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Osteogenic potential of periodontal ligament stem cells are unaffected after exposure to lipopolysaccharides

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Abstract: Periodontitis develops as a result of a continuous interaction between host cells and subgingival pathogenic bacteria. The periodontium has a limited capacity for regeneration, probably due to changes in periodontal ligament stem cells (PDLSCs) phenotype. The aim of this study was to evaluate the effects of lipopolysaccharides from *Porphyromonas gingivalis* (PgLPS) on mesenchymal phenotype and osteoblast/cementoblast (O/C) potential of PDLSCs. PDLSCs were assessed for Toll-like receptor 2 (TLR2) expression by immunostaining technique. After, cells were exposed to PgLPS, and the following assays were carried out: (i) cell metabolic activity using MTS; (ii) gene expression for *IL-1*β, *TNF-*α and *OCT-4* by real-time polymerase chain reaction (RT-qPCR); (iii) flow cytometry for STRO-1 and CD105, and (iv) osteogenic differentiation. PDLSCs were positive for TLR2. PgLPS promoted cell proliferation, produced *IL-1*β and *TNF-*α, and did not affect the expression of stem cell markers, STRO-1, CD105 and *OCT-4*. Under osteogenic condition, PDLSCs exposed to PgLPS showed a similar potential to differentiate toward osteoblast/cementoblast phenotype compared to control group as revealed by mineralized matrix deposition and levels of transcripts for *RUNX2*, *ALP* and *OCN*. These results provide evidence that PgLPS induces pro-inflammatory cytokines, but does not change the mesenchymal phenotype and osteoblast/cementoblast differentiation potential of PDLSCs.

Keywords: Lipopolysaccharides; Periodontal Ligament; Stem Cells, Osteoblasts.

Introduction

Periodontal disease develops as a result of a continuous interaction between host cells and subgingival pathogenic bacteria, such as the gram-negative anaerobe *Porphyromonas gingivalis* (Pg), a major etiological agent of periodontitis.[1] Once damaged, the periodontium has a limited capacity for regeneration, which is an extremely complex process, mostly resulting in repair rather than regeneration during the healing process.

Currently, a wide variety of regenerative therapies have been proposed to promote the regeneration of periodontal supporting tissues, relying almost entirely on the use of implantation of structural substitutes and focusing specially on regenerating lost alveolar bone. However, the

clinical results are hard to predict, and histologically, the regenerative potential of these techniques has proved limited.[2]

The complex series of events associated with periodontal regeneration involves recruitment of locally derived progenitor cells to the affected site, and their subsequent differentiation into periodontal ligament and mineralized tissues.[3] The limited regeneration potential of current techniques may be a result of poor innate ability of damaged periodontal tissues to regenerate.[4] This may occur after the prolonged inflammatory process caused by pathogenic bacteria. Periodontopathic bacteria possess a number of potential virulence factors and induce host inflammatory mediators, eventually leading to connective tissue degradation and alveolar bone resorption.[5]

Studies have shown that the presence of chronic inflammation might compromise the migration, proliferation and availability of stem cells in multiple sclerosis[6] and atherosclerosis.[7] Regarding periodontal disease, an *in vitro* study showed that exposure to the etiological factors induced apoptosis in periodontal ligament cells.[8]

P. gingivalis presents several bioactive components. Lipopolysaccharide (LPS) is a major constituent of the bacteria outer membrane, and is considered a potential inducer of pro- and anti-inflammatory cytokines and chemokines.[9] These proteins interact with Toll-like receptors (TLRs), which play key roles in innate immune recognition and cellular activation in response to pathogens.[1] Among these receptors, TLR2 and TLR4 function as main sensors for innate cell wall components of gram-negative bacteria, and may be related in the progression of periodontitis.[1,10]

Based on current available evidence, exposure to an enriched endotoxin environment may affect mesenchymal stem cell (MSC) properties such as self-renewal, differentiation potential, and production of cytokines and extracellular matrix compounds. Thus, this study investigated the effects of lipopolysaccharides from *Porphyromonas gingivalis* (PgLPS) on cell metabolic activity, expression of pro-inflammatory cytokines and stem cell markers, and osteoblast/cementoblast differentiation potential of periodontal ligament stem cells (PDLSCs).

