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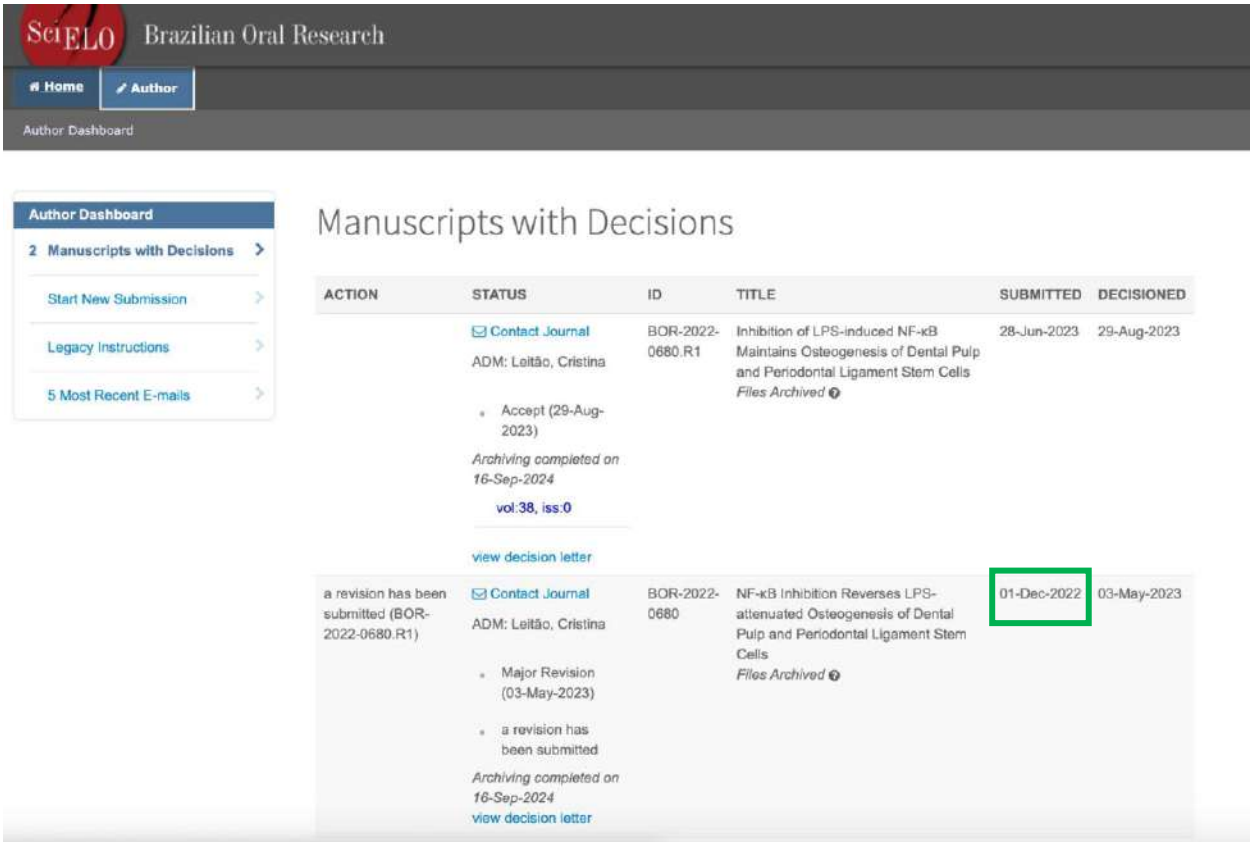

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**NF-κB Inhibition Reverses LPS-attenuated Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells**

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**Abstract**

Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PLSCs) can be differentiated into osteoblasts, suggesting that both stem cells are potential candidates for bone tissue engineering. Osteogenesis process is influenced by many environmental factors, including lipopolysaccharide (LPS). The role of LPS in regulating osteogenic differentiation of mesenchymal stem cells (MSCs) is still unclear. LPS might affect osteogenic differentiation of both stem cells through different mechanisms. The present study aimed to investigate and compare the effect of LPS supplementation on the osteogenic differentiation in DPSCs and PLSCs. Passage 5 DPSCs and PLSCs were harvested and characterized using flow cytometer. DPSCs and PLSCs were then cultured in an osteogenic medium with/without LPS and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor Bay 11-7082. Bone nodule formation was assessed by alizarin red staining and documented under an inverted light microscope. NF-κB p65 transcription factor binding assay was performed to determine NF-κB induction by LPS. Measurement of alkaline phosphatase (ALP) activity was performed to examine the effect of LPS supplementation on bone nodule formation by DPSCs and PLSCs. LPS significantly increased NF-κB activity ($p<0.05$) and significantly reduced ALP activity ($p<0.05$), which impaired bone nodule formation in both DPSCs and PLSCs. Bay 11-7082 inhibited LPS-induced NF-κB activity, partially improved ALP activity, and reversed osteogenic differentiation ability of DPSCs and PLSCs. Taken together, NF-κB pathway plays a key role in osteogenesis and should be inhibited to achieve optimal osteogenesis.

**Keywords:** Stem Cells; Dental Pulp; Periodontal Ligament; Lipopolysaccharides; NF-kappa B.

## Introduction

Mesenchymal stem cells (MSCs) have been reported to have potential uses in tissue engineering and regenerative medicine[1–3], including in the field of dentistry.[4] Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PLSCs) are oral tissue-derived stem cells that have MSCs properties.[4–6] Under specific culture conditions, DPSCs and PLSCs can be differentiated into various cell lineages, including osteoblasts.[7,8] DPSCs and PLSCs have higher growth potential compared to bone marrow mesenchymal stem cells (BMMSCs).[9] Moreover, DPSCs and PLSCs have been reported to have an immunomodulatory activity.[2,3,10] Hence, DPSCs and PLSCs are suggested as potential candidates for bone tissue engineering and regeneration applications, such as alveolar bone repair.[4]

Osteogenesis process is influenced by many environmental factors, including inflammatory factors produced by bacteria. The most common inflammatory factors produced by bacteria is lipopolysaccharide (LPS). LPS is an essential cell wall component of Gram-negative bacteria and it is known to induce inflammatory responses in the oral cavity with insufficient dental hygiene.[11] LPS is generally recognized by host toll-like receptor 4 (TLR4). LPS binding to TLR4 recruits myeloid differentiation primary response gene 88 (MyD88) to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which leads to overexpression of genes encoding proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF).[12–14]

LPS has been reported to inhibit bone tissue formation by MSCs.[15,16] In contrast, other studies also showed that LPS did not alter or even stimulate the osteogenic ability of MSCs.[17,18] Therefore, the role of LPS in regulating osteogenic differentiation of MSCs is still unclear. LPS might affect osteogenic differentiation of DPSCs and PLSCs through different mechanisms. Study of LPS in inhibiting osteogenic differentiation in both DPSCs and PLSCs

has not been investigated. The present study aimed to investigate and compare the effect of LPS supplementation on the osteogenic differentiation in DPSCs and PLSCs.

## Methodology

### Cells Thawing and Culture

DPSCs and PLSCs cell culture was performed as previously described[6] with modification. Cryopreserved passage 5 DPSCs and PLSCs reported in previous research[6,10] were thawed and cultured in MesenCult™ MSC Basal Medium (StemCell™ Technologies, Vancouver, Canada) supplemented with MesenCult™ MSC Stimulatory Supplement (StemCell™ Technologies), fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 200 U/mL penicillin, 200 $\mu$g/mL streptomycin, and 0.5 $\mu$g/mL amphotericin (Gibco). The cultured DPSCs and PLSCs were used in the following experiments. Ethical approval of this project was obtained from the Ethics Committee of **xxx** (No. **xxx**). Written informed consent was obtained for the collection of human samples for this experiment.

### Flow Cytometric Analysis

To confirm whether DPSCs and PLSCs had MSC markers, flow cytometric analysis was conducted using BD Stemflow hMSC Analysis Kit (BD Biosciences, Franklin Lakes, NJ, USA) as previously described.[10] DPSCs or PLSCs ($1 \times 10^7$ cells) were incubated with/without marker-specific antibodies as well as their isotypes for positive (CD90, CD105, and CD73) and negative (CD45, CD34, CD11b, CD19, and HLA-DR) markers. The labeled DPSCs and PLSCs were analyzed on FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). Minimal surface marker criteria for defining MSCs proposed by the International Society for Cellular Therapy (ISCT) was used to confirm MSCs characteristics of DPSCs and PLSCs.[19]

### *In vitro* Osteogenic Functional Assay

*In vitro* osteogenic functional assay was performed as previously described[6] with modification. DPSCs and PLSCs were cultured using osteogenic medium containing 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich), and 50 $\mu$g/mL L-ascorbic acid (Sigma-Aldrich). DPSCs and PLSCs cultured in the osteogenic medium added with 10 $\mu$g/mL LPS (Wako, Osaka, Japan) or 10 $\mu$g/mL LPS and 100 $\mu$M NF-κB inhibitor Bay 11-7082 (Sigma-Aldrich) were used as the experimental group. The medium was aspirated from each plate on day 7, 14 and 21. The plates were washed twice with PBS and fixed for 2 min in 4% paraformaldehyde (Wako) in PBS, then treated with glycerol (Bio-Rad, Hercules, CA, USA) at room temperature for 5 min. After removing the fixative, the cells were washed three times with distilled water. After that, the cells were stained with 2% alizarin red solution (Sigma-Aldrich) for 20 min. The plates were washed three times with distilled water after alizarin red was removed. Finally, the cells were observed and documented under an inverted light microscope (Zeiss, Jena, Germany).

