

# *Porphyromonas gingivalis* lipopolysaccharide enhances the proliferation of human periodontal ligament cells via upregulation of cyclin D1, cyclin A and cyclin B1

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**Abstract.** Human periodontal ligament cells (hPDLs) play a notable role in periodontal tissue homeostasis and regeneration. However, the effect of *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS) on the proliferation of hPDLs remains unclear. The present study investigated the effects of *Pg*-LPS on the proliferation profile of hPDLs, and the involvement of cyclins and cyclin-dependent kinases in the process. hPDLs were treated with *Pg*-LPS, and cell proliferation and cycle were detected using Cell Counting Kit-8 assays and flow cytometry. The mRNA expression levels of the cyclins and cyclin-dependent kinases (CDKs), including cyclins A, B1, D1 and D2 and CDK1, 2 and 4, were detected using reverse transcription-quantitative PCR. The protein expression levels of cyclins A, B1 and D1 were analysed using western blotting. The proliferation of hPDLs was significantly increased after

treatment with *Pg*-LPS at the concentrations of 0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$  for 24, 36 and 48 h compared with the cells cultured without LPS ( $P < 0.01$ ). The proliferation index of hPDLs was significantly enhanced after treatment with *Pg*-LPS (0.0001, 0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ) for 24 h ( $P < 0.01$ ). However, the S-phase fraction (SPF) only significantly increased after treatment with *Pg*-LPS at 0.01  $\mu\text{g/ml}$  for 24 h ( $P < 0.05$ ), while the G<sub>2</sub>/M-phase fraction increased ( $P < 0.01$ ) and the G<sub>0</sub>/G<sub>1</sub>-phase fraction decreased ( $P < 0.01$ ) compared with the controls. The proliferation index and SPF increased, peaked at 24 h and then decreased at 48 h in both *Pg*-LPS-stimulated and control groups. Notably, *Pg*-LPS significantly upregulated the expression levels of cyclins D1, A and B1 after 24 h compared with those in the controls. Overall, the present study indicated that *Pg*-LPS may enhance the proliferation of hPDLs, potentially through upregulation of cyclins D1, A and B1.

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**Abbreviations:** hPDLs, human periodontal ligament cells; *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide; CCK-8, Cell Counting Kit-8; CDK, cyclin-dependent kinase; SPF, S-phase fraction; PDL, periodontal ligament;  $\alpha$ -MEM, modified Eagle's medium  $\alpha$ ; RT-qPCR, reverse transcription-quantitative PCR; WB, western blotting; ANOVA, analysis of variance

**Key words:** *Porphyromonas gingivalis*, lipopolysaccharide, human periodontal ligament cells, cell proliferation, cell cycle

## Introduction

Periodontal ligaments (PDL) connect the cementum to the alveolar bone and provide mechanical support to the periodontium (1). Human periodontal ligament cells (hPDLs), largely composed of fibroblasts, are involved in PDL homeostasis and regeneration (2). The proliferation of hPDLs may be required for the ability of the PDL to maintain a normal cell population and space under physiological conditions (3,4). Cell proliferation is known to be activated by injury to the PDL, such as infection or overload force (5,6). Therefore, the proliferation capacity of hPDLs may be needed for the renewal and repair of the PDL.

Proliferation is controlled by cell cycle progression, which is a complex and stepwise process. Cyclins and cyclin-dependent kinases (CDKs) are the predominant proteins that regulate the progression of the cell cycle. Cyclin D1 plays a notable role in G<sub>1</sub> phase progression of the cell cycle via CDK4 activation (7).

Cyclin D2 and cyclin A bind and activate CDK2, appearing in the G<sub>1</sub>/S and S phases (8,9). Cyclin B1, in partnership with CDK1, is associated with the transition from the G<sub>2</sub> phase to mitosis (10).

*Porphyromonas gingivalis* (*Pg*) is a major periodontal pathogen whose lipopolysaccharide (LPS) induces the production of inflammatory cytokines, such as IL-1 $\beta$ , -6, -8 and TNF- $\alpha$  (11-14). *Pg*-LPS has been identified as an important contributor to periodontal inflammation, destruction and alveolar bone resorption (11,15-17). Numerous studies that investigated the interactions between *Pg*-LPS and hPDLCs have demonstrated that *Pg*-LPS can promote the immuno-inflammatory response of hPDLCs (16-18). Cell proliferation and inflammatory cytokine release are the two major inflammatory responses observed in this setting (11,16,17). Given that pathological alterations caused by inflammatory insults can impact the regenerative capacities of hPDLCs, the mechanism of how LPS affects the proliferation of hPDLCs needs to be investigated. However, the impact of *Pg*-LPS on hPDLC proliferation has not been clearly resolved and remains a debated subject. Yu *et al* (19) reported that LPS-induced inflammation inhibits cell proliferation. By contrast, studies by Kato *et al* (11) and Takemura *et al* (20) have suggested that *Pg*-LPS promotes cell proliferation and induces pro-inflammatory cytokines. At present, to the best of our knowledge, there are few reports on the role of CDKs and cyclins in *Pg*-LPS-induced inflammation, and the effects of *Pg*-LPS on the cell cycle in the process of cell proliferation remain unclear. Therefore, the present study aimed to investigate the effects of *Pg*-LPS on the proliferation of hPDLCs and regulation of the cell cycle.