Methodology

Cell culture

This study was approved by the Institutional Review Board of Piracicaba Dental School – University of Campinas (#022/2011). Three populations of mesenchymal progenitor cells (STRO1$^+$, CD105$^+$, CD34$^-$, and CD45$^-$ cells) from periodontal ligament (PDL) of permanent teeth were obtained and characterized in a previous study.[11] Briefly, CD105$^+$-enriched cell subsets from PDL were isolated by magnetic cell sorting, and characterized by flow cytometry and immunostaining. All three PDLSC populations were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% L glutamine and 1% penicillin/streptomycin (GIBCO BRL, Life Technologies, Carlsbad, USA) (standard media) at 37°C in atmosphere containing 5% CO_2, frozen with *Recovery™ Cell Culture Freezing Medium* (Gibco BRL) and kept in liquid nitrogen for subsequent experiments.

Preparation of LPS solution from Porphyromonas gingivalis

Bacterial LPS was suspended in 1 mL of pure, sterile and endotoxin-free water as a stock solution (concentration 1 mg/mL) of *Porphyromonas gingivalis* (InvivoGen, San Diego, USA). At the time of each experiment, the concentrations of 0, 0.1, 1 and 10 μg/mL[12] were obtained by diluting LPS in DMEM from the stock solution.

Immunostaining for TLR2

To evaluate the expression pattern of TLR2, PDLSCs were seeded at 4×10^4 cells/well on glass coverslips (13 mm, Knittel® GmbH - Braunschweig, Germany) placed in 24-well plates (Falcon, BD Labware, Franklin Lakes, USA), and cultured for 24 hours in standard media. Afterward, cells were fixed in 4% paraformaldehyde for 10 minutes, blocked with 3% bovine serum albumin (BSA, Sigma, St Louis, USA) for 30 minutes, followed by a standard protocol for immunostaining. Cells were incubated with a mouse anti-human TLR2 antibody (1:50; Abcam, Cambridge, USA) followed by a secondary antibody Goat anti-mouse IgG Alexa Fluor 488 (1:1000) (Invitrogen, Carlsbad, USA) for one hour. Samples were

counterstained with TO-PRO®-3 iodide 642/661 (1:2000) (Invitrogen) for 15 minutes. For negative control, only secondary antibody was used. The samples were analyzed by confocal laser scanning microscopy (Leica TCS SP5AOBS, Mannheim, Germany).

Cell metabolic activity assay

To determine the cytotoxicity of 0, 0.1, 1 or 10 µg/mL concentrations of PgLPS, MTS assay was carried out as described previously.[13] At time points of 1, 3, 7, and 10 days, cells were washed with phosphate buffered saline (GIBCO BRL). Then, 20 µL of CellTiter96®AQ_{ueous} One Solution Reagent – MTS assay (PromegaCo., Ltd. Madison, USA) was added to each well and cells were incubated for 2 hours. Absorbance at 490nm was measured.

Pro-inflammatory cytokine gene expression

To verify whether PDLSCs responded to 0, 0.1, 1 or 10 µg/mL of PgLPS, a real time quantitative polymerase chain reaction (RT-qPCR) was performed to assess gene expression changes on pro-inflammatory cytokines interleukin-1 beta (*IL-1*β) and tumor necrosis factor alpha (*TNF*-α). PDLSCs were seeded (2×10^5/60-mm dishes) and cultured in standard media for 24 hours. Subsequently, media was changed and supplemented with PgLPS. After 24 hours, total RNA was obtained using TRIzol® reagent (Invitrogen) and mRNA levels were determined by RT-qPCR.