**NF-κB p65 Transcription Factor Binding Assay**

To determine NF-κB induction by LPS in DPSCs and PLSCs, NF-κB p65 transcription factor binding assay was performed using NF-kB p65 Transcription Factor Assay Kit (Abcam, Cambridge, UK) following the procedure described in the instruction manual. DPSCs and PLSCs were nuclear extracted using Nuclear Extraction Kit (Abcam) according to the manufacturer's instructions prior to determination of NF-κB activity. The nuclear extracts containing NF-kB were loaded into 96-well plates containing dsDNA with NF-κB response element sequence. After that, rabbit anti-NF-κB primary antibody and HRP-linked goat anti-rabbit IgG secondary antibody were added sequentially. Results were measured at $OD_{450}$ nm using a spectrophotometer (Bio-Rad). Each experimental group was measured in triplicate.

### Alkaline Phosphatase (ALP) Activity Assay

The effect of LPS supplementation on bone nodule formation by DPSCs and PLSCs after 3 weeks was examined by measuring alkaline phosphatase (ALP) activity with colorimetric Alkaline Phosphatase Assay Kit (Abcam) according to the manufacturer's protocol. Briefly, homogenized DPSCs or PLSCs ($1 \times 10^5$ cells) and p-nitrophenyl phosphate (pNPP) were loaded into 96-well plates. After incubating the plates in the dark, stop solution was added and the samples were measured at $OD_{405}$ nm using a spectrophotometer (Bio-Rad) and activity of ALP (U/L) was calculated. Each experimental group was measured in triplicate.

### Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test, while independent samples t-test were used to analyze differences between paired experimental groups. *p*-values <0.05 were considered statistically significant.

## Results

### Phenotypic Characterization of DPSCs and PLSCs

DPSCs and PLSCs showed high expression of CD90, CD105 and CD73 (>95%), while expressions of negative markers were <2% (Figure 1, Figure 2). These surface biomarkers characteristics matched the standard criteria to define MSCs proposed by ISCT, suggesting that the cultured DPSCs and PLSCs were having the property of MSCs.

### LPS Impaired Osteogenic Differentiation of DPSCs and PLSCs

Under an inverted light microscope, bone nodules, which were displayed by Alizarin positive-red mineralized deposits, were observed in DPSCs on the third week culture, while bone nodules were observed in PLSCs on the second week culture. Upon supplementation of

10 μg/mL LPS, DPSCs and PLSCs lost their osteogenic potential although cultured in osteogenic medium. No bone nodules were observed in LPS-supplemented DPSCs and PLSCs after 1, 2 and 3 weeks (Figure 3).

**LPS Induced NF-κB Pathway in DPSCs and PLSCs**

Average basal NF-κB DNA binding activity of DPSCs and PLSCs average that were measured at $OD_{450}$ were 0.236±0.005 and 0.253±0.008, respectively. Upon supplementation of LPS, average $OD_{450}$ values of DPSCs was 0.580±0.029, which significantly increased compared to the control group ($p$=0.000). Meanwhile, the average $OD_{450}$ values of PLSCs after LPS supplementation was 0.667±0.051, which significantly increased compared to the control group ($p$=0.000). These results indicated an increase in NF-κB DNA binding activity that was associated with the activation of NF-κB. The elevated LPS-induced NF-κB activity was confirmed by addition of Bay 11-7082. The average $OD_{450}$ values of DPSCs supplemented with LPS and Bay 11-7082 (0.349±0.037) was significantly lower compared to LPS only group ($p$=0.001), but significantly higher than control group ($p$=0.006). Similarly, the average $OD_{450}$ values of PLSCs supplemented with LPS and Bay 11-7082 (0.420±0.022) was significantly lower compared to LPS only group ($p$=0.002), but significantly higher than control group ($p$=0.000) (Figure 4). This data suggested that Bay 11-7082 specifically inhibited NF-κB pathway activated by LPS.

**LPS Reduced ALP Activity and Bone Nodule Formation in DPSCs and PLSCs**

ALP activity of DPSCs and PLSCs were 60.893±6.516 U/mL and 70.637±4.902 U/mL, respectively. ALP activity of DPSCs after LPS supplementation was 5.333±0.323, which significantly reduced after three weeks of culture with osteogenic medium compared to the control group ($p$=0.000). ALP activity of PLSCs (6.277±2.026) also significantly reduced compared to the control group ($p$=0.000) (Figure 5). Lower ALP activity was associated with

inhibited bone nodule formation in LPS-supplemented DPSCs and PLSCs (Figure 6). Supplementation with LPS and Bay 11-7082 significantly increased ALP activity of DPSCs compared to LPS only group ($p$=0.000). Similarly, PLSCs supplemented with LPS and Bay 11-7082 exhibited a significant increase in ALP activity compared to LPS only group ($p$=0.000). ALP activity of Bay 11-7082-supplemented DPSCs was significantly lower than the control group ($p$=0.028). Furthermore, ALP activity of Bay 11-7082-supplemented PLSCs was also significantly lower than the control group ($p$=0.017). This data showed that Bay 11-7082 partially improved ALP activity in both DPSCs and PLSCs (Figure 5). Moreover, Bay 11-7082 also reversed osteogenic differentiation ability of LPS-supplemented DPSCs and PLSCs (Figure 6).

**Discussion**

Current study demonstrated that LPS inhibited bone nodule formation in both DPSCs and PLSCs by stimulating NF-κB activity and reducing ALP activity. It has been reported that NF-κB pathway induced by LPS is initiated by inhibitor of kappa-B kinase beta (IKK-β) activation. IKK-β catalyzes inhibitor of kappa-B alpha (IκBα) phosphorylation, which triggers polyubiquitination and degradation of IκBα by the 26S proteasome, hence allowing NF-κB translocation to nucleus.[20,21] Meanwhile, one of the many pathways that is involved in bone tissue formation is bone morphogenetic protein/Smad (BMP/Smad). Crosstalk between BMP/Smad and NF-κB pathway has been reported. Thus, NF-κB pathway is involved in regulating bone tissue formation through BMP/Smad pathway modulation. Active NF-κB binds to common-partner Smad/receptor-regulated Smad (Co-Smad/R-Smad) complex formed in BMP/Smad pathway, preventing this complex from regulating the expression of target genes[22], such as *Runx2*.[23] Therefore, LPS-induced NF-κB pathway in this research could inhibit bone nodule formation.

*Runx2* encodes a transcription factor which regulates transcription of genes involved in osteoblast differentiation, such as ALP-encoding gene, which plays an important role in bone mineralization.[24] Expression of this gene could be suppressed through the activation of LPS-induced NF-κB pathway. Downregulation of *Runx2* causes reduction of ALP activity (Figure 5), which leads to failure of bone nodule formation (Figure 3).

NF-κB signaling can be blocked by several substances, such as Bay 11-7082. NF-κB in various types of stem cells, for instance BMMSCs[25,26], adipose derived mesenchymal stem cells (AdMSCs)[26], and neural stem cells (NSCs)[27] has been reported to be inhibited by Bay 11-7082. Present study discloses the role of Bay 11-7082 and its mechanism in reversing osteogenic differentiation regulated by NF-κB in both DPSCs and PLSCs. Upon Bay 11-7082 supplementation, the NF-κB activity was suppressed, which simultaneously enhanced ALP activity and partially reversed osteogenic potential in DPSCs and PLSCs. Bay 11-7082's inhibition of LPS-activated NF-κB pathway in both DPSCs and PLSCs could be targeted on IκBα phosphorylation, hence preventing NF-κB activation and translocation.[28] The NF-κB inhibition by Bay 11-7082 might trigger upregulation of *Runx2* expression, leading to elevation of ALP activity, which reverses the formation of mineralized bone nodules in DPSCs and PLSCs.

Extracellular LPS has been known to activate canonical TLR4-mediated NF-κB pathway.[29] However, a study revealed that cytosolic LPS can also induce inflammatory responses via activation of caspase-4/5/11. Caspase-4/5/11 directly binds to cytosolic LPS, which comes from intracellular Gram-negative bacteria or possible extracellular LPS endocytosis by the host cell. This interaction induces oligomerization and activation of caspase-4/5/11, resulting in cell pyroptosis[30] as well as IL-1β/18 production and release.[31] Thus, the TLR4-independent pathway might be involved in affecting osteogenic potential of LPS-supplemented DPSCs and PLSCs as well.

## Conclusion

Activation of NF-κB by LPS causes the reduction of ALP activity, hence inhibits osteogenic differentiation process in DPSCs and PLSCs. Inhibition of NF-κB activity can elevate ALP activity, hence reverse the osteogenic differentiation ability of DPSCs and PLSCs. Taken together, NF-κB pathway plays a key role in osteogenesis and should be inhibited to achieve optimal osteogenesis.

## Declaration of Interest

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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## Figure Legends

**Figure 1.** Flow cytometric results of DPSCs. DPSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of DPSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

**Figure 2.** Flow cytometric results of PLSCs. PLSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of DPSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

**Figure 3.** LPS inhibited osteogenic differentiation of DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PLSCs were stained with alizarin red as described in Methodology. Black bar: 100 $\mu$m.