## Materials and methods

**Culture of hPDLCs.** Ethical approval for the present study was granted by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (approval no. IRB UW13-120; Hong Kong, China). hPDLCs were isolated from the non-decayed, healthy teeth of three donors (two females aged 13 and 15 years old separately; one male aged 14 years old) who had undergone premolar extraction for orthodontic treatment at the Division of Paediatric Dentistry and Orthodontics in The Faculty of Dentistry, The University of Hong Kong from 10th January 2017 to 31st December 2018. Written consent to use the samples in scientific research was signed by the parents or legal guardians of the donors. The PDL tissues of the root were scraped and collected from the middle third of the premolar root surfaces to avoid contamination by cells derived from the gingiva and dental germ. The primary hPDLCs were cultured in modified Eagle's medium- $\alpha$  ( $\alpha$ -MEM) containing 10% foetal bovine serum (HyClone; Cytiva) and 1% antibiotic solution (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After achieving 80% confluence, cells were detached by treatment with 0.25% trypsin (Thermo Fisher Scientific, Inc.) and sub-cultured in fresh  $\alpha$ -MEM. The hPDLCs were characterised by immunocytochemical staining for vimentin and cytokeratin. The 3rd to 5th passages of the hPDLCs were used as the test (treated with *Pg*-LPS) and control groups (without *Pg*-LPS). The hPDLCs were synchronised in

serum-free  $\alpha$ -MEM culture medium at 37°C for 24 h. After serum starvation, the cells in the test and control groups were synchronised to the same stages of the cell cycle. This was based on previous studies demonstrating that serum starvation can arrest cells at the G<sub>0</sub>/G<sub>1</sub> phase with high efficiency and no toxic effects (5,21-24).

**Cell viability and proliferation detection using cell counting kit-8 (CCK-8) assay.** The hPDLCs were seeded into 96-well plates at 5x10<sup>3</sup> cells/well. After serum starvation, the cells were treated with *Pg*-LPS (InvivoGen) at various concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10  $\mu$ g/ml) in the medium of  $\alpha$ -MEM containing 10% FBS for different durations (0, 6, 12, 18, 24, 36 and 48 h, respectively) at 37°C *in vitro*. Afterwards, 10  $\mu$ l CCK-8 solution (APEx-BIO Technology LLC) was added to each well of the plates. After incubation for 4 h at 37°C, the plates were measured for absorbance at 450 nm using a microplate reader (Molecular Devices, LLC).

**Flow cytometry for cell proliferation and cell cycle analysis.** The dose- and time-dependent effects of *Pg*-LPS were assessed separately by flow cytometry to monitor cell proliferation and cell cycle. To test the dose-dependent effects, hPDLCs were seeded into six-well plates at a density of 1.2x10<sup>5</sup> cells/well. After serum starvation, the cells were treated with *Pg*-LPS at various concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10  $\mu$ g/ml) for 24 h at 37°C *in vitro*. To investigate the time-dependent effects, hPDLCs were seeded into six-well plates at a density of 0.6x10<sup>5</sup> cells/well. After serum starvation, the cells were stimulated with the appropriate concentration (based on the previous result) of *Pg*-LPS for different durations (0, 6, 12, 18, 24, 36 and 48 h) at 37°C *in vitro*. Cells were harvested by trypsinisation, washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol on ice for 15 min. After the ethanol was washed out with PBS, the cells were re-suspended in 500  $\mu$ l DNA staining solution containing 50  $\mu$ g/ml propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.), 0.05% Triton X-100 and 0.1% mg/ml RNase A in PBS. The samples were kept in the dark at 37°C for 40 min. A FACSVerse flow cytometer (BD Biosciences) acquired 10,000 events for each sample, and the percentage of cells in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle were determined using FlowJo software (version 10.0.7; Tree Star, Inc.). The proliferation index and S-phase fraction (SPF) were analysed using flow cytometry. The formulae used to calculate the above were as follows (25): Proliferation index (%)=[(S + G<sub>2</sub>/M)/(G<sub>0</sub>/G<sub>1</sub> + S + G<sub>2</sub>/M)] x100%; SPF (%)=[S/(G<sub>0</sub>/G<sub>1</sub> + S + G<sub>2</sub>/M)] x100%; G<sub>0</sub>/G<sub>1</sub>-phase fraction (%)=[(G<sub>0</sub>/G<sub>1</sub>)/(G<sub>0</sub>/G<sub>1</sub> + S + G<sub>2</sub>/M)] x100%; G<sub>2</sub>/M-phase fraction (%)=[(G<sub>2</sub>/M)/(G<sub>0</sub>/G<sub>1</sub> + S + G<sub>2</sub>/M)] x100%.