Mesenchymal stem cell markers expression

Flow cytometry analysis of cell surface

Fluorescence-active cell sorting was used to evaluate the expression of cell surface markers STRO-1 and CD105 (Endoglin, SH2 antigen) under PgLPS exposure. PDLSCs were plated in 100-mm tissue culture dishes and cultured in standard media for 24 hours. Subsequently, media was changed to 10% DMEM supplemented with 1 µg/mL PgLPS. After 7 days, cell suspension was obtained by detaching monolayers of PDLSC populations with 5 mg/mL of Collagenase IV (Gibco) and 5mM EDTA (Applied Biosystems, Foster City, USA), and blocking with 10% normal donkey serum (Sigma) for 20 minutes. Cells (10^6) were incubated with mouse anti-human monoclonal antibodies against STRO-1-fluorescein isothiocyanate (FITC) (BioLegend, San Diego, USA) and CD105-allophycocianin (APC) (BD Bioscience, San Jose, USA) for 40 minutes at 4°C, washed and resuspended in phosphate buffered saline (pH 7,4). As a negative control, FITC and APC-conjugated non-specific mouse IgG1 antibodies (BD Bioscience) were used. Quantitative fluorescence-activated cell sorter (FACS) analysis was performed on a FACScan instrument (BD FACSCalibur™; BD Bioscience Pharmigen, San Jose, USA), and the results were processed using CELLQUEST software (BD Bioscience Pharmigen).

OCT-4 gene expression

To determine whether LPS exposure could change the gene expression of octamer-binding transcription factor 4 (*OCT-4*), PDLSCs were cultured in standard media supplemented with 1 µg/mL PgLPS. After 7 days, total RNA was obtained using TRIzol® reagent (Invitrogen) and the mRNA levels for *OCT-4* were determined by RT-qPCR.

Osteogenic induction

To evaluate the ability of PDLSCs to differentiate along the osteoblast/cementoblast lineage under PgLPS exposure, cells were seeded with standard media for gene expression (2×10^5/60-mm dishes) and mineral nodule formation (24-well plates) *in vitro*. After 24 hours, it was added osteogenic-inducing medium (OM) (DMEM 10% FBS, 50 µg/mL ascorbic acid, 10 mM β-glycerol-phosphate, 10^{-5} M dexamethasone) supplemented or not with 1 µg/mL PgLPS. Total RNA was obtained using TRIzol® reagent (Invitrogen) at days 3, 7, and 14, followed by the expression analysis of run-related transcription factor-2 (*RUNX2*), alkaline phosphatase (*ALP*) and osteocalcin (*OCN*). In parallel, *in vitro* mineral nodule formation was assessed at day 21 using the Alizarin Red staining (AR-S, Sigma).[13]

Gene expression analysis

Transcription expression of cytokines *IL-1*β and *TNF*-α; osteogenic markers *RUNX2, ALP* and *OCN*; and *OCT-4* were examined by RT-qPCR. Samples were prepared as described previously [13]. Primers sequences for *ALP, OCN, RUNX2, IL-1*β, *TNF*-α, *OCT-4* and glyceral-dehyde-3-phosphate dehydrogenase (*GAPDH*) are shown in Table 1.

Statistical analysis

All experiments were performed in triplicate. Means and standard deviations were obtained to establish statistical comparisons. To assess cell proliferation, *OCT-4* and osteoblast gene expression, a two-way Analysis of Variance test was performed, followed by Tukey test. To assess the effect of LPS on cytokines' gene expression non-parametric Kruskal-Wallis test was performed followed by the Student-Newman-Keuls test, using Bioestat 5.0 software (Belém, Brazil). *P* values less than 0.05 were considered significant.

Results

PDLSCs express PgLPS receptor

Immunofluorescence analysis revealed that PDLSCs exhibited positive staining for TLR2, confirming that these cells are capable of recognizing PgLPS (Figures 1A and B).

PgLPS partially induces PDLSCs response

To verify whether PDLSCs responded to PgLPS, a metabolic activity, as an indicator for cell proliferation, was measured using MTS assay. All cell populations

Table 1. Primer sequences used for PCR amplification in real time-PCR.

Gene	Forward Primer	Reverse Primer	Amplicon
ALP	5′-cgggcaccatgaaggaaa-3′	5′-ggccagaccaaagatagagtt-3′	184
OCN	5′-agctcaatccggactgt-3′	5′-ggaagaggaaagaagggtgc-3′	150
RUNX2	5′-ccgtccatccactctaccac-3′	5′-atgaaatgcttgggaactgc-3′	139
IL-1β	5′-cttcttcgacacatgggataac-3′	5′-tttgggatctacactctccagc-3′	283
TNF-α	5′-tccacccatgtactcctcac-3′	5′-cctcccagatagatgggctcata-3′	155
OCT-4	5′-agcttagcttcaagaacatgtgta -3′	5′-gttgcctctcactcggt -3′	161
GAPDH	5′-acatcatccctgcctctac-3′	5′-ccaccttcttgatgtcatcatatttg-3′	171