**Figure 4.** LPS induced NF-κB activity in DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium and treated with/without 10 $\mu$g/mL LPS and 100 $\mu$M Bay 11-7082 for 3 weeks. NF-κB activity was measured as described in Methodology. The data are expressed as mean ± standard deviation (n = 3). *$p$-value <0.05, independent samples t-test.

**Figure 5.** LPS decreased ALP activity of DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium and treated with/without 10 $\mu$g/mL LPS and 100 $\mu$M Bay 11-7082 for 3 weeks. ALP activity was measured as described in Methodology. The data are expressed as mean ± standard deviation (n = 3). *$p$-value <0.05, independent samples t-test.

**Figure 6.** Bay 11-7082 regained osteogenic differentiation of DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium and treated with/without 10 $\mu$g/mL LPS and 100 $\mu$M Bay 11-7082 for 3 weeks. DPSCs and PLSCs were stained with alizarin red as described in Methodology. Black bar: 100 $\mu$m.

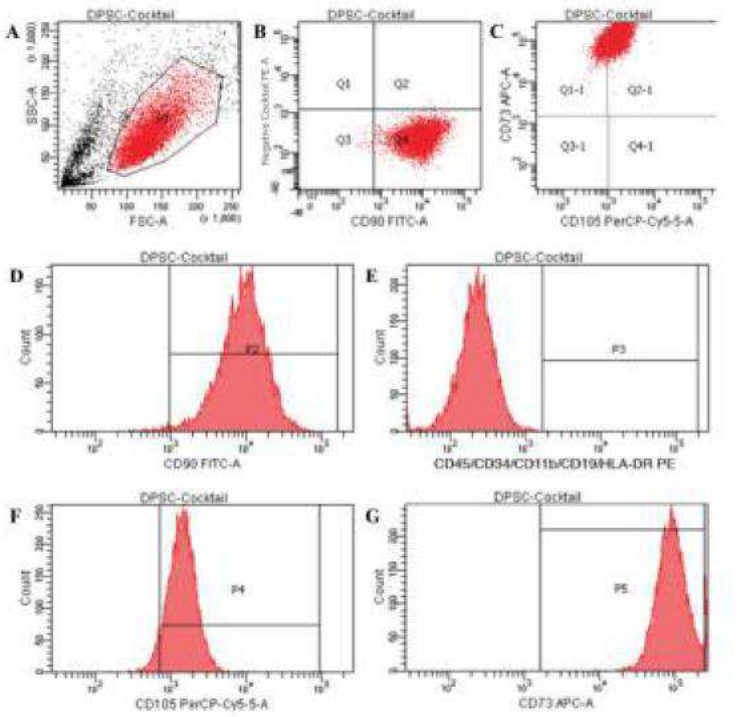


**Figure 1.** Flow cytometric results of DPSCs. DPSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of DPSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

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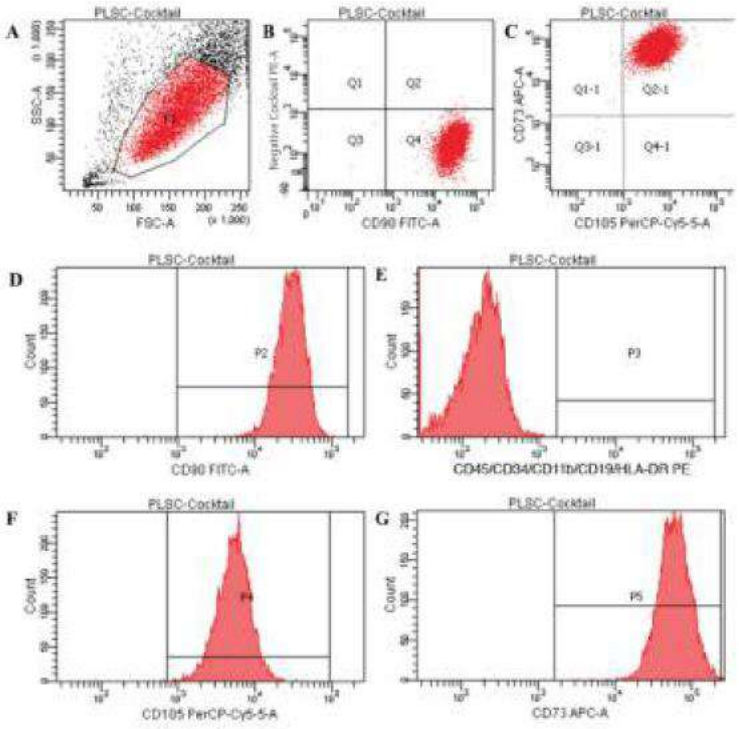



**Figure 2.** Flow cytometric results of PLSCs. PLSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of DPSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

163x164mm (300 x 300 DPI)

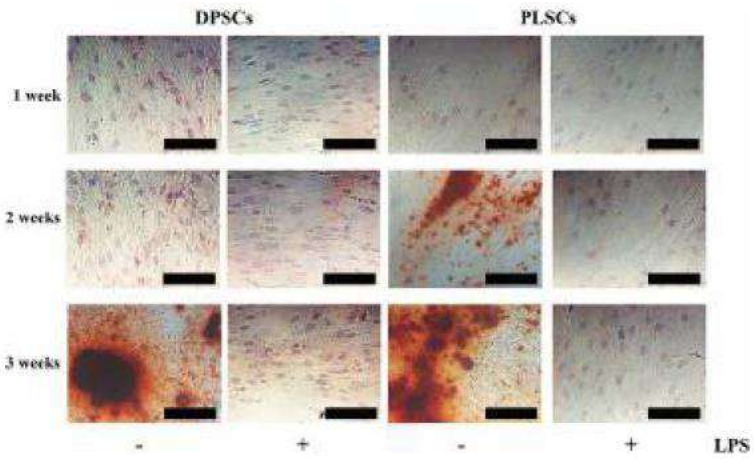
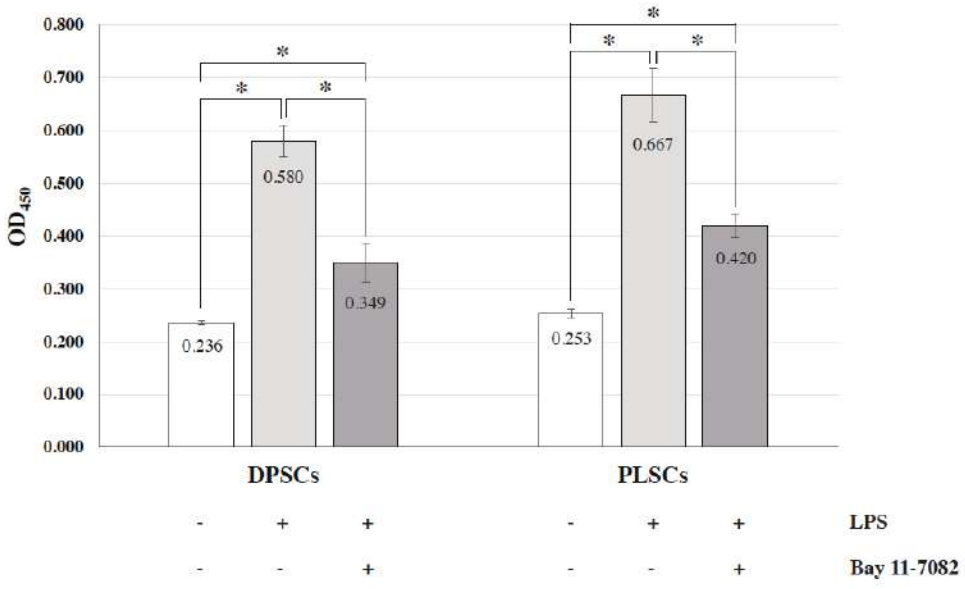



**Figure 3.** LPS inhibited osteogenic differentiation of DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PLSCs were stained with alizarin red as described in Methodology. Black bar: 100 μm.

199x121mm (300 x 300 DPI)


0.800
0.700
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*
DPSCs
PLSCs
- + + - + + LPS
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**Figure 4**

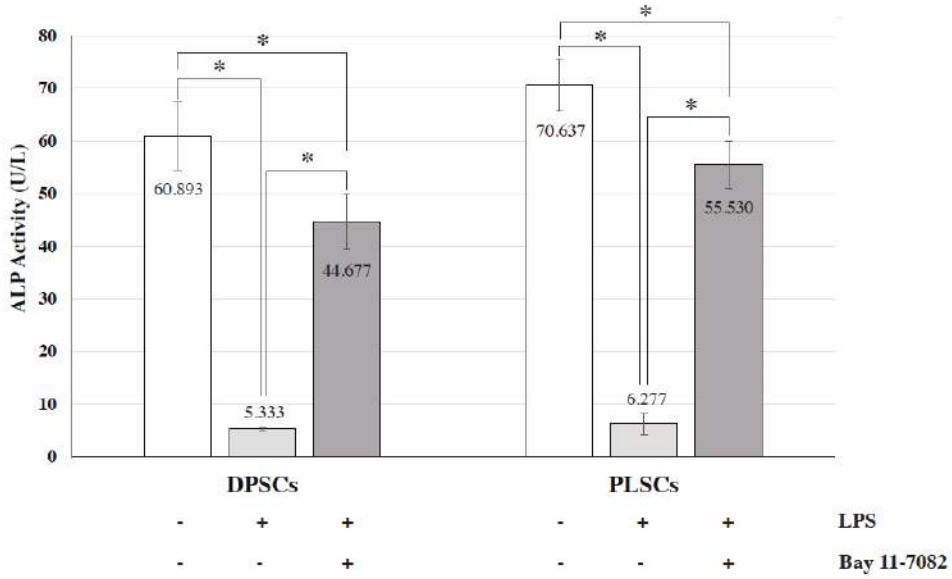

ALP Activity (U/L)
80
70
60
50
40
30
20
10
0
60.893
5.333
44.677
70.637
6.277
55.530
*
DPSCs
PLSCs
- + + - + + LPS
- - + - - + Bay 11-7082

