

Inflammatory Response and Proliferation of Stem Cells Isolated from Human Exfoliated Deciduous Teeth to Lipopolysaccharide from *Porphyromonas Gingivalis*

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Abstract

The effects of various concentrations of 0.01, 0.1, and 1.0 µg/mL of lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P. gingivalis*) on inflammatory response and proliferation of stem cells isolated from human exfoliated deciduous teeth (SHEDs) were compared. The MTT test was utilized to assess cell growth. Our research indicated that *P. gingivalis* LPS administration did not influence the proliferation of SHEDs. The expression levels of *IL1B*, *IL6*, and *IFNG* escalated in a dose-dependent way. Statistical significance was noted in the expression of *IL1B* at a concentration of 0.1 µg/mL of *P. gingivalis* LPS ($p < 0.05$) and in the expression of *IL6* at a concentration of 1.0 µg/mL of *P. gingivalis* LPS ($p < 0.05$). Moreover, treatment with *P. gingivalis* LPS at doses of 0.01 and 0.1 µg/mL resulted in enhanced TNF gene expression. Notably, at the maximum concentration (1.0 µg/mL), the gene expression level substantially diminished. Statistical significance was observed just in the expression of TNF at a concentration of 0.1 ($p < 0.05$). In conclusion, within the limitation of this study, findings indicate that 0.1 µg/mL *P. gingivalis* LPS is the optimal concentration for subsequent experiments involving SHEDs.

Keywords: Cell proliferation, *P. gingivalis* lipopolysaccharide (*P. gingivalis* LPS), Pro-inflammatory cytokines, SHEDs

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Introduction

Pulpitis is among the most common dental infections. Teeth affected by pulpitis frequently lead to various physiological and pathological changes, such as localized inflammation, edema, and congestion of the pulp tissue.¹ Moreover, pulpal infection that extends beyond the periodontium in deciduous teeth may adversely affect the development of permanent tooth germs. *Porphyromonas gingivalis* (*P. gingivalis*) is a gram-negative anaerobic bacillus that is frequently encountered as a pathogen in dental pulp infections and has been recognized as the causative

agent in the first stages of pulp and periodontal disease.^{2,3} A critical virulence factor of *P. gingivalis* is lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria and serves as a strong endotoxin exhibiting a wide range of biological action.⁴ The host response to LPS is primarily mediated through its recognition by Toll-like receptor 4 (TLR4), which, upon activation, initiates intracellular signaling cascades such as the NF- κ B and MAPK pathways via Myeloid differentiation primary response 88 (MyD88) dependent signaling.^{5,6} NF- κ B plays a critical role

in the migration and adhesion of various cell types.^{7,8} Moreover, IFNG regulates the odonto/osteogenic differentiation of dental pulp stem cells (DPSCs) through both NF- κ B and MAPK signaling pathways.⁹ MAPK has also been associated with the proliferation of endothelial progenitor cell-like cells generated from the periodontal ligament (PDL) via MEK/ERK and JNK-mediated signaling pathways.¹⁰ *Porphyromonas gingivalis* lipopolysaccharides (*P. gingivalis* LPS) initiates the inflammatory process by inducing the expression of pro-inflammatory cytokines and chemokines such as interleukin 1 beta (*IL1B*), *IL6*, *IL8*, *TNF* in periodontal ligament stem cells (PDLSCs)¹¹ and human monocytic cell.¹² Consequently, DNA methylation or the expression of microRNAs alters gene expression.⁶ Considering this, there is growing interest in understanding how *P. gingivalis* LPS affects various stem cell populations relevant to dental tissue repair and regeneration, particularly stem cells from human exfoliated deciduous teeth (SHEDs).