ALP: alkaline phosphatase; OCN: osteocalcin; RUNX2: runt-related transcription factor 2; IL-1β: interleukin-1 beta; TNF-α: tumor necrosis factor alpha; OCT-4: octamer-binding transcription factor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

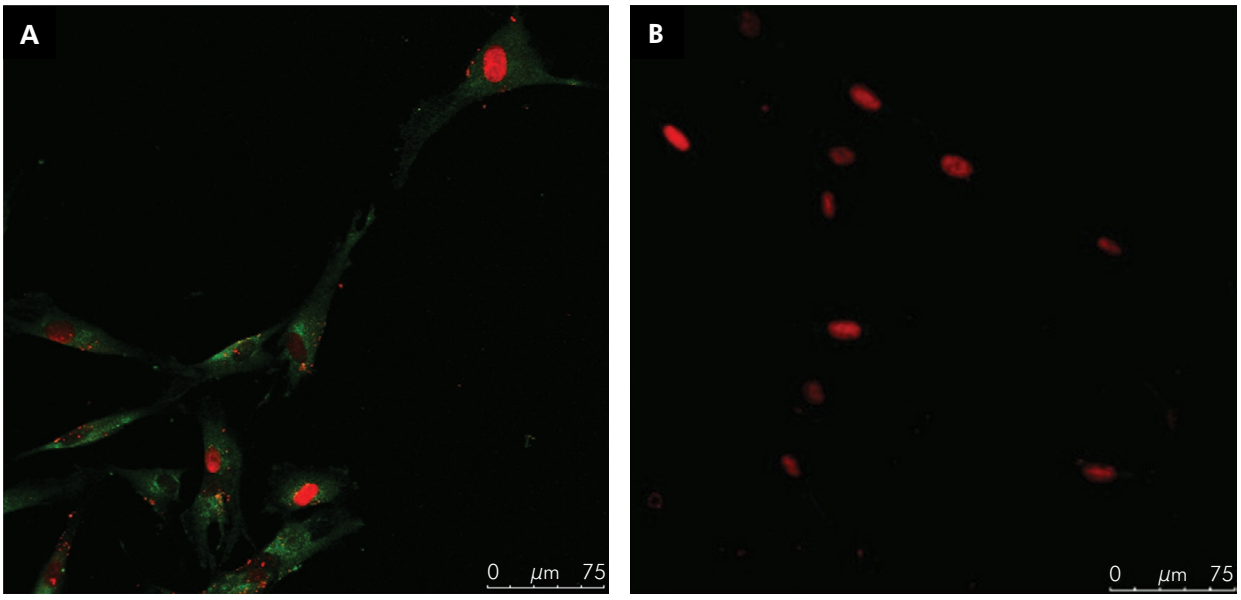

Figure 1. Periodontal ligament progenitor cells express TLR2. (A) Positive immunofluorescence for the TLR2. (B) Control is represented by PDLSCs incubated with secondary antibody only.

were able to retain the capacity to form adherent colonies and spindle-shaped fibroblasts after exposure to PgLPS (data not shown). As shown in Figure 2A, LPS did not affect the proliferation of PDLSCs during the 10-day period, even after incubation with a relatively high concentration of toxin (1 µg and 10 µg/mL). In addition, MTS values showed a time-dependent increase of cell proliferation for all groups, with an increasing number of cells between 1 and 7 days in culture, stabilizing after 7 days.

Gene expression changes on pro-inflammatory cytokines showed that PDLSCs only expressed *IL-1*β and *TNF*-α mRNA after PgLPS challenge. The expression of *TNF*-α was upregulated for all PgLPS concentrations, however only at a concentration of 1 µg/mL a significant difference was found compared to non-exposed cells ($p < 0.05$) (Figure 2B). Even with increased *IL-1*β transcripts after 24 hours of LPS challenge, the difference was not significant between groups, probably due to the greater variability of *IL-1*β expression among the three cell populations (Figure 2C).