**Figure 5**

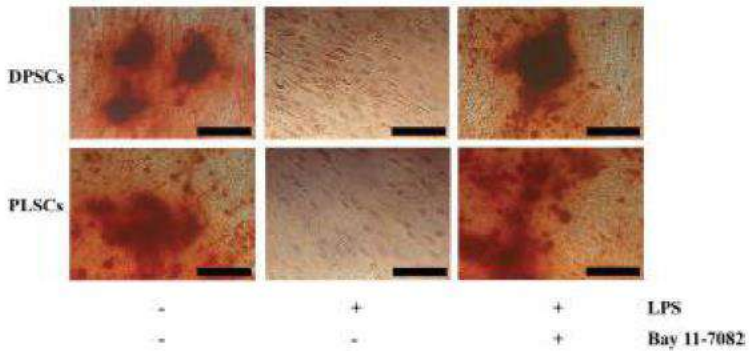
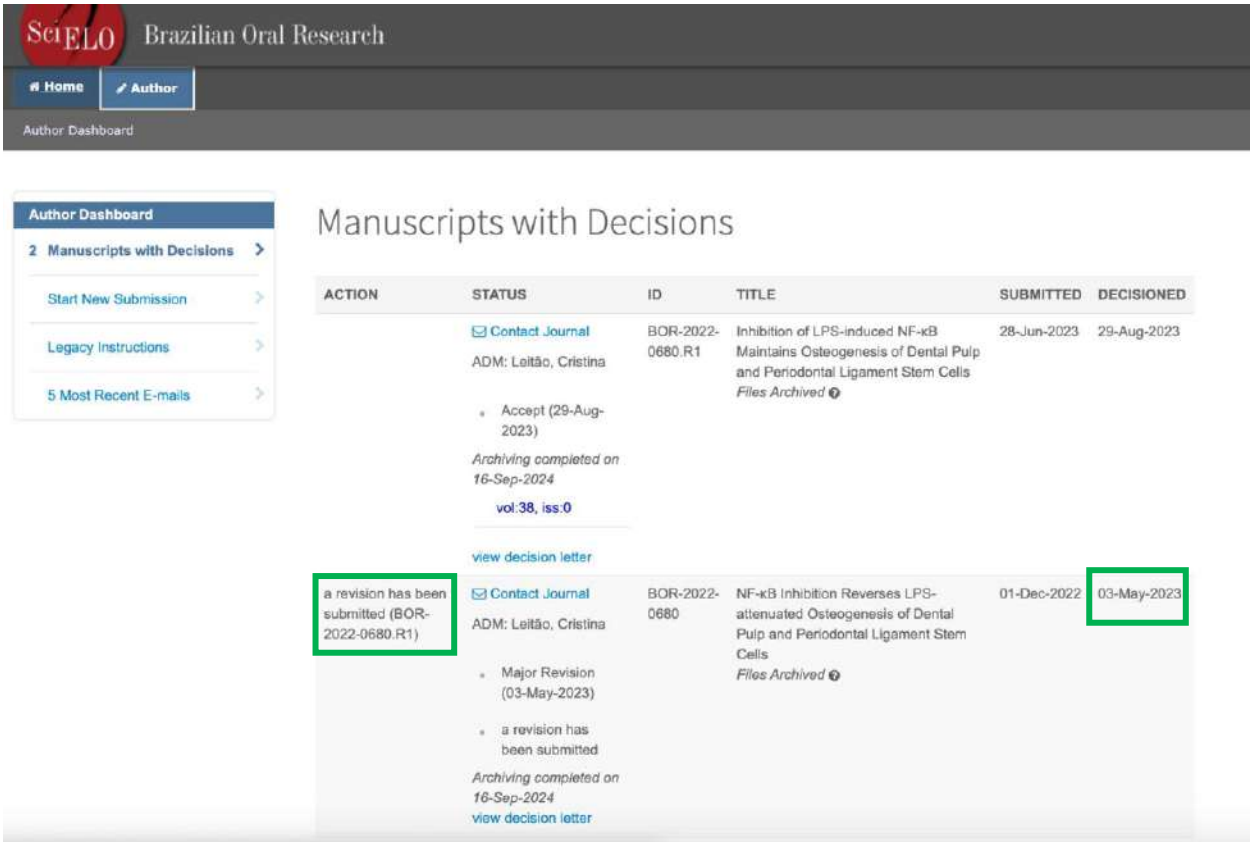



**Figure 6.** Bay 11-7082 regained osteogenic differentiation of DPSCs and PLSCs. DPSCs and PLSCs were cultured in osteogenic medium and treated with/without 10 μg/mL LPS and 100 μM Bay 11-7082 for 3 weeks. DPSCs and PLSCs were stained with alizarin red as described in Methodology. Black bar: 100 μm.

199x95mm (300 x 300 DPI)

## 2. Bukti hasil *review* dari *reviewer* jurnal Brazilian Oral Research (3 Mei 2023)

Bukti *review* terlihat pada baris bawah *Author Dashboard* (kotak hijau)



Brazilian Oral Research

**Decision Letter (BOR-2022-0680)**

**From:** smpaiva@uol.com.br

**To:** ferry@trisakti.ac.id

**CC:**

**Subject:** Brazilian Oral Research - Decision on Manuscript ID BOR-2022-0680

**Body:** 03-May-2023

Dear Dr. Sandra:

Manuscript ID BOR-2022-0680 entitled "NF-κB Inhibition Reverses LPS-attenuated Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells" which you submitted to the Brazilian Oral Research, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into https://mc04.manuscriptcentral.com/bor-scielo and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You may also click the below link to start the revision process (or continue the process if you have already started your revision) for your manuscript. If you use the below link you will not be required to login to ScholarOne Manuscripts.

*** PLEASE NOTE: This is a two-step process. After clicking on the link, you will be directed to a webpage to confirm. ***

https://mc04.manuscriptcentral.com/bor-scielo?URL_MASK=b9d6d3f8218948d7a6687116133de6e2

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text.

Once the revised manuscript is prepared, you can upload it and submit it through your Author Center.

When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to the Brazilian Oral Research, your revised manuscript should be submitted by 03-Jul-2023. If it is not possible for you to submit your revision by this date, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Brazilian Oral Research and I look forward to receiving your revision.

Sincerely,
Dr. Saul Paiva
Editor-in-Chief, Brazilian Oral Research
smpaiva@uol.com.br

Associate Editor Comments to Author:

Associate Editor
Comments to the Author:

Dear Dr. Ferry,

Thank you for submitting your manuscript to Brazilian Oral Research.

We have completed the evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following MAJOR REVISION. We invite you to resubmit your manuscript after addressing the comments below.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please highlight every change made in response to reviewer comments in the text, provide a point-by-point response to the reviewers comments, and provide suitable rebuttals for any comments not addressed.
Please note that your revised submission will need to be re-reviewed.

Reviewer 1#
This paper aimed to investigate and compare the effect of LPS supplementation on the osteogenic differentiation in DPSCs and PLSCs.
The following points should be considered to improve the manuscript.

Title
"NF-κB Inhibition Reverses LPS-attenuated Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells."
Aim
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Conclusion
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When this reviewer places "title, aim, and conclusion" side by side, the aim seems incomplete. Please rewrite the objective more cohesively.
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P2 L35 "LPS is generally recognized by host toll-like receptor 4 (TLR4). LPS binding to TLR4 recruits myeloid differentiation primary response gene 88 (MyD88) to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which leads to overexpression of genes encoding proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)."
Please add a figure to make the mechanism easier for the reader to understand. Also, add your main result.

Methodology
Have ethical principles been established in accordance with the Declaration of Helsinki? If yes, please put it in the text.

P3 L14 "DPSCs and PLSCs cell culture was performed as previously described6 with modification."
Please specify the modification.

P3 L17 " passage 5 DPSCs and PLSCs..."
The low passage is an essential characteristic of the self-renewal of stem cells, why was passage 5 used in this work?
P4 L3 "...was performed as previously described6 with modification".
In the understanding of this reviewer, the citation of the paper https://doi.org/10.18585/inabj.v9i2.286 appears once again in the text, unnecessarily.
If the protocol is not identical, it does not justify the citation of the paper. It is better to write the modified protocol as a new protocol.
Please readjust the text in the methodology and if you want to keep the citation, establish the comparison in the discussion, not in the methodology.

P4 L12 "10 μg/mL LPS and 100 μM Bay NK-kV inhibitor Bay ... were used as the experimental group."
Please specify better how the inhibition was performed.