**Detection of genes via reverse transcription-quantitative PCR (RT-qPCR).** A RT-qPCR assay was carried out to analyse the mRNA expression of genes associated with different stages of proliferation. After hPDLCs were stimulated with 0.01  $\mu$ g/ml *Pg*-LPS for 18 or 24 h, the mRNA expression levels of cyclins A, B1, D1, D2 and CDK1, 2 and 4 were detected by RT-qPCR. The sequences of the primers used are listed in Table I. Total RNA was immediately extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Cyclin A	GCCTTTCATTTAGCACTC	TGAAGGTCCATGAGACAA
Cyclin B1	GGAAACATGAGAGCCATCCT	TTCTGCATGAACCGATCAAT
Cyclin D1	CAAACAGATCATCCGCAAAC	GCGTGTGAGGCGGTAGT
CDK1	TGAAACTGCTCGCACTTG	ATGGTAGATCCCGGCTTATT
CDK2	CAGAAACAAGTTGACGGGAGA	GACATCCAGCAGCTTGACAATA
CDK4	ACAGCTACCAGATGGCACTTACA	CAAAGATACAGCCAACACTCCAC
Cyclin D2	GTGTGATGCCATATCAAGTCC	TCGCATACACTGATCATGC
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT

CDK, cyclin-dependent kinase.

reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. qPCR was then performed using Power SYBR<sup>®</sup>-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used as the housekeeping gene for the internal control. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. The relative mRNA expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (26) and normalized to the internal reference gene GAPDH.

**Detection of proteins by western blotting (WB).** After the hPDLs were incubated with 0.01 μg/ml Pg-LPS for 24 h, the cells were washed with cold PBS and lysed using a RIPA buffer (50 mM Tris; pH, 8.0; 150 mM NaCl; 5 mM EDTA, pH 8.0; 0.8% Triton X-100) containing 1% protease inhibitor. Protein concentrations were determined using the bicinchoninic acid protein assay. Equal quantities of protein extracts (30 μg/lane) were separated via 10% SDS-PAGE. After transferring the proteins to PVDF membranes. The membranes were blocked for 1 h at room temperature with 5% non-fat milk in TBST (10 mM Tris, 100 mM NaCl and 0.1% Tween-20), after which they were probed overnight at 4°C with primary antibodies against cyclin A (1:500; cat. no. sc596; Santa Cruz Biotechnology, Inc.), cyclin B1 (1:500; cat. no. sc245; Santa Cruz Biotechnology, Inc.), cyclin D1 (1:1,000; cat. no. ab134175; Abcam) and β-actin (1:1,000; cat. no. 8457; Cell Signaling Technology, Inc.). The membranes were washed with TBST and incubated with HRP-conjugated anti-IgG secondary antibodies (anti-rabbit IgG-HRP-linked antibody, 1:3,000; cat. no. cst7074; Cell Signaling Technology, Inc.; anti-mouse IgG-HRP-linked antibody, 1:3,000; cat. no. sc2055; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. After washing, the blots were developed with enhanced chemiluminescence (WesternBright<sup>®</sup> ECL HRP substrate; Advansta, Inc.) and exposed to X-ray film (Kodak). The densities of western blotting bands were measured by Quantity One 4.6.9 software (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Statistical analysis was carried out using SPSS 19.0 software (IBM Corp.). A one-way analysis of

variance (ANOVA) and Tukey's post hoc test were conducted for the data of flow cytometry and WB. A two-way ANOVA followed by Bonferroni test was performed for the CCK-8 and RT-qPCR data. All data were expressed as the mean ± standard deviation from four independent experiments. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Cell viability and proliferation detection by CCK-8 assay.** As presented in Fig. 1A, the hPDLs were stimulated with Pg-LPS at different concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10 μg/ml) for various durations (6, 12, 18, 24, 36 and 48 h) *in vitro*. The results showed that Pg-LPS could not inhibit the cell viability of hPDLs. The proliferation of hPDLs was significantly enhanced in the culture medium at concentrations of 1 and 10 μg/ml Pg-LPS for 6 (P<0.05) and 18 h (P<0.01) compared with the cells cultured without LPS. Pg-LPS at the concentrations of 0.001, 0.01, 0.1, 1 and 10 μg/ml for 24, 36 and 48 h significantly promoted the proliferation of hPDLs compared with controls (P<0.01). The proliferation of hPDLs was also significantly increased with Pg-LPS at the concentration of 0.0001 μg/ml for 48 h compared with the cells cultured without LPS (P<0.01). These results suggested that Pg-LPS could not inhibit the cell viability of hPDLs. Instead, Pg-LPS can increase hPDL proliferation.