SHEDs are a type of mesenchymal stem cells (MSCs) capable of multipotential differentiation, including osteoblastic/odontogenic, adipogenic, neurogenic, and angiogenic differentiation after induction.¹³ SHEDs are isolated from dental pulp tissues that remain in naturally shedding deciduous teeth, making them easily accessible without requiring invasive procedures for cell collection. SHEDs offer distinct advantages over other MSC sources, including non-invasive collection, high proliferation, and potent regenerative capacity, supporting their relevance for inflammation-related studies in dental tissues. Several studies have examined the effect of the inflammatory microenvironment on the proliferative potential, multilineage differentiation, migration, and inflammatory cytokine secretion of oral MSCs. Notably, SHEDs have been shown to express Toll-like receptors TLR2 and TLR4, enabling them to respond to LPS.¹⁴ Of particular interest is the response of SHEDs to *P. gingivalis* LPS, although current findings remain inconsistent due to variations in MSC sources, inflammatory stimuli types and concentrations, and experimental designs.¹⁵ Previous studies have reported that *P. gingivalis* LPS can promote cell proliferation in PDLSCs¹¹ and fibroblasts,¹⁶ whereas other reports suggest

that *P. gingivalis* LPS inhibit proliferation in dental follicle progenitor cells (DFPCs).¹⁷ A similar inhibitory effect was observed in PDLSCs, where *P. gingivalis* LPS suppressed cell proliferation via TLR4 activation, increased reactive oxygen species (ROS) production, induced apoptosis, and altered the cell cycle through upregulation of cyclins D1, A, and B1.¹⁸ Furthermore, *P. gingivalis* LPS has been shown to stimulate bone marrow mesenchymal stem cell (BMMSC) proliferation at low concentrations while inhibiting it at higher doses.¹⁹ These conflicting results may stem from differences in LPS concentration, exposure duration, or donor variability, suggesting that the cellular response to *P. gingivalis* LPS may be dose- and context-dependent.

Despite this growing body of evidence, no studies have specifically investigated the effects of *P. gingivalis* LPS on SHEDs, representing a critical gap in understanding their roles in tooth tissue regeneration. Therefore, the purpose of this study is to examine how *P. gingivalis* LPS affects SHEDs, including the expression of pro-inflammatory cytokine genes and cell proliferation. The results of this study will facilitate the establishment of SHEDs for dental tissue regeneration and may contribute to the development of a new valuable pulp treatment protocol in the future.

Materials and Methods

Cell Isolation and culture of SHEDs

SHEDs were obtained from healthy pediatric patients aged 7-10 years, from deciduous teeth with no carious lesions or pathologic lesions. The teeth were extracted following the treatment plan such as prolonged retention teeth and stored in a culture medium. Ethical approval was submitted by the Ethics Committee, Faculty of Dentistry, Chulalongkorn University (approval no. 018/2023). The study protocol and informed consent were provided to the child's parent. To clarify, extracted teeth were washed three times with sterile phosphate buffer saline (PBS). The pulp tissues were carefully removed from the teeth and cut into small pieces with a surgical blade and placed on a 35-mm tissue culture plate (Corning, New York, NY, USA) containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum

(HyClone, USA (Gibco, USA), 2mM L-glutamine (Gibco, USA), 100 Units/ml Penicillin (Gibco, USA), 100 µg/mL Streptomycin (Gibco, USA), and 5 µg/mL Amphotericin B (Gibco, USA). The cell explants were incubated at 37°C in a humidified atmosphere of 5 %CO₂ in the air. The medium was changed every two days. When cell confluence was achieved, cells were detached with 0.25 % trypsin-EDTA (Gibco, USA) and subcultured at a 1:3 ratio into 60-mm tissue culture plates for the first passage. Cells from passages 3-6 were used for subsequent experiments.

Characterization of SHEDs

To characterize the cells, cultured cells were tested for properties as multipotent mesenchymal stem cells by plastic adhesion, shape, and morphology, as well as for their multipotential differential differentiation ability, using *in vitro* mineralization. Flow cytometry was used to determine the expression of cell surface antigens in SHEDs cells. Single cell suspensions were obtained by detaching cells with 0.25 % trypsin-EDTA solution. Cells were centrifuged and the supernatant culture medium was discarded. Then, cells were rinsed with PBS and stained with primary antibodies conjugated to fluorescent dye, including FITC-conjugated anti-human CD44 (BD Bioscience Pharmingen, USA), FITC-conjugated anti-human CD90 (BD Bioscience Pharmingen, USA), and PerCP-conjugated anti-human CD45 (BD Bioscience Pharmingen, USA). Stained cells were analyzed using a FACS^{Calibur} flow cytometer using the CellQuest software (BD Biosciences, USA).