P4 L17 "The medium was aspirated from each plate on day 7, 14 and 21"
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The peak of ALP activity occurs at 14 days. This reviewer did not understand why the

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I would recommend the manuscript be published in the Brazilian Oral Research. But before this can be accepted for publication it needs to be edited to improve the clarity and readability for the English-speaking readership of the Journal to the issues addressed below:

1. A professional English edit is required for the manuscript.
2. The main novelty of the study should be clearer. Chen et al (2022, doi: 10.1080/21655979.2022.2051690) have already shown the effects of nuclear factor-κB signaling pathway on periodontal ligament stem cells under lipopolysaccharide-induced inflammation/ and, the literature such as Son et al (2017, https://doi.org/10.1111/jcmm.12988), Yuan et al. (2019, https://doi.org/10.1111/1440-1681.13059) demonstrated the effects of LPS in DPSCs.
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4. Page 3 lines 35-45: the study did not analyze toll-like receptor4 or MyD88. Why have these details been highlighted in the introduction?
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17. The conclusions are too broad.
18. Page 16, lines 46–47: the statistical test seems inadequate. The experiment has two types of cells with 3 treatments. 2-way ANOVA should be performed. please explain. Was a sample calculation performed? n = 3 seems too low.
19. Figures 1 and 2: acronyms need to be better described for a full understanding of the article. SSC-A? CD73 APC-A? CD105 PerCP-Cy5-5-A?
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22. Figure 6: these figures should be together with Figure 3. It should be presented together with the control group and with the graph in Figure 5. Which experimental period would these images be from?

Entire Scoresheet:
Reviewer: 1

Recommendation: Major Revision

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The peak of ALP activity occurs at 14 days. This reviewer did not understand why the experiment was performed after 3 weeks. Please justify.

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: Yes

Does the Abstract (Summary) clearly and accurately describe the content of the article?: No

Is the problem significant and concisely stated?: Yes

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Is the problem significant and concisely stated?: No

Are the methods described comprehensively?: No

Are the interpretations and conclusions justified by the results?: Yes

Is adequate reference made to other work in the field?: Yes

Is the language acceptable?: No

Please rate the priority for publishing this article (1 is the highest priority, 10 is the lowest priority): 5

Length of article is: Adequate

Number of tables is: Too few

Number of figures is: Too few

Please state any conflict(s) of interest that you have in relation to the review of this paper (state "none" if this is not applicable).: None

Rating:

Interest: 3. Average

Quality: 4. Below Average

Originality: 4. Below Average

Overall: 4. Below Average

**Date Sent:** n/a

Are the methods described comprehensively?: No

Are the interpretations and conclusions justified by the results?: Yes

Is adequate reference made to other work in the field?: Yes

Is the language acceptable?: Yes

Please rate the priority for publishing this article (1 is the highest priority, 10 is the lowest priority): 9

Length of article is: Adequate

Number of tables is: Too few

Number of figures is: Too few

Please state any conflict(s) of interest that you have in relation to the review of this paper (state "none" if this is not applicable).: None

Rating:

Interest: 4. Below Average

Quality: 4. Below Average

Originality: 3. Average

Overall: 5. Poor

Reviewer: 2

Recommendation: Major Revision

Comments:
Title: NF-κB Inhibition Reverses LPS-attenuated Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells

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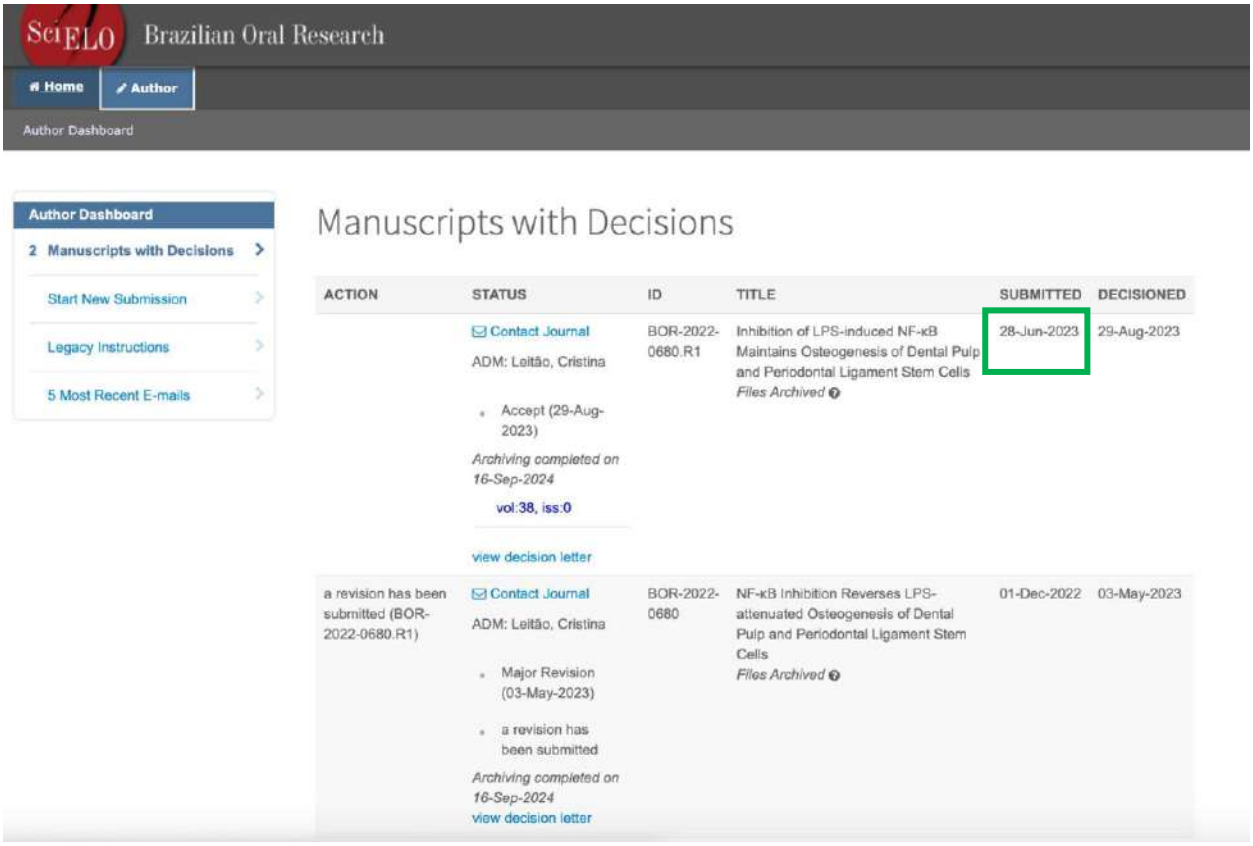
**3. Bukti revisi artikel yang dikirimkan kembali ke jurnal Brazilian Oral Research (28 Juni 2023)**

Bukti revisi artikel terlihat pada baris atas *Author Dashboard* (kotak hijau)



Brazilian Oral Research

**Preview (BOR-2022-0680)**

**From:** office.bor@ingroup.srv.br

**To:** ferry@trisakti.ac.id

**CC:** ferry@trisakti.ac.id, jantish@trisakti.ac.id, angliana@prostem.co.id, maria.celinna@prodia.institute, nurrani.mustika.dewi@prodia.institute, melanie.hendriaty@gmail.com

**Subject:** Brazilian Oral Research - Manuscript ID BOR-2022-0680.R1

**Body:** 28-Jun-2023

Dear Dr. Sandra:

Your manuscript entitled "Inhibition of LPS-induced NF-κB Maintains Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells" has been successfully submitted online and is presently being given full consideration for publication in the Brazilian Oral Research.

Your manuscript ID is BOR-2022-0680.R1.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc04.manuscriptcentral.com/bor-scielo and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to https://mc04.manuscriptcentral.com/bor-scielo.

Thank you for submitting your manuscript to the Brazilian Oral Research.

Sincerely,
Brazilian Oral Research Editorial Office

**Date Sent:** n/a

Artikel yang telah direvisi dan dikirimkan kembali adalah sebagai berikut:

Brazilian Oral Research


**Inhibition of LPS-induced NF-κB Maintains Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells**

| | |
|---|---|
| Journal: | *Brazilian Oral Research* |
| Manuscript ID | BOR-2022-0680.R1 |
| Manuscript Type: | Original Research Report |
| Specialties: | Pulp Biology |
| Category--Select your categories from the <A HREF='http://www.nlm.nih.gov/mesh/MBrowser.html' target='_new'> MeSH</a> or <A HREF='http://decs.bvs.br/' target='_new'> DeCS</a> lists.: | Stem Cells, Dental Pulp, Periodontal Ligament, Lipopolysaccharides, NF-kappa B |
| | |

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Manuscripts

# Inhibition of LPS-induced NF-κB Maintains Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells


## Abstract

Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) can be differentiated into osteoblasts, suggesting that both stem cells are potential candidates for bone tissue engineering. Osteogenesis is influenced by many environmental factors, including lipopolysaccharide (LPS). LPS-induced NF-κB activity might give different effects on the osteogenic potency of different MSCs types. Therefore, the present study was conducted to evaluate the effect of LPS-induced NF-κB activity and its inhibition in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without NF-κB inhibitor Bay 11-7082, and treated with/without LPS. Bone nodule formation was assessed by alizarin red staining and documented under an inverted light microscope. NF-κB and alkaline phosphatase (ALP) activities were measured to examine the effect of Bay 11-7082 pretreatment and LPS supplementation on osteogenic differentiation of DPSCs and PDLSCs. LPS significantly induced NF-κB activity ($p$=0.000) and significantly reduced ALP activity ($p$=0.000), which inhibited bone nodule formation in both DPSCs and PDLSCs. Bay 11-7082 inhibited LPS-induced NF-κB activity, partially maintained ALP activity and osteogenic potency of LPS-supplemented DPSCs and PDLSCs. Taken together, inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.