**Cell proliferation and cell cycle analysis by flow cytometry.** After serum starvation for 24 h, the majority of cells were at the G<sub>0</sub>/G<sub>1</sub> stage and few were in the S or G<sub>2</sub>/M phases of the cell cycle (Fig. S1). To assess the dose-dependent effect of Pg-LPS, hPDLs were cultured with different concentrations of LPS (0.0001, 0.001, 0.01, 0.1, 1 and 10 μg/ml); the proliferation index of these treated cells was significantly enhanced compared with cells cultured without LPS (P<0.01), while there were no significant differences among the groups stimulated with Pg-LPS at all concentrations tested (Fig. 1B). However, the SPF was only significantly increased with Pg-LPS at a concentration of 0.01 μg/ml (P<0.05), compared with cells cultured without LPS (Figs. 1B and S2). As presented in Figs. 2 and S3, when hPDLs were incubated with 0.01 μg/ml Pg-LPS for 24 h, the G<sub>2</sub>/M-phase fraction of the hPDLs was higher compared with that in the control group (P<0.01), whereas the G<sub>0</sub>/G<sub>1</sub>-phase

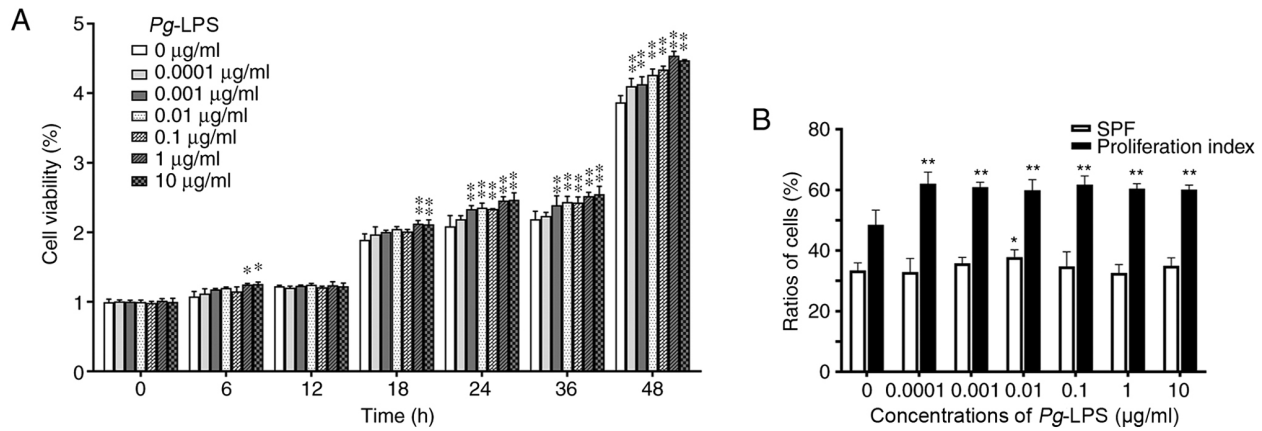


Figure 1. Effects of *Pg*-LPS on the proliferation and cell cycle of hPDLCS. (A) Cell viability and proliferation detected using Cell Counting Kit-8 assay. Proliferation of hPDLCS was significantly enhanced in culture medium at the concentrations of 1 and 10  $\mu\text{g/ml}$  *Pg*-LPS compared with the cells cultured without LPS at 6 and 18 h. *Pg*-LPS at the concentrations of 0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$  for 24, 36 and 48 h significantly promoted the proliferation of hPDLCS compared with controls. The proliferation of hPDLCS was also significantly increased with *Pg*-LPS at the concentration of 0.0001  $\mu\text{g/ml}$  for 48 h compared with the cells cultured without LPS. (B) Cell proliferation and cell cycle analysis using flow cytometry. After treatment with different concentrations of *Pg*-LPS (0.0001, 0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ) for 24 h, the proliferation index of hPDLCS was significantly enhanced at concentrations ranging from 0.0001-10  $\mu\text{g/ml}$ , but the SPF only increased significantly at 0.01  $\mu\text{g/ml}$  compared with that of cells without LPS. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control group (0  $\mu\text{g/ml}$  *Pg*-LPS) at each time point. *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide; hPDLCS, human periodontal ligament cells; SPF, S-phase fraction.