Neither *OCT-4*, STRO-1 or CD105 are influenced by PgLPS exposure

Since the results showed that *TNF*-α was upregulated at a concentration of 1 µg/mL ($p < 0.05$), this concentration was chosen to proceed with subsequent experiments. First, the possible role of PgLPS on the MSC phenotype was investigated. Flow cytometry analysis showed that the proportion of STRO-1$^+$ and CD105$^+$ cells remain unaffected after exposure to PgLPS for 7 days (Table 2). In addition, gene expression analysis by qRT-PCR revealed similar mRNA levels of the pluripotent stem cell marker *OCT-4* for exposed and non-exposed cells (Figure 3A).

PgLPS challenge does not change osteoblast/cementoblast differentiation of PDLSCs

PDLSCs exposed and non-exposed to PgLPS were induced to differentiate into osteoblast/cementoblast phenotype for 21 days. Both groups were able to promote mineral nodule deposition *in vitro*, as visualized by AR-S (Figure 3B). No mineralized nodules were observed in cells cultured in the DMEM used as a

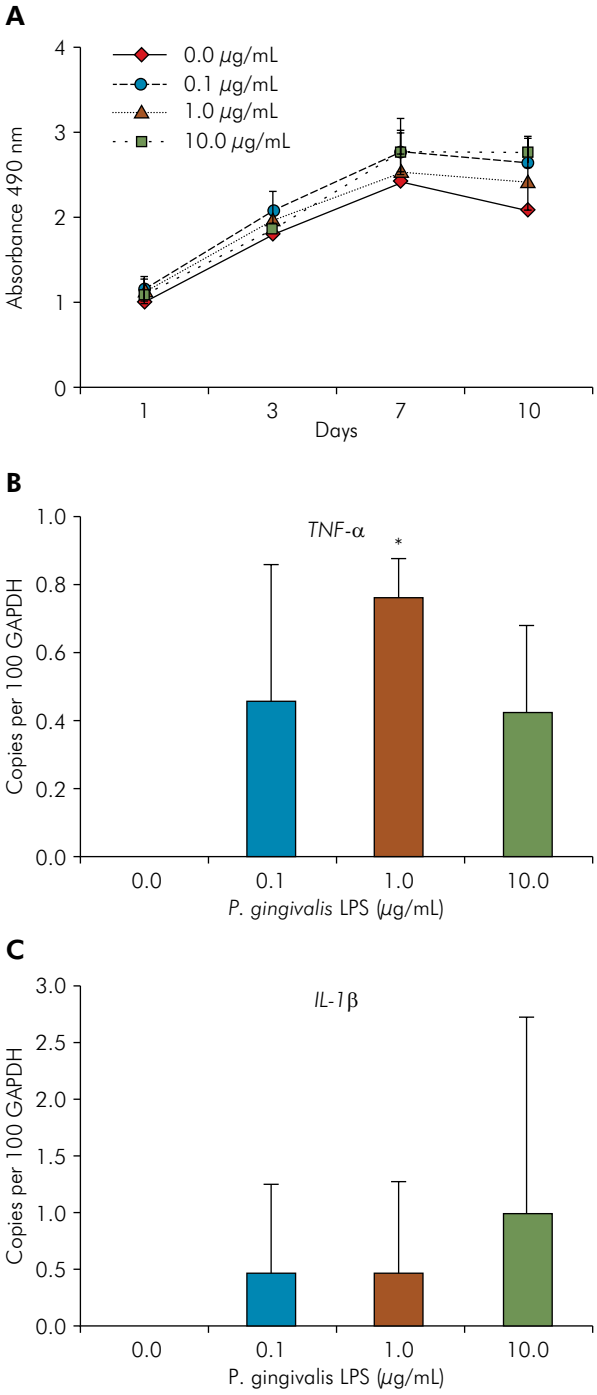

Figure 2. *P. gingivalis* lipopolysaccharides (PgLPS) effect on metabolic activity and cytokines expression. (A) Periodontal ligament stem cells (PDLSCs) were cultured in the presence of PgLPS at concentrations of 0, 0.1, 1 and 10 µg/mL. Metabolic activity as an indicator for cell proliferation was measured with MTS assay at 1, 3, 7 and 10 days. (B and C) RT-qPCR showed that PgLPS enhanced the expression of *IL-1*β and *TNF*-α, respectively, in PDLSCs after 24 hours of exposure. Representative data of three independent experiments are shown. * Statistical significance intergroup ($p < 0.05$).