**Keywords:** stem cells; dental pulp; periodontal ligament; lipopolysaccharides; NF-kappa B.

## Introduction

Mesenchymal stem cells (MSCs) have been reported to have potential uses in tissue engineering and regenerative medicine[1–3], including in the field of dentistry.[4] Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) are oral tissue-derived stem cells that have MSCs properties.[4–6] Under specific culture conditions, DPSCs and PDLSCs can be differentiated into mesenchymal lineages, including osteoblasts.[7,8] DPSCs and PDLSCs have higher growth potential compared to bone marrow mesenchymal stem cells (BMMSCs).[9] Moreover, DPSCs and PDLSCs have been reported to have an immunomodulatory activity.[2,3,10] Hence, DPSCs and PDLSCs are suggested as potential candidates for bone tissue engineering and regeneration applications, such as alveolar bone repair.[4]

Osteogenesis process is influenced by many environmental factors, including inflammatory factors produced by bacteria.[11,12] The most common inflammatory factor is lipopolysaccharide (LPS), which is continuously shed from Gram-negative bacteria colonizing the periodontal tissues and may cause inflammatory diseases, such as periodontitis.[13] This substance induces inflammatory responses through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway.[14,15] Inhibition studies on the LPS-induced NF-κB activity in PDLSCs have been reported, so that the osteogenesis could be undisrupted.[11,12] However, in other types of MSCs, such as BMMSCs, LPS induced the NF-κB activity but did not alter the osteogenic differentiation.[11] In addition, in adipose derived mesenchymal stem cells (AdMSCs), LPS induced NF-κB activity as well as stimulated the osteogenic differentiation.[16] Therefore, NF-κB inhibition might give different effects on the osteogenic potency of different MSCs types. The present study was conducted to evaluate the effect of LPS-induced NF-κB activity and its inhibition using a specific inhibitor, Bay 11-7082, in DPSCs and PDLSCs.

## Methodology

### Cells Thawing and Culture

Cryopreserved passage 5 DPSCs and PDLSCs reported in the previous research[6,10] were thawed and cultured in MesenCult MSC Basal Medium (StemCell Technologies, Vancouver, Canada) supplemented with MesenCult MSC Stimulatory Supplement (StemCell Technologies), 200 U/mL penicillin, 200 $\mu$g/mL streptomycin, and 0.5 $\mu$g/mL amphotericin (Gibco). Upon reaching confluency, DPSCs and PDLSCs were harvested and used in the following experiments. This study was performed in accordance with the Declaration of Helsinki. Approval was granted by the Ethics Committee of xxx (No. xxx). Written informed consent was obtained for the collection of human samples for this experiment.

### Flow Cytometric Analysis

To confirm whether DPSCs and PDLSCs had MSC markers, flow cytometric analysis was conducted using BD Stemflow hMSC Analysis Kit (BD Biosciences, Franklin Lakes, NJ, USA) as previously described.[10] DPSCs ($1\times10^7$ cells) and PDLSCs ($1\times10^7$ cells) were incubated with/without marker-specific antibodies as well as their isotypes for positive (CD90, CD105, and CD73) and negative (CD45, CD34, CD11b, CD19, and HLA-DR) markers. The labeled DPSCs and PDLSCs were analyzed on FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). Minimal surface marker criteria for defining MSCs proposed by the International Society for Cellular Therapy (ISCT) was used to confirm MSCs characteristics of DPSCs and PDLSCs.[17]

### *In vitro* Osteogenic Functional Assay

*In vitro* osteogenic functional assay was performed as previously described.[6] DPSCs ($8\times10^4$ cells) and PDLSCs ($8\times10^4$ cells) were cultured in a 6-well plate using osteogenic medium containing 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 100

nM dexamethasone (Sigma-Aldrich), and 50 $\mu$g/mL L-ascorbic acid (Sigma-Aldrich). DPSCs and PDLSCs were pretreated with/without 100 $\mu$M NF-κB inhibitor Bay 11-7082 (Sigma-Aldrich) for 30 min and supplemented with/without 10 $\mu$g/mL *Porphyromonas gingivalis* LPS (Wako, Osaka, Japan) for 1, 2, or 3 weeks. The medium was then removed, and the plates were washed twice with PBS and fixed for 2 min in 4% paraformaldehyde (Wako) in PBS, then treated with glycerol (Bio-Rad, Hercules, CA, USA) at room temperature for 5 min. After removing the fixative, the cells were washed three times with distilled water. After that, the cells were stained with 2% alizarin red solution (Sigma-Aldrich) for 20 min. The plates were washed three times with distilled water after alizarin red was removed. Finally, the cells were observed and documented under an inverted light microscope (Zeiss, Jena, Germany). Experiment was performed twice in triplicate.

**NF-κB Activity Assay**

After Bay 11-7082 pretreatment for 30 min and LPS supplementation for 3 weeks, NF-κB activity in DPSCs ($2\times10^6$ cells) and PDLSCs ($2\times10^6$ cells) was determined using NF-κB p65 Transcription Factor Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. Treated DPSCs and PDLSCs were nuclear extracted using Nuclear Extraction Kit (Abcam) according to the manufacturer's instructions prior to determination of NF-κB activity. The nuclear extracts containing NF-κB were loaded into 96-well plates containing dsDNA with NF-κB response element sequence. After that, rabbit anti-NF-κB primary antibody and HRP-linked goat anti-rabbit IgG secondary antibody were added sequentially. Results were measured at $OD_{450}$ nm using a spectrophotometer (Bio-Rad). Experiment was performed twice in triplicate.

**Alkaline Phosphatase (ALP) Activity Assay**

After Bay 11-7082 pretreatment for 30 min and LPS supplementation with/without Bay 11-7082 for 3 weeks, ALP activity in DPSCs and PDLSCs was measured with colorimetric Alkaline Phosphatase Assay Kit (Abcam) according to the manufacturer's protocol. Briefly, homogenized DPSCs or PDLSCs ($1\times10^5$ cells) and *p*-nitrophenyl phosphate (pNPP) were loaded into 96-well plates. After incubating the plates in the dark, stop solution was added and the samples were measured at $OD_{405}$ nm using a spectrophotometer (Bio-Rad) and activity of ALP (U/L) was calculated. Experiment was performed twice in triplicate.

**Statistical Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (SPSS IBM, Armonk, NY, USA). Shapiro-Wilk test was used as a normality test, while two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) was used to compare NF-κB and ALP activities of DPSCs and PDLSCs in different treatment groups. *p*-values <0.05 were considered as statistically significant.

**Results**

**Phenotypic Characterization of DPSCs and PDLSCs**

DPSCs and PDLSCs exhibited high expression of CD90, CD105 and CD73 (>95%), while expressions of negative markers were <2% (Figure 1, Figure 2). These surface biomarkers characteristics matched the standard criteria to define MSCs proposed by ISCT, suggesting that the cultured DPSCs and PDLSCs were having the property of MSCs.

**LPS Inhibited Osteogenic Differentiation of DPSCs and PDLSCs**

Under an inverted light microscope, bone nodules, which were displayed by Alizarin positive-red mineralized deposits, were observed in DPSCs on the third week culture, while

bone nodules were observed in PDLSCs on the second week culture. Meanwhile, no bone nodules were observed in 10 $\mu$g/mL LPS-supplemented DPSCs and PDLSCs after 1, 2 and 3 weeks (Figure 3).

**LPS Induced NF-κB Activity in DPSCs and PDLSCs**

NF-κB activities of untreated DPSCs and PDLSCs were 0.236±0.005 AU and 0.253±0.008 AU, respectively. Upon three weeks of LPS supplementation, NF-κB activities of DPSCs and PDLSCs were 0.580±0.029 AU and 0.667±0.051 AU. By pretreatment of Bay 11-7082, NF-κB activities of LPS-supplemented DPSCs and PDLSCs were 0.349±0.037 AU and 0.420±0.022 AU (Figure 4).

A two-way ANOVA did not show a significant interaction between the types of stem cells and treatments on the NF-κB activity ($p$=0.148). There were significant differences of NF-κB activity in different treatment groups ($p$=0.000). The 3-weeks-LPS-supplemented NF-κB activities of both DPSCs and PDLSCs were significantly higher than those of untreated DPSCs and PDLSCs ($p$=0.000) as well as those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p$=0.000). The NF-κB activities of untreated DPSCs and PDLSCs were significantly lower than those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p$=0.000). These results indicated that LPS induced NF-κB activation in both DPSCs and PDLSCs, and Bay 11-7082 partially inhibited LPS-induced NF-κB pathway.