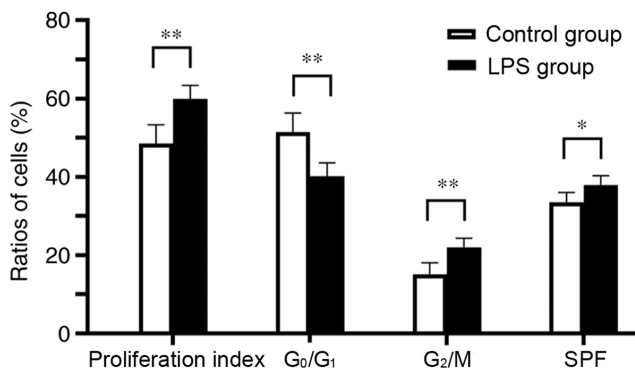


Figure 2. Effects of 0.01  $\mu\text{g/ml}$  *Pg*-LPS on the proliferation index, the G<sub>0</sub>/G<sub>1</sub>-phase fraction, the G<sub>2</sub>/M-phase fraction and SPF of hPDLCS. When hPDLCS were incubated with 0.01  $\mu\text{g/ml}$  *Pg*-LPS for 24 h, the proliferation index, SPF and G<sub>2</sub>/M-phase fraction of hPDLCS were higher compared with the control group, whereas the G<sub>0</sub>/G<sub>1</sub>-phase fraction was lower. \* $P < 0.05$ , \*\* $P < 0.01$ . *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide; hPDLCS, human periodontal ligament cells; SPF, S-phase fraction.

fraction was lower ( $P < 0.01$ ). Figs. 3 and S4 present the data for time-dependent effects. Both the proliferation index and SPF of the hPDLCS increased, reached a peak at 24 h and then decreased from 24 to 48 h in both the *Pg*-LPS-stimulated and control groups. The proliferation index and SPF of hPDLCS were significantly increased after treatment with *Pg*-LPS at 24 h compared with the cells cultured without LPS ( $P < 0.01$  and  $P < 0.05$ , respectively), while they were significantly decreased after treatment with *Pg*-LPS for 36 h compared with the cells cultured without LPS ( $P < 0.01$  and  $P < 0.05$ , respectively). There were no significant differences between *Pg*-LPS-stimulated and control groups at 0, 6, 12, 18 and 48 h. These results suggest that changes in PI and SPF were not dose-dependent, but were time-dependent.

*mRNA expression levels of cyclins and CDKs in hPDLCS treated with Pg-LPS.* The mRNA expression levels of cyclin A

and cyclin D1 were significantly increased in the LPS-treated hPDLCS at 18 and 24 h compared with their control groups at the same time point ( $P < 0.01$ ), and the levels of cyclin B1 were also increased at 24 h ( $P < 0.01$ ; Fig. 4). There were no significant differences in CDK1, CDK2 and CDK4 mRNA expression levels at 18 and 24 h between the LPS-treated and control groups at the same time points (Fig. 4). By tracking the trends of change, the levels of cyclin A, cyclin B1, CDK1 and cyclin D2 in the LPS-treated group were revealed to have risen sharply at 24 h compared with those at 18 h, whereas that of cyclin D1 decreased ( $P < 0.01$ ; Fig. 4). The expression levels of cyclin A, cyclin B1, CDK1 and CDK2 in the control group were higher at 24 h compared with those at 18 h ( $P < 0.05$ ; Fig. 4). These findings suggest that *Pg*-LPS can mainly promote the mRNA expression levels of cyclins D1, A and B1.

*Cyclin protein expression in hPDLCS treated with Pg-LPS.* hPDLCS were incubated in the presence or absence of 0.01  $\mu\text{g/ml}$  *Pg*-LPS. After 24 h of treatment, the protein expression levels of cyclins A, B1 and D1 were analysed using western blotting. Total cell lysates were normalised to  $\beta$ -actin. The levels of cyclin B1 and cyclin D1 in the LPS-treated groups significantly increased after 24 h compared with control groups at the same time point ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 5). These results were in concordance with the mRNA expression levels. There were no significant differences in the expression levels of cyclin A between the control and LPS group. These results revealed that *Pg*-LPS can upregulate the protein expression levels of cyclins D1 and B1.

## Discussion

The results of the present study indicated that high concentrations of *Pg*-LPS (1 and 10  $\mu\text{g/ml}$ ) stimulated the proliferation of hPDLCS at early stages (6 and 18 h), and that low concentrations of *Pg*-LPS (0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ) stimulated the proliferation of hPDLCS at later stages (24, 36 and 48 h).