LPS media preparation and treatment of SHEDs with various LPS concentrations

P. gingivalis LPS powder purified by phenol extraction (Sigma-Aldrich, USA) was dissolved in endotoxin-free water and homogenized to prepare the *P. gingivalis* LPS stock solution of 1 mg/mL. From this, a series of concentrations namely 0.01, 0.1, and 1.0 µg/mL were prepared via serial solution.

Pro-inflammatory cytokine gene expression

SHEDs were seeded at a density of 100,000 cells/well in 12-well plates in culture medium and incubated for 24 hours to allow cell attachment. The medium was changed to serum-free medium with various *P. gingivalis* LPS concentrations for 24 hours. The expression levels

of pro-inflammatory cytokines were determined by quantitative RT-PCR (qRT-PCR). The extraction of cellular RNA was performed using Trizol Reagent, following the guidelines provided by the manufacturer and converted into complementary DNA (cDNA) by using reverse transcriptase iScript cDNA Synthesis Kits (Bio-Rad, USA). After that, qPCR was performed on MiniOpticon real-time PCR system (Bio-Rad, USA) using FastStart Essential DNA Green Master kit (Roche Diagnostic, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used for normalization of each sample as the housekeeping gene. The primer sequences for qRT-PCR are listed in Table 1.

Table 1 Primer sequences of pro-inflammatory cytokine genes used in this study

Gene	Primer sequence
<i>GAPDH</i>	Forward: 5' CAC TGC CAA CGT GTC AGT GGT G' Reverse: 5' GTA GCC CAG GAT GCC CTT GAG 3'
<i>IL1B</i>	Forward: 5' GCA GAA GTA CCT GAG CTC GC 3' Reverse: 5' CTT GCT GTA GTG GTG GTC GG 3'
<i>IL6</i>	Forward: 5' CCT GAA CCT TCC AAA GAT GGC 3' Reverse: 5' CTG ACC AGA AGA AGG AAT GCC 3'
<i>IFNG</i>	Forward: 5' CCA ACT AGG CAG CCA ACC TAA 3' Reverse: 5' AGC ACT GGC TCA GAT TGC AG 3'
<i>TNF</i>	Forward: 5' CAC AGT GAA GTG CTG GCA AC 3' Reverse: 5' ACA TTG GGT CCC CCA GGA TA 3'

Cell proliferation assay

SHEDs were seeded at a density of 25,000 cells/well in 24-well plates in culture medium. After 24 hours, the culture medium was replenished with fresh medium containing the respective concentrations of *P. gingivalis* LPS, and this process was repeated three times for each concentration. On days 1, 3, and 7, changes in cell growth viability were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The existing medium in every well were removed and replaced with 300 µL MTT solution (USB Corporation, USA) for 15 minutes at 37°C in a humidified atmosphere of 5 % CO₂ to allow formazan crystal formation. Next, the formazan was dissolved using eluting agents that contained dimethylsulfoxide and glycine buffer. The solutions were determined for optical density (OD) by a microplate reader

(ELx800; BIO-TEK®) at 570 nm. using the vehicle-treated group as the baseline for comparison.

Statistical analysis

The experiments were duplicated using cells obtained from at least four different donors (n=4). Data were reported in the form of mean $\pm$ standard deviation (SD). The statistical analysis of the results was performed using the Kruskal-Wallis test and Dunn's multiple comparison test. The statistical analyses were created using GraphPad Prism 10.2.3 (GraphPad Software, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Isolation and characterization of SHEDs

Cells isolated from primary teeth were characterized by spindle-shaped, fibroblast-like morphology and formed distinguishable colonies (Fig. 1a). Flow cytometry analysis demonstrated the presence of mesenchymal stem cell markers CD44 (99.36) and CD90 (99.87), while the hematopoietic cell marker CD45 was absent (2.69) (Fig. 1b). Upon osteogenic induction, these cells exhibited increased mineral deposition compared to undifferentiated control cells, indicating their osteoblastic potential (Fig. 1c).