**LPS Reduced ALP Activity and Inhibited Bone Nodule Formation in DPSCs and PDLSCs**

A two-way ANOVA did not show a significant interaction between the types of stem cells and treatments on the ALP activity ($p$=0.148). There were significant differences of ALP activity in different treatment groups ($p$=0.000). ALP activities of untreated DPSCs and

PDLSCs were 60.893±6.516 U/mL and 70.637±4.902 U/mL, respectively. After three weeks of LPS supplementation, ALP activities of both DPSCs (5.333±0.323 U/mL) and PDLSCs (6.277±2.026 U/mL) were significantly lower compared with those of untreated DPSCs and PDLSCs ($p$=0.000) (Figure 5). Lower ALP activities were associated with inhibition of bone nodule formation in LPS-supplemented DPSCs and PDLSCs (Figure 6). By pretreatment of Bay 11-7082, ALP activities of LPS-supplemented DPSCs (44.677±5.193 U/mL) and PDLSCs (55.530±4.478 U/mL) were significantly higher compared with those of supplemented with LPS merely ($p$=0.000), but significantly lower than those of untreated ($p$=0.000). These results showed that Bay 11-7082 partially maintained ALP activity in both DPSCs and PDLSCs (Figure 5). Moreover, Bay 11-7082 pretreatment partially maintained osteogenic potency of LPS-supplemented DPSCs and PDLSCs (Figure 6).

## Discussion

NF-κB activation, which could be induced by LPS, has been reported to play an important role in inflammatory responses and bone loss in periodontitis.[19] The present study demonstrated that *P. gingivalis*-derived LPS induced NF-κB activity and inhibited bone nodule formation in both DPSCs and PDLSCs. These findings are consistent with a previous study showed that LPS-induced NF-κB activity impaired the osteogenic potency of GMSCs.[20] LPS supplementation could also inhibit osteogenic differentiation in dental follicle stem cells (DFSCs).[21]

Not only targeting κB site, the activated NF-κB has been reported to inhibit Smad in regulating *Runx2*[22], thus ALP production could be inhibited.[23] In the present study, bone nodule formation was observed clearly after 3 weeks culture for both DPSCs and PDLSCs. In accordance, ALP activity was observed in the 3-weeks-culture, which was reduced by LPS supplementation. Taken together, NF-κB activity induced by LPS, could reduce ALP activity in both DPSCs and PDLSCs, leading to the inhibition of bone nodule formation. This finding

is in accordance with a previous study revealed that LPS-induced NF-κB activity downregulated mRNA and protein expressions of ALP in GMSCs.[20] Furthermore, LPS was reported to reduce ALP activity in DFSCs.[21]

NF-κB signaling can be blocked by several substances, one of which is Bay 11-7082. This substance has been reported to inhibit NF-κB activity in various types of stem cells, including BMMSCs[24,25], AdMSCs[25], and neural stem cells (NSCs)[26]. Present study disclosed the role of Bay 11-7082 and its mechanism in maintaining osteogenic differentiation in LPS-stimulated DPSCs and PDLSCs. Upon Bay 11-7082 supplementation, the NF-κB activity was suppressed, which partially maintained ALP activity and osteogenic potency in DPSCs and PDLSCs.

LPS could induce inflammatory signaling pathway via NF-κB and other molecules, such as AP-1.[27] Therefore, Bay 11-7082 could only suppress the inflammatory signaling pathway partially *via* NF-κB, meanwhile AP-1 could still inhibit the osteogenic differentiation of DPSCs and PDLSCs. Consequently, other inhibitors should be investigated further to suppress LPS-induced inflammatory signaling pathway fully so that osteogenic differentiation of DPSCs and PDLSCs could be undisrupted.

## Conclusion

Inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.

## Declaration of Interest

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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## Figure Legends

**Figure 1.** Flow cytometric results of DPSCs. DPSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of DPSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73. APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin5.5 area.

**Figure 2.** Flow cytometric results of PDLSCs. PDLSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of PDLSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73. APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin5.5 area.

**Figure 3.** LPS inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in osteogenic medium and treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in Methodology. Black bar: 100 $\mu$m.

**Figure 4.** LPS induced NF-κB activity in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in osteogenic medium, pretreated with/without 100 $\mu$M Bay 11-7082 for 30 min, and treated with/without 10 $\mu$g/mL LPS for 3 weeks. NF-κB activity was measured as described

in Methodology. The data are expressed as mean ± standard deviation (n=6). *$p$<0.05, Tukey's HSD.

**Figure 5.** Bay 11-7082 prevented LPS-decreased ALP activity of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in osteogenic medium, pretreated with/without 100 $\mu$M Bay 11-7082 for 30 min, and treated with/without 10 $\mu$g/mL LPS for 3 weeks. ALP activity was measured as described in Methodology. The data are expressed as mean ± standard deviation (n=6). *$p$<0.05, Tukey's HSD.

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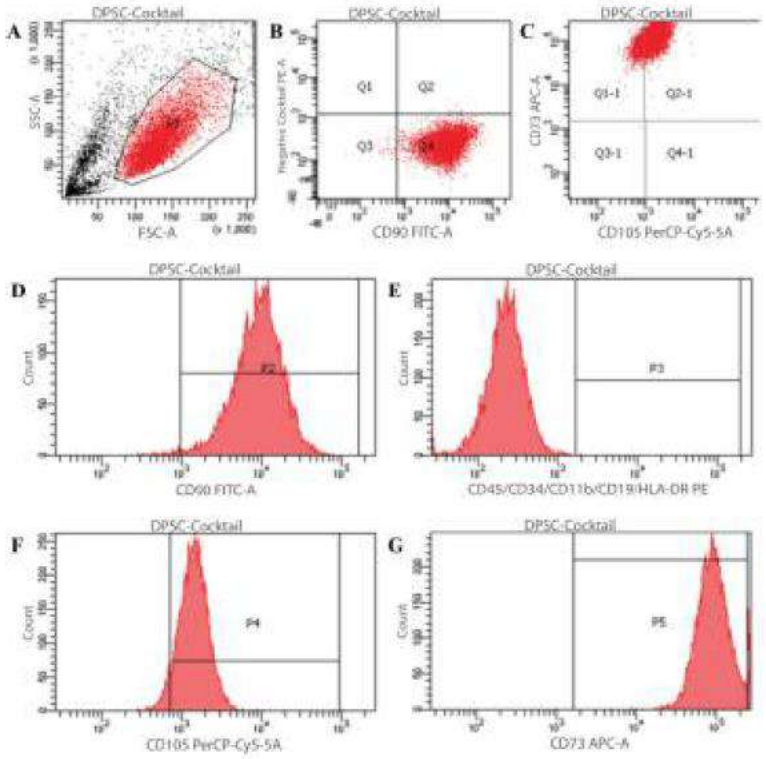


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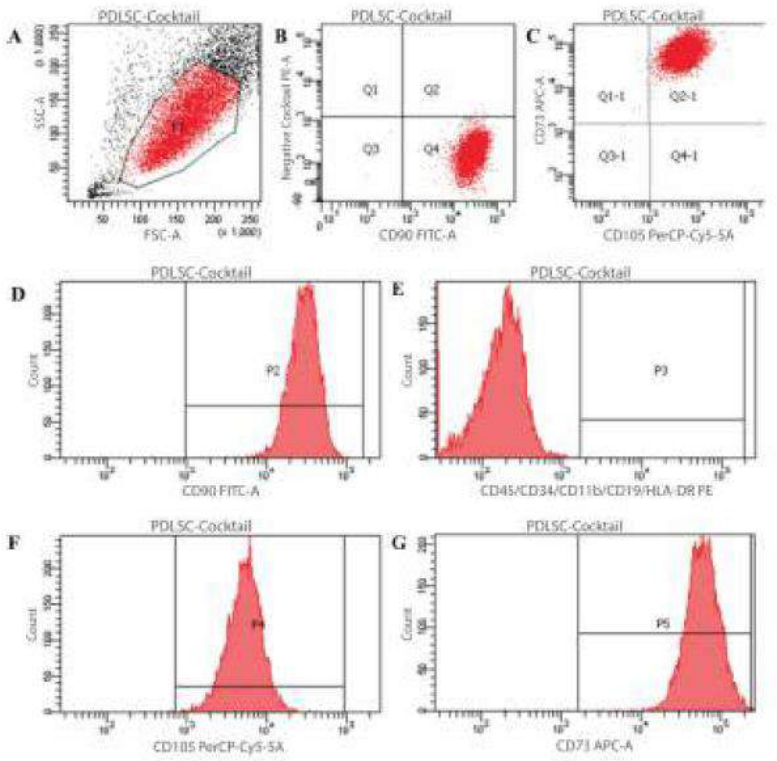


**Figure 2.** Flow cytometric results of PDLSCs. PDLSCs were harvested and labeled with specific antibodies for MSC markers as described in Methodology. (A) Granularity and size of PDLSCs. (B) Dot plot for negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73. APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin5.5 area.

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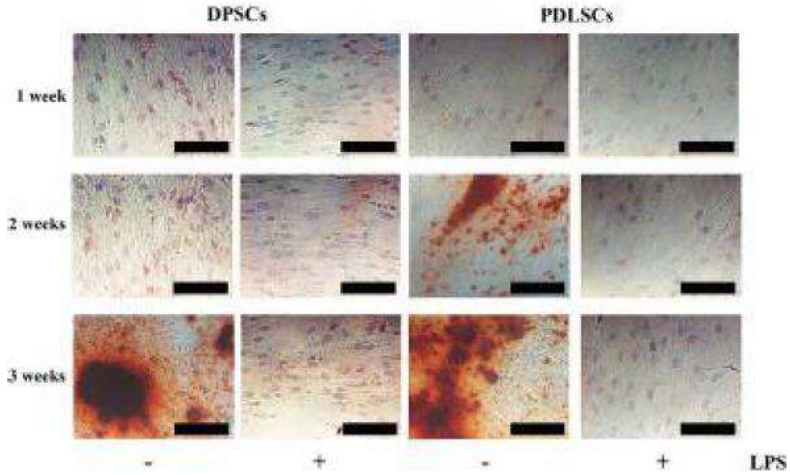


**Figure 3.** LPS inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in osteogenic medium and treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in Methodology. Black bar: 100 μm.