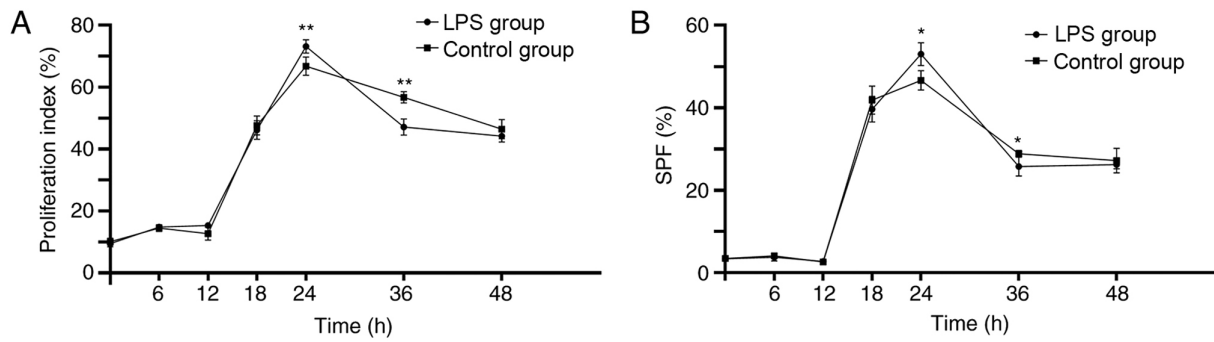


Figure 3. Changes of the proliferation index and SPF in hPDLCs induced by 0.01  $\mu\text{g/ml}$  *Pg*-LPS for 48 h. (A) Proliferation index and (B) SPF of hPDLCs increased, reached a peak at 24 h and then reduced from 24 to 48 h in *Pg*-LPS-stimulated and control groups. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control group at each time point. SPF, S-phase fraction; hPDLCs, human periodontal ligament cells; *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide.

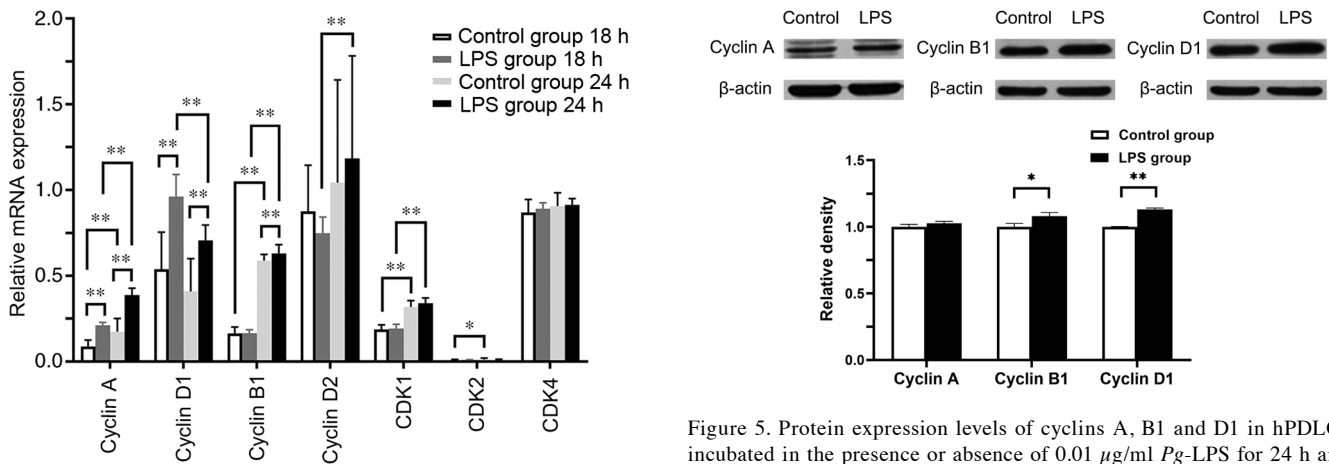


Figure 4. mRNA expression levels of cyclins A, B1, D1 and D2, and CDK1, 2 and 4 in hPDLCs treated with or without 0.01  $\mu\text{g/ml}$  *Pg*-LPS at 18 and 24 h. mRNA levels of cyclin A and cyclin D1 were significantly increased in the LPS-treated group at 18 h compared with those in the control group. mRNA levels of cyclins A, D1 and B1 were significantly increased in the LPS-treated group at 24 h compared with those in the control group. mRNA levels of cyclin A, cyclin B1, CDK1 and CDK2 in the control group were higher at 24 h compared with at 18 h. mRNA levels of cyclin A, cyclin B1, cyclin D2 and CDK1 in the LPS-treated group rose sharply, whereas that of cyclin D1 fell from 18 h to 24 h. \* $P < 0.05$ , \*\* $P < 0.01$ . CDK, cyclin-dependent kinase; hPDLCs, human periodontal ligament cells; *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide.

Figure 5. Protein expression levels of cyclins A, B1 and D1 in hPDLCs incubated in the presence or absence of 0.01  $\mu\text{g/ml}$  *Pg*-LPS for 24 h and assessed using western blotting. Protein levels of cyclin B1 and cyclin D1 in the LPS-treated group significantly increased compared with those in the control group. \* $P < 0.05$ , \*\* $P < 0.01$ . *Pg*, *Porphyromonas gingivalis*; LPS, lipopolysaccharide.