Table 2. Percentage of STRO-1$^+$ and CD105$^+$ cells in the periodontal ligament stem cells populations after *P. gingivalis* lipopolysaccharide (1 μg/mL) challenge.

PDL-CD105$^+$ populations	STRO-1		CD105	
	control	PgLPS	control	PgLPS
1	0.47	0.5	97.67	95.94
2	1.67	0	98.58	94.68
3	1.1	1.65	94.52	75.22
Mean ± SD	1.08 ± 0.6	0.71 ± 0.84	96.92 ± 2.13	88.61 ± 11.61

PgLPS: *Porphyromonas gingivalis*.

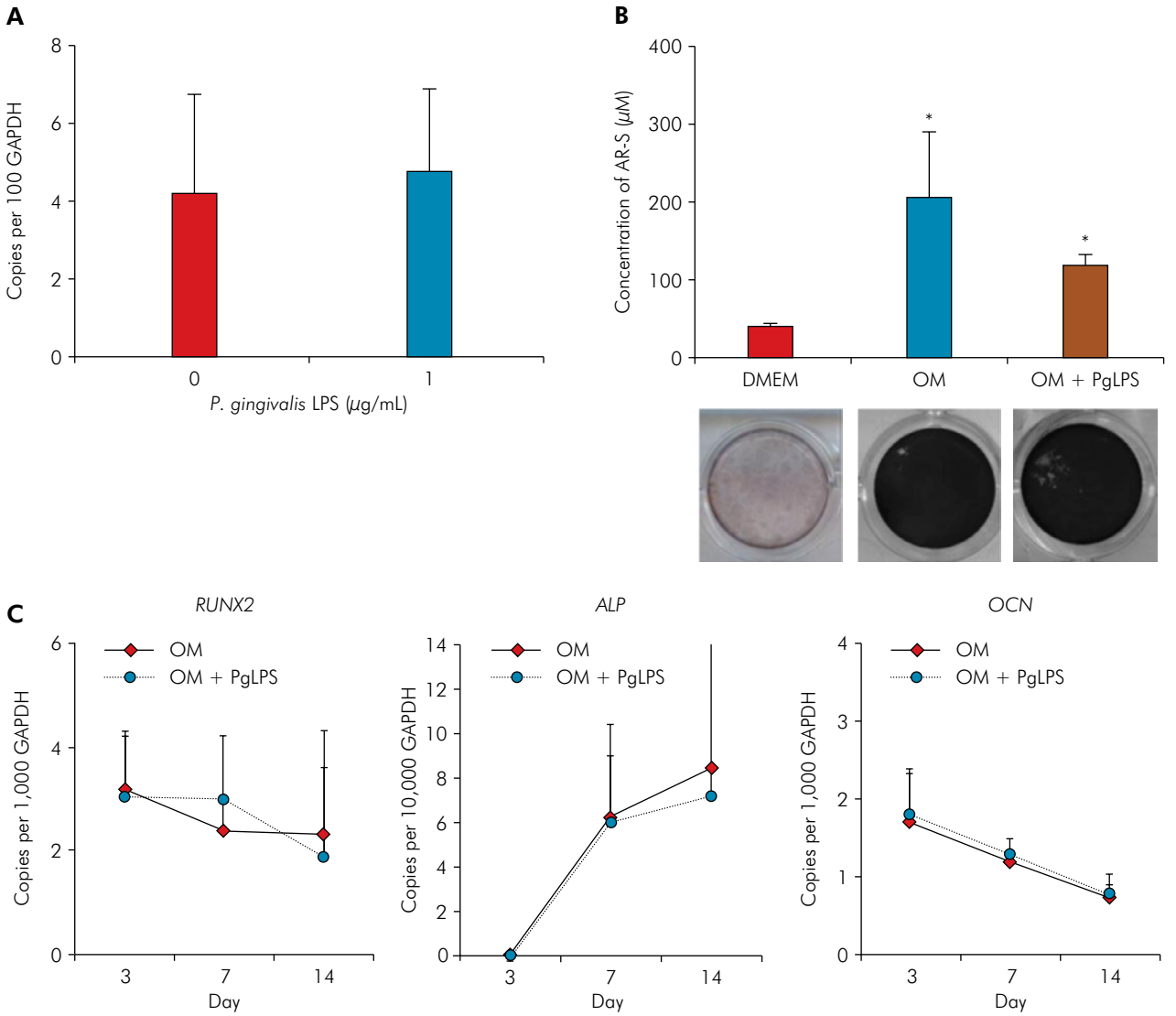

Figure 3. Stem cell markers expression and osteoblast/cementoblast differentiation of periodontal ligament stem cells (PDLSCs) after *P. gingivalis* lipopolysaccharides (PgLPS) exposure. (A) Gene expression for *OCT-4* after 24 hours of 1 μg/mL PgLPS exposure. (B) Quantification of AR-S showed that 1 μg/mL PgLPS did not affect PDLSCs mineralization at 21 days. (C) PDLSCs were cultured in Osteogenic-inducing media (OM), or OM + 1 μg/mL PgLPS for 3, 7 and 10 days. RT-qPCR analysis indicated that PgLPS did not alter the expression pattern of mRNAs for *RUNX2*, *ALP* and *OCN*. Representative data of three independent experiments are shown. * Statistical significance intergroup ($p < 0.05$).

control. The potential for osteoblast/cementoblast differentiation was further confirmed by qRT-PCR analysis, showing an increased expression of a key osteoblast transcription factor, *RUNX2*, and two osteoblast-specific early/late differentiation transcripts (*ALP* and *OCN*, respectively) in osteogenically-induced cells compared to non-induced (Figure 3C). When cells were cultured in OM supplemented with PgLPS, levels of transcripts for *RUNX2*, *ALP*, and *OCN* showed a similar behavior compared to the positive control group, in all studied periods (Figure 3C).

Discussion

MSCs play an important role on periodontal regeneration, so understanding the factors and mechanisms modulating their regenerative capacity is important to increase treatment predictability.[14] Some studies suggest that periodontal ligament cells may alter their phenotype in response to inflammation promoted by bacterial lipopolysaccharides.[15,16,17] *Porphyromonas gingivalis* is known to produce a repertoire of virulence factors such as fimbriae, capsules, LPS, lipoteichoic acids, hemagglutinins, gingipains, outer membrane proteins and vesicles.[1] Lipopolysaccharide is one of the most abundant virulence factors from *P. gingivalis*, as it activates the host inflammatory response and disrupts bone remodeling process.