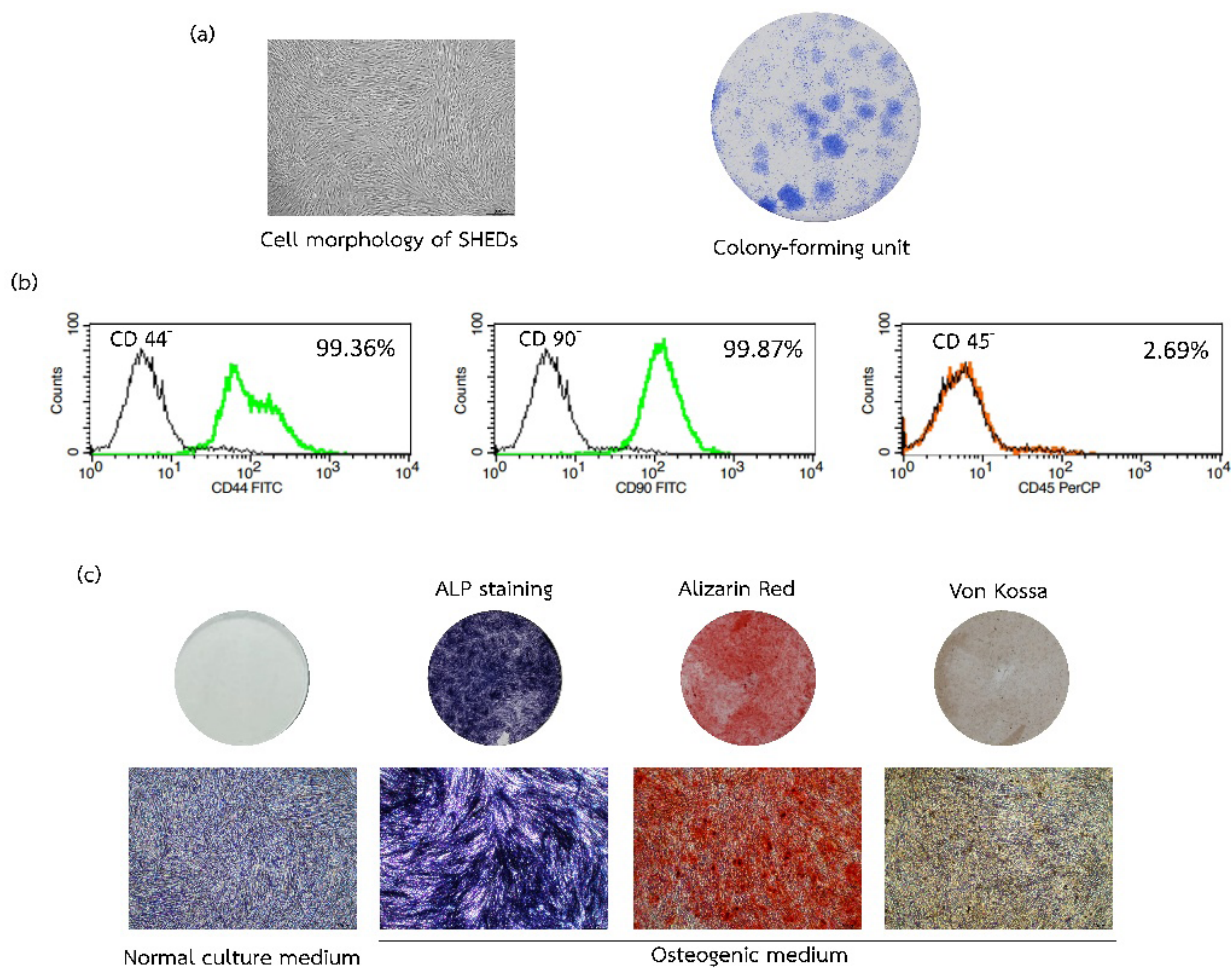


Figure 1 Characterization of the cells isolated from dental pulp tissues. (a) Cell morphology of isolated cells from primary teeth exhibited a spindle-like morphology under phase-contrast microscopy (left) (scale bar at 4X = 300 μ m.) and colony forming unit ability (right). (b) Presence of mesenchymal stem cell markers CD44 and CD90 by flow cytometric analysis, and the absence of the hematopoietic marker CD45. (black line: negative control) (c) The osteogenic differentiation was examined using alkaline phosphatase on day 7, Alizarin Red S and Von Kossa staining on day 14 after osteogenic induction (Scale bar at 4X = 300 μ m.)