Moreover, *Pg*-LPS at a concentration of 0.01  $\mu\text{g/ml}$  prominently increased the proliferation of hPDLCs by affecting the  $G_1$ , S and  $G_2/M$  phases. A number of studies have reported different results concerning the effects of LPS on the proliferation of hPDLCs. Jönsson *et al* (27) demonstrated that PDL cell proliferation is unaffected by stimulation for 72 h with a high concentration (10  $\mu\text{g/ml}$ ) of *Escherichia coli* LPS. Huang *et al* (28) reported that stimulation with *Pg*-LPS (0.01, 0.1  $\mu\text{g/ml}$ ) for either 3 or 5 days has no effect on PDL cell proliferation. Jung *et al* (29) also demonstrated similar results when treating cells with *Pg*-LPS at different concentrations (0.1, 0.5, 1, 5, 10 and 20  $\mu\text{g/ml}$ ) for 7 days. By contrast, other studies have indicated that treatment with *Pg*-LPS at lower concentrations such as 0.01 and 0.1  $\mu\text{g/ml}$  stimulates the proliferation of hPDLCs on day 3 (30), whereas high concentrations of LPS, such as 1 (28), 10 and 100  $\mu\text{g/ml}$  (30), have been demonstrated inhibit the proliferation of hPDLCs.

These aforementioned findings highlight differences in both the dose- and time-dependent effects of *Pg*-LPS on the proliferation of hPDLCs. These contradictory results may be due to several reasons. First, hPDLCs in culture consist of several subpopulations (31). Second, there is a large amount of inter-individual heterogeneity in the responses of hPDLCs to *Pg*-LPS (32). Third, the properties of hPDLCs may change with different passages (33). Fourth, the proliferation capability of hPDLCs is a function of the patient's age and health status (23). Fifth, these results depend on certain characteristics of hPDLCs, the chemical structures of LPS and a number of culture environmental factors, including primary cell separation methods, culture medium composition, oxygen concentration, culture time and other physical or chemical stimulation (34). Finally, in the majority of the aforementioned studies, cell proliferation was detected using an MTT assay (28-30). However, using quantitative measurements, such as the CCK-8 assay, which is reported to be more stable and sensitive than MTT, improves the quality of results (35,36).

In the present study, the 3rd to 5th passages of hPDLCs from young patients with good oral health were selected to eliminate the influence of ageing or diseases on proliferative ability (5,37). *Pg*-LPS was used instead of *E. coli* LPS as *Pg* is a major periodontal pathogen that is capable of promoting cell

proliferation and inflammatory cytokine production (5,18). The present study detected both dose- and time-dependent effects using CCK-8 assay and flow cytometry to monitor cell proliferation and cell cycle.

As the cell cycle progresses, the preparatory  $G_0/G_1$  phase ensures that everything is ready for DNA synthesis, after which DNA replication occurs during the S phase (38). The  $G_2/M$  phase is the nuclear fission stage, which focuses on orderly division of the cell into two daughter cells (39). Proliferation index and SPF are two factors of flow cytometry indicative of cell proliferation activity. They provide an approximation of the growth fraction and usually represent the velocity of cell division (40). The present study revealed that the proliferation index of hPDLCS increased significantly after treatment with *Pg*-LPS (0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ) for 24 h which was consistent with the results of CCK-8 assay. However, there were no significant differences in the proliferation indices of hPDLCS among the groups stimulated with *Pg*-LPS at different concentrations (0.0001, 0.001, 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$ ). The SPF of hPDLCS only significantly increased when treated with *Pg*-LPS at a concentration of 0.01  $\mu\text{g/ml}$ , whilst there were no significant differences after treatment with *Pg*-LPS at the other concentrations (0.0001, 0.001, 0.1, 1 and 10  $\mu\text{g/ml}$ ) compared with cells cultured without LPS. These results indicated that changes in the proliferation index and SPF were not dose-dependent. After the hPDLCS were stimulated with 0.01  $\mu\text{g/ml}$  *Pg*-LPS for 24 h, both the SPF and  $G_2/M$ -phase fraction were increased compared with those in the control group, whereas the  $G_0/G_1$ -phase fraction was significantly decreased. This suggested that *Pg*-LPS enhanced the proliferation of hPDLCS by affecting both the S and  $G_2/M$  phases, while simultaneously reducing the proportion of cells in the  $G_0/G_1$  phase.