[1,18] Since the effect of PgLPS on PDLSCs remains unknown, the aim of the present study was to investigate whether this virulence factor would affect the biological properties of PDL progenitor cells. PDL heterogeneous cell population was purified to CD105 surface marker, the most frequently reported positive surface marker in MSCs.[19]. The PDL progenitor cell populations were previously characterized by our group as being pluripotent mesenchymal progenitor cells by expression of stem cell markers such as CD105, CD166, OCT-4, and STRO-1.[11]

Studies have shown that the TLRs family is involved in the recognition of bacterial cell wall components[20] and a current study described that PgLPS triggers an inflammatory response by binding to TLR2 and TLR4.[10] In the present study, TLR2 expression in PDLSC populations was verified, emphasizing that these cells can recognize bacterial virulence factors. Furthermore, a previous study published by our research group reported the expression of TLR4 in these same cell populations.[13] To our knowledge, this is the first study to describe the expression of these receptors by PDL CD105$^+$ progenitor cells, although previous studies have reported the expression of TLR4 in cementoblasts,[15] and of TLR2 and TLR4 in the heterogeneous PDL cell population.[9,21]

To evaluate the cytotoxicity effect of PgLPS, PDLSCs were initially cultured in the presence of different concentrations of this bacterial toxin. The data obtained in this study indicate that cells remained viable and proliferating regardless of the PgLPS concentration. The presence of the active proliferation stage was also found in studies that assessed the effect of *P. gingivalis* endotoxin in pre-osteoblastic,[22] PDL,[23] and PDLSC cells.[18]

Cytokines' gene expression was then performed to determine PgLPS effect on cell inflammatory response. Two pro-inflammatory cytokines that affect bone formation,[24] *TNF-*α and *IL-1*β, were investigated. As observed in PDL cells,[9,23,25] PDLSCs,[25] and human monocytic cells,[26] our cell populations showed an increase of transcripts for *TNF-*α and *IL-1*β after PgLPS exposure. These results suggest that PDL progenitor cells present a pro-inflammatory response to stimulation by *P. gingivalis* similar to the active immune cells.