P. gingivalis LPS Upregulates Pro-inflammatory Cytokine Gene Expression

Upon *P. gingivalis* LPS treatment, qRT-PCR revealed an upregulation in the expression of *IL1B*, *IL6* and *IFNG* in a dose-dependent manner. Statistical significance was observed in the expression of *IL1B* when cells were treated with 0.1 µg/mL of *P. gingivalis* LPS ($p < 0.05$) (Fig. 2a) and the expression of *IL6* at concentration of 1.0 µg/mL of

P. gingivalis LPS ($p < 0.05$) (Fig. 2b). Furthermore, exposure to *P. gingivalis* LPS also induced increased gene expression levels of *TNF* when treated with concentrations of 0.1 µg/mL (Fig. 2c). Interestingly, at the highest concentration (1.0 µg/mL), the gene expression level gradually decreased (Fig. 2c). However, statistical significance was only observed in the expression of *TNF* at concentrations of 0.1 ($p < 0.05$) (Fig. 2c).

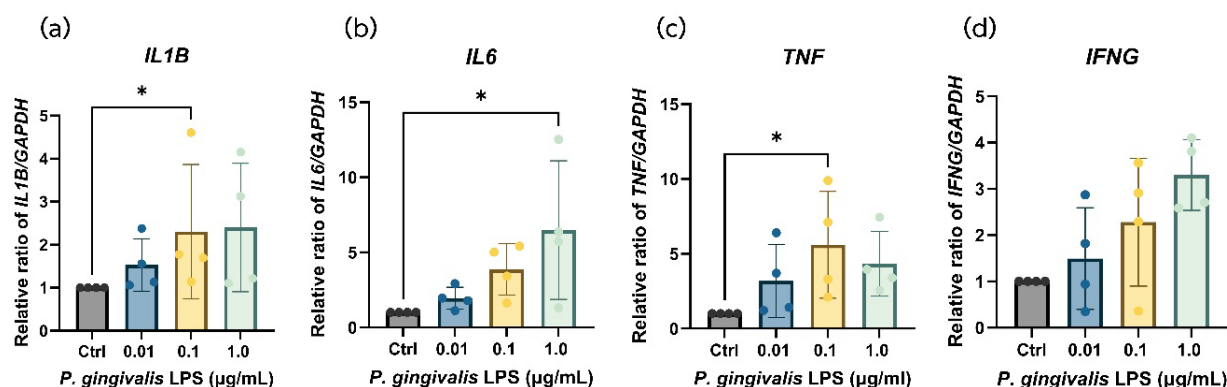


Figure 2 The effect of *P. gingivalis* LPS on the expression of pro-inflammatory cytokines of SHEDs. The relative gene expression of (a) *IL1B*, (b) *IL6*, (c) *TNF*, and (d) *IFNG* in SHEDs after treatment with *P. gingivalis* LPS at concentrations of 0.01, 0.1, and 1.0 µg/mL were compared to SHEDs without *P. gingivalis* LPS treatment. Data are shown as mean $\pm$ SD. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's multiple comparison ($n=4$); * $p < 0.05$

Effects of *P. gingivalis* LPS on the proliferation of SHEDs

Results revealed no statistically significant difference in cell proliferation ability between the control group and

the experimental groups treated with *P. gingivalis* LPS at concentrations of 0.01, 0.1, and 1.0 µg/mL ($p > 0.05$) (Fig. 3).