The cell cycle is a complex process that is needed for the proliferation of cells. CDKs and cyclins are central to this process. Extensive work on gene knockout mouse models of cell cycle regulators has revealed compensatory mechanisms that regulate the interactions among cyclins and CDKs (41,42). In addition, CDK2 has been revealed to be dispensable in the regulation of the mitotic cell cycle, as both CDK4 and CDK1 can cover for its functions (43). In the present study, the results of western blotting demonstrated that *Pg*-LPS treatment increased the expression levels of cyclin D1 and cyclin B1, but not of cyclin A. This implied that the CDK4/cyclin D1 and CDK1/cyclin B1 complexes, as potential master regulators, may compensate for the function of the CDK2/cyclin A complex in cell cycle control upon exposure to *Pg*-LPS. In the current study, the mRNA expression levels were consistent with those of protein expression for cyclin D1 and cyclin B1, but the cyclin A mRNA and protein expression levels were not concomitant. The inconsistency between cyclin A mRNA and protein expression levels may arise from several factors. First, the relationship between mRNA and protein expression levels is not strictly linear, as different regulatory mechanisms, such as gene transcription, post-transcriptional regulation, translation and protein modification, act on the synthesized mRNA and protein (44). Second, transcription and translation can be regulated in a spatiotemporal manner, and protein expression is known to lag behind mRNA transcription (45). Therefore, protein expression levels may not concur with mRNA

transcription levels at a given time point. Third, the detection sensitivities are different for RT-qPCR and western blotting, with RT-qPCR having increased sensitivity. Overall, the results of RT-qPCR and western blotting analyses confirmed that *Pg*-LPS was required for proliferation and acted by upregulating cyclin D1 and cyclin B1, which were involved in the  $G_1$  and  $G_2/M$  phases, respectively.

Upon further investigating the time dependency of cell proliferation after exposure to *Pg*-LPS, the present study revealed that *Pg*-LPS significantly augmented the proliferation index and SPF of hPDLCS at the 24-h time point and significantly reduced both at the 36-h time point but not at the other time points (6, 12, 18 or 48 h). The proliferation of hPDLCS, as indicated by SPF and proliferation index, indicated the same trends in change for both the test and control groups, suggesting that *Pg*-LPS did not change the overall time-dependency. Both the proliferation index and SPF of hPDLCS gradually increased, peaked at 24 h and then decreased from 24 to 48 h in both the *Pg*-LPS-stimulated and control groups. Maintaining the proper incubation period is therefore critical for *in vitro* studies involving hPDLCS. The present study demonstrated that when control hPDLCS (without *Pg*-LPS) were incubated for durations of 18 to 24 h, cyclin A, cyclin B1, CDK1 and CDK2 significantly increased. When hPDLCS in the test group (treated with 0.01  $\mu\text{g/ml}$  *Pg*-LPS) were incubated for the same durations, cyclin D2 levels also rose significantly and cyclin D1 levels fell. These observations may help explain why *Pg*-LPS significantly augmented the proliferation index and SPF of hPDLCS at the 24 h time point but not at the 18 h time point, and why, when progressing from 18 to 24 h, cyclin D2 increased to accelerate the  $G_1/S$  transition and cyclin D1 decreased to slowed down  $G_1$  progression. Changes that occur over longer durations will require further investigations in the future.

Currently, the intrinsic mechanisms by which *Pg*-LPS affects cell proliferation and regulates the cell cycle are unknown. Numerous studies have suggested that *Pg*-LPS produces pro-inflammatory cytokines mainly through the Toll-like receptor 4 pathway, which influences cell proliferation (5,19). However, the effect of *Pg*-LPS on the cell cycle and the concentration of *Pg*-LPS that affects hPDLCS proliferation are still matters of debate (18,46–48). The present study demonstrated that *Pg*-LPS promoted the proliferation of hPDLCS and that *Pg*-LPS prominently affected the  $G_1$ , S and  $G_2/M$  phases at a concentration of 0.01  $\mu\text{g/ml}$ . *Pg*-LPS was also demonstrated to likely affect the cell cycle through the actions of cyclin D1 and cyclin B1. However, the exact role of cyclin A in the cell cycle control is not yet clear as the result of inconsistencies between the RT-qPCR and WB data in the present study.

In summary, *Pg*-LPS may significantly stimulate the proliferation of hPDLCS through the upregulation of cyclins D1, A and B1. The mechanism of *Pg*-LPS on the proliferation and cell cycle regulation of hPDLCS remains unclear, and further studies are required. The results from the current study only address the short-term effects of *Pg*-LPS on the proliferation of hPDLCS. However, periodontal disease is a chronic inflammatory condition that can occur due to multiple and complex factors. Hence, more investigations conducted over longer durations are warranted to further understand periodontal pathogenesis and devise more effective therapeutic strategies.

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## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

YY, CZ and LJ made substantial contributions to the overall structural design of the study, methodology and data analysis. JL, YH and ZT performed the experiments. JL detected the cell proliferation and cell cycle by flow cytometry and analysed mRNA expression levels of the cyclins and CDKs by RT-qPCR. YH performed the western blotting of the cyclins. ZT and MG were major contributors in the CCK-8 assay for cell proliferation. JL and YH analyzed the data, drafted and revised the manuscript under the guidance of CZ, LJ and YY. JL and YH confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

Ethical approval for the present study was granted by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (approval no. IRB UW13-120; Hong Kong, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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