation of toll-like receptor 3 for amplification of trophic factors. *J Endod* 2022;48:872—9.
42. Zhan Q, Song R, Zeng Q, et al. Activation of TLR3 induces osteogenic responses in human aortic valve interstitial cells through the NF-kB and ERK1/2 pathways. *Int J Biol Sci* 2015;11: 482—93.
43. Khokhani P, Belluomo R, Croes M, Gawlitta D, Kruyt MC, Weinans H. An in vitro model to test the influence of immune cell secretome on mesenchymal stromal cell osteogenic differentiation. *Tissue Eng C Methods* 2022;28:420—30.
44. Sismey-Durrant HJ, Hopps RM. Effect of lipopolysaccharide from Porphyromonas gingivalis on prostaglandin E2 and interleukin-1-beta release from rat periosteal and human gingival fibroblasts in vitro. *Oral Microbiol Immunol* 1991;6:378—80.
45. Ko YJ, Kwon KY, Kum KY, et al. The anti-inflammatory effect of human telomerase-derived peptide on P. gingivalis lipopolysaccharide-induced inflammatory cytokine production and its mechanism in human dental pulp cells. *Mediat Inflamm* 2015;2015:385127.
46. Jung JY, Woo SM, Kim WJ, et al. Simvastatin inhibits the expression of inflammatory cytokines and cell adhesion molecules induced by LPS in human dental pulp cells. *Int Endod J* 2017;50:377—86.
47. Osaki J, Yamazaki S, Hikita A, Hoshi K. Hematopoietic progenitor cells specifically induce a unique immune response in dental pulp under conditions of systemic inflammation. *Heliyon* 2022;8:e08904.
48. Chang MC, Chang HH, Lee MY, et al. Prostaglandin F(2alpha)-induced interleukin-8 production in human dental pulp cells is associated with MEK/ERK signaling. *J Endod* 2009;35:508—12.
49. Chang MC, Chen YJ, Lee MY, et al. Prostaglandin F(2alpha) stimulates MEK-ERK signaling but decreases the expression of alkaline phosphatase in dental pulp cells. *Int Endod J* 2010;43: 461—8.
50. Chang MC, Lin SI, Lin LD, et al. Prostaglandin E2 stimulates EP2, adenylate cyclase, phospholipase C and intracellular calcium release to mediate cyclic adenosine monophosphate production in dental pulp cells. *J Endod* 2016;42:584—8.
51. Lorencetti-Silva F, Pereira PAT, Meirelles AFG, Faccioli LH, Paula-Silva FWG. Prostaglandin E2 induces expression of mineralization genes by undifferentiated dental pulp cells. *Braz Dent J* 2019;30:201—7.
52. Ohkura N, Yoshiba K, Yoshiba N, et al. Prostaglandin E2-transporting pathway and its roles via EP2/EP4 in cultured human dental pulp cells. *J Endod* 2023;49:410—8.