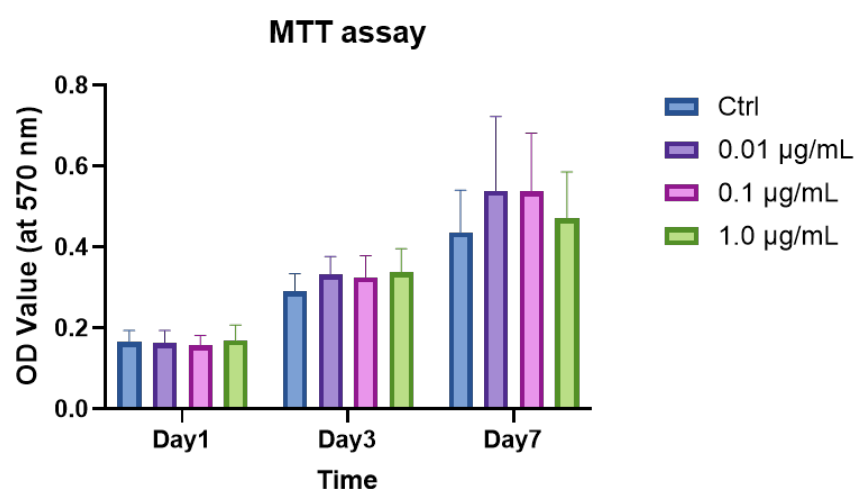


Figure 3 The effect of *P. gingivalis* LPS on the proliferation of SHEDs. Increasing *P. gingivalis* LPS concentration did not result in statistically different cell proliferative ability between the control group and the experimental groups ($p > 0.05$). Data are shown as mean $\pm$ SD. Statistical analysis was conducted using the Kruskal-Wallis test with Dunn's multiple comparison ($n=4$)

Discussion

This study is the first to explore the effects of *P. gingivalis* LPS on SHEDs, particularly at low concentrations. Our findings offer valuable insight into how clinically relevant LPS stimuli may influence SHEDs behavior in the context of dental inflammation and regeneration.

Dental caries is a chronic infectious condition driven by oral bacterial invasion into enamel and dentin. As the infection progresses, bacterial toxins such as LPS induces inflammation, triggering a host immune response and initiating tissue repair through the formation of tertiary dentin mediated by dental stem cells.²⁰⁻²² Among the various LPS sources studied, *P. gingivalis* LPS stands out due to its relevance in mimicking the pathophysiological environment of the carious pulp.²³ While *Escherichia coli* (*E. coli*) LPS has been shown to be a potent stimulator of immune responses in dental stem cells, its clinical relevance remains debated.²⁴⁻²⁷ In contrast, although *P. gingivalis* LPS is less potent, it more accurately reflects the natural inflammatory processes occurring in carious lesions and may therefore serve as a superior model for studying pulpal inflammation.^{24,28}

Our preliminary results confirmed that *P. gingivalis* LPS at concentration lower than 0.01 µg/mL had minimal impact on SHEDs proliferation, while higher concentration (10 µg/mL) had deleterious effects. Based on these observations, we focused on a dose range of 0.01–1.0 µg/mL for subsequent experiments. *P. gingivalis* LPS exposure led to a dose-dependent increase in pro-inflammatory cytokine gene expression, specifically *IL1B*, *IL6*, and IFNG. Statistically significant upregulation of *IL1B* and *IL6* was observed at 0.1 µg/mL and 1.0 µg/mL, respectively ($p < 0.05$) (Fig. 2a-b), consistent with previous findings in PDLSCs treated with 1 µg/mL *P. gingivalis* LPS.¹¹ TNF expression also increased at 0.1 µg/mL *P. gingivalis* LPS, similar to trends reported in DPSCs and stem cell from the apical papilla (SCAPs) exposed to *P. gingivalis* LPS.^{29,30} Interestingly, TNF levels declined at 1.0 µg/mL but remained elevated compared to control levels, indicating a possible threshold effect or feedback regulation. Notably, while these cytokines are known for their pro-inflammatory roles, they also contribute to regenerative signaling cascades, particularly

those involved in odontoblastic differentiation and reparative dentinogenesis. This dual role highlights the complexity of interpreting inflammatory cytokine profiles, as they may simultaneously mediate damage and repair.^{31,32}