Although PDLSCs have shown a pro-inflammatory response after exposure to the bacterial toxins, there is no evidence about the effect of bacterial challenge on the stem cell phenotype. The findings of the present study demonstrated that the percentage of CD105$^+$ cells remained high in PDLSCs population even in the presence of PgLPS. Another important stem cell marker, STRO-1, was also evaluated. Flow cytometry analysis revealed that, on average 1.08% of PDLSCs were STRO-1$^+$. This low expression of STRO-1 is in agreement with other reports in PDLSCs,[27] PDL cells,[28] dental pulp and dental follicle stem cells.[29] After exposure to bacterial toxin, on average 0.71% of PDLSCs remained positive for STRO-1. Stem cell markers expression showed that there was no significant difference in exposed *versus* non-exposed cells, however it is important to highlight the distinct

behavior of each PDLSC population. As shown by our data, PDLSC population #3 presented a lower positivity for CD105 and STRO-1 after LPS challenge. On the other hand, the positivity of both stem cell markers remained unaffected in the population #1.

The multipotential capability of PDLSCs may also be associated with the expression of *OCT-4*. As observed in dental pulp MSCs,[30] our findings revealed that the endotoxin challenge did not affect *OCT-4* mRNA levels in PDL progenitor cells. To the best of our knowledge, the present study is the first to assess the expression of stem cell-related markers (CD105, STRO-1 and *OCT-4*) in PDLSCs after bacterial endotoxin challenge. The findings that stem cell markers expression are not affected in the presence of *P. gingivalis* LPS, raises the possibility that even under an inflammatory condition, periodontal ligament MSCs are capable of maintaining their pluripotential and undifferentiated state.

MSCs are also characterized by the capacity of differentiating into multiple types of skeletal tissues.[31,32] As periodontal regeneration requires formation of new bone and cementum, it is important to understand the osteoblastic/cementoblastic differentiation pattern of PDLSCs challenged with PgLPS. PDLSCs maintained the ability of maturation towards osteoblast/cementoblast phenotype even under exposure to bacterial endotoxin, as shown by the deposition of mineralized matrix *in vitro* and the expression of three osteogenic gene markers, *RUNX2*, *ALP* and *OCN*. However, previous studies observed that *P. gingivalis* induced a negative effect on osteogenesis, characterized by inhibition of pre-osteoblast differentiation and suppression of bone formation.[22,33,34] Additionally, *P. gingivalis* LPS has been shown to decrease the expression of osteogenic gene markers in cementoblasts,[15] PDL fibroblasts,[8] mouse bone marrow MSC[35] and heterogeneous PDLSCs.[18]

Some factors could contribute to the discrepancies in these results such as heterogeneity of the cell source, distinct stages of differentiation and lineage commitment of cells, and experimental conditions. In addition, the method used to purify bacterial products may influence cell behavior. In this study, a commercially available PgLPS was used while other studies purified LPS directly from *P. gingivalis* strain.[15,33,34] Cell lineage is another important factor; among the cited studies, only one investigated the effects of *P. gingivalis* LPS on PDLSCs.[18] However, this population was not isolated and highly $CD105^+$ purified periodontal ligament progenitor cells were employed. Since it was already demonstrated that MSCs from PDL harbors a heterogeneous stem-cell-enriched population,[32,36,37] it is possible that PDLSCs composed by $CD105^+$ cell subsets are less susceptible to *P. gingivalis* LPS challenge compared to heterogeneous PDLSC populations. However, further studies are required to elucidate the response of PDLSCs to other *P. gingivalis* virulence factors.

Conclusion

Our findings provide evidence that *P. gingivalis* LPS induces cytokines' pro-inflammatory response in PDLSCs purified for CD105 marker. However, this response was not able to change the ability of PDLSCs to differentiate towards osteoblast/cementoblast phenotype, suggesting that these cells could develop a mechanism of resistance, which may be very important for periodontal tissue regeneration.

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