In terms of cell proliferation, *P. gingivalis* LPS at concentration below 1.0 µg/mL produced a mild increase in SHEDs proliferation by day 7; however, this effect did not reach statistical significance (Fig. 3). The concentration of 0.1 µg/mL *P. gingivalis* LPS was found to preserve SHEDs viability, suggesting it may represent a biologically relevant dose for mimicking mild inflammatory conditions *in vitro*. These results are partially aligned with earlier studies demonstrating enhanced PDLSCs proliferation at 10 µg/mL *P. gingivalis* LPS¹¹ and increased BMMSC proliferation at 0.1 µg/mL, whereas higher concentrations were inhibitory.¹⁹ A similar biphasic response was also noted in DPSCs treated with *E. coli* LPS.³³ However, other cell types such as SCAPs, DFPCs, and BMMSCs have shown no significant proliferative response to *P. gingivalis* LPS^{34,35}, reinforcing the notion that LPS effects are highly context-dependent. These variations may also reflect inherent differences between permanent and deciduous tooth-derived stem cells, as SHEDs possess higher proliferative capacity and neurotrophic potential compared to other dental stem cell types. Thus, SHEDs offer a unique and underutilized *in vitro* model for examining host–pathogen interactions specific to primary dentition, which is especially relevant in pediatric oral health research. A limitation of this study is that it focused solely on cell proliferation; the potential effects of *P. gingivalis* LPS on SHEDs differentiation were not evaluated. As LPS may influence differentiation without affecting viability, this warrants further investigation in future studies.

The discrepancies observed among different studies likely stem from several factors, including heterogeneity in donor cell sources (genetic background, age, gender), the stage of cellular differentiation, and inter-individual variability that may affect stem cell bioactivity.²⁴ Methodological variables such as culture duration, frequency of medium changes, and experimental timelines also influence outcomes. Importantly, differences in the molecular structure

of LPS especially the lipid A moiety and variations in receptor binding affinity further complicate interpretations. For instance, The activation of *E. coli* LPS in dental pulp cells is initiated through pattern recognition receptors (PRRs), such as TLR4 and TLR2, located on the cell membrane.²⁴ These receptors detect pathogen-associated molecular patterns (PAMPs) like LPS and trigger downstream inflammatory signaling pathways. These include the MyD88-dependent pathway, NF- κ B signaling, MAPK activation via TIR domain-containing adaptors, and the PI3K-Akt pathway, all of which contribute to the production of pro-inflammatory cytokines.³² However, the interaction of *P. gingivalis* LPS with TLR2 and TLR4 remains controversial. While *E. coli* LPS increases TLR4 expression without affecting TLR2 in DPSCs, *P. gingivalis* LPS does not appear to alter the expression of either receptor in these cells.²⁴ Therefore, examining the effects of *P. gingivalis* LPS on TLR2 and TLR4 expression in SHEDs may help determine whether *P. gingivalis* LPS activates similar signaling pathways, providing further insight into immune response mechanisms in dental pulp-derived stem cells. Whether *P. gingivalis* LPS activates SHEDs via TLR2, TLR4, or alternative signaling pathways remains unresolved and warrants further investigation. It is also important to acknowledge the potential variability introduced by differences in LPS purification methods across suppliers and batches. These inconsistencies may influence cellular responses and highlight the need for standardization in future studies.

Despite the absence of statistically significant proliferation changes, our study suggests that low-dose *P. gingivalis* LPS may subtly promote SHEDs proliferation and inflammatory cytokine production in a dose-dependent manner. This finding may have implications for modeling chronic pulpal inflammation and studying early tissue responses in regenerative endodontic research. Further investigations are needed to delineate the molecular pathways involved and to evaluate the long-term regenerative capacity of SHEDs under sustained *P. gingivalis* LPS stimulation.

Conclusions

This study demonstrates that Porphyromonas gingivalis LPS induces a dose-dependent inflammatory

response in SHEDs, characterized by upregulation of *IL1B*, *IL6*, *IFNG*, and *TNF*. While low concentrations of *P. gingivalis* LPS did not significantly enhance SHEDs proliferation, a subtle promotive trend was observed, suggesting a potential role in early-stage tissue responses. These results support the use of *P. gingivalis* LPS as a clinically relevant stimulus to model chronic pulpal inflammation and highlight SHEDs as a promising *in vitro* system for investigating host-pathogen interactions and inflammation-mediated regenerative processes in the primary dentition.

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