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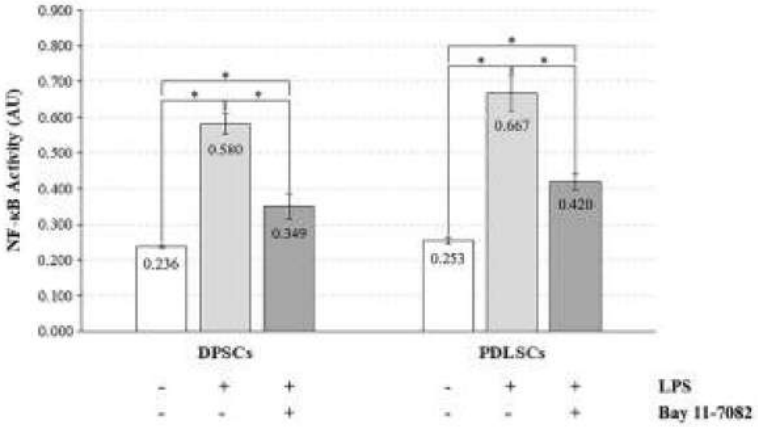


**Figure 4.** LPS induced NF-κB activity in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in osteogenic medium, pretreated with/without 100 μM Bay 11-7082 for 30 min, and treated with/without 10 μg/mL LPS for 3 weeks. NF-κB activity was measured as described in Methodology. The data are expressed as mean ± standard deviation (n=6). *$p$<0.05, Tukey's HSD.

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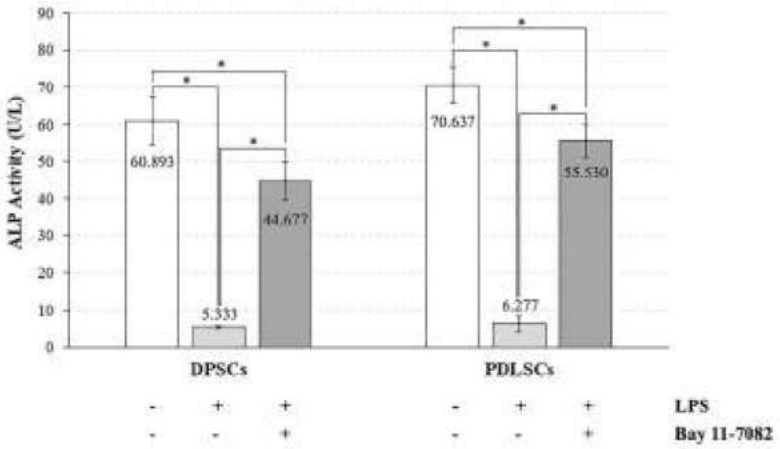


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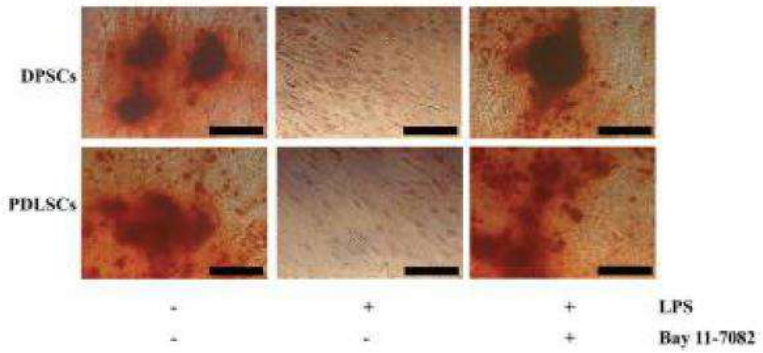


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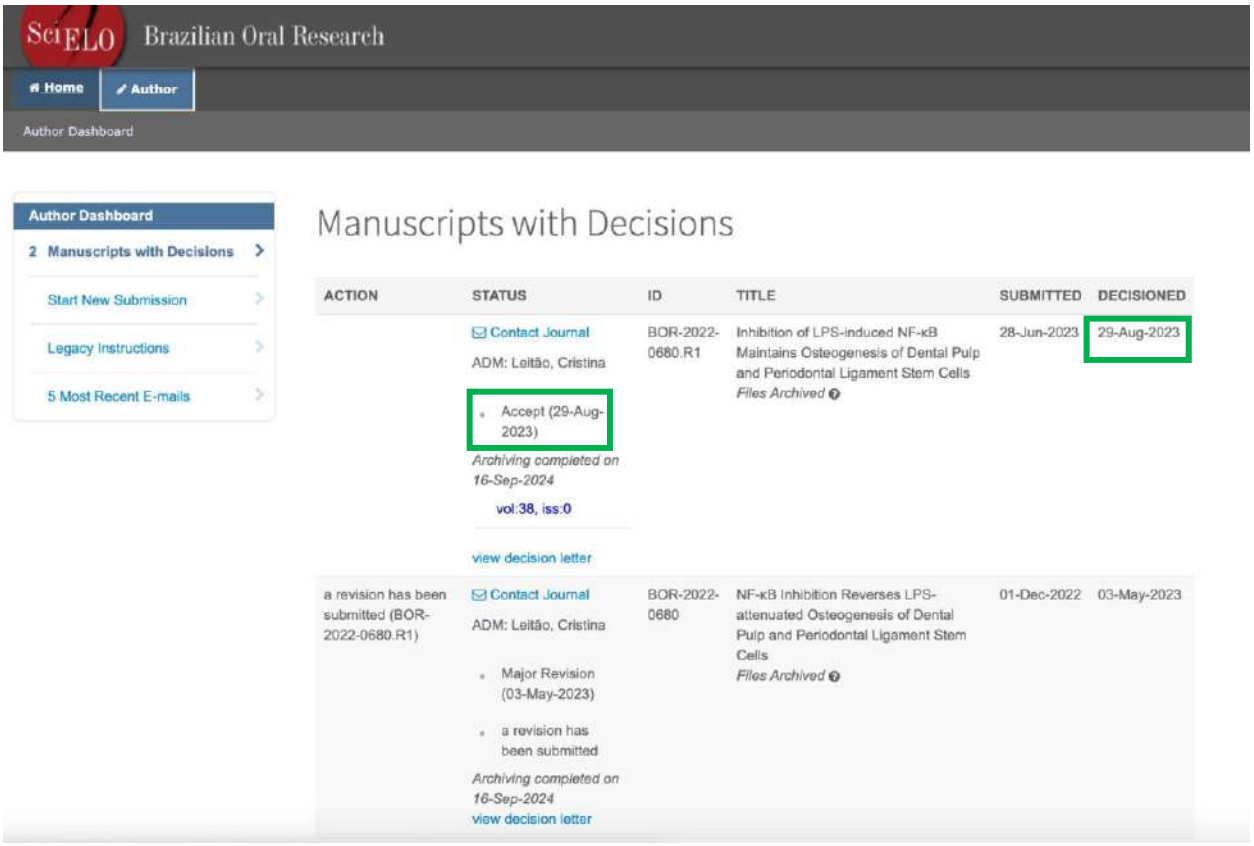

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# Inhibition of Lipopolysaccharide-Induced NF-κB Maintains Osteogenesis of Dental Pulp and Periodontal Ligament Stem Cells


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**Abstract**

Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) can differentiate into osteoblasts, indicating that both are potential candidates for bone tissue engineering. Osteogenesis is influenced by many environmental factors, one of which is lipopolysaccharide (LPS). LPS-induced NF-κB activity affects the osteogenic potencies of different types of MSCs differently. This study evaluated the effect of LPS-induced NF-κB activity and its inhibition in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without NF-κB inhibitor Bay 11-7082, and treated with/without LPS. Alizarin red staining was performed to assess bone nodule formation, which was observed under an inverted light microscope. NF-κB and alkaline phosphatase (ALP) activities were measured to examine the effect of Bay 11-7082 pretreatment and LPS supplementation on osteogenic differentiation of DPSCs and PDLSCs. LPS significantly induced NF-κB activity ($p = 0.000$) and reduced ALP activity ($p = 0.000$), which inhibited bone nodule formation in DPSCs and PDLSCs. Bay 11-7082 inhibited LPS-induced NF-κB activity, and partially maintained ALP activity and osteogenic potency of LPS-supplemented DPSCs and PDLSCs. Thus, inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.

**Keywords:** stem cells; dental pulp; periodontal ligament; lipopolysaccharides; NF-kappa B.

## Introduction

Several studies have reported that mesenchymal stem cells (MSCs) have potential uses in tissue engineering and regenerative medicine,[1–3] including the field of dentistry.[4] Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) are oral tissue-derived stem cells that possess the properties of MSCs.[4–6] Under specific culture conditions, DPSCs and PDLSCs can be differentiated into mesenchymal lineages, including osteoblasts.[7–9] DPSCs and PDLSCs have higher growth potential compared with bone marrow mesenchymal stem cells (BMMSCs)[10], and possess immunomodulatory activity.[2,3,11] Hence, DPSCs and PDLSCs are potential candidates for bone tissue engineering and regeneration applications, such as alveolar bone repair.[4]

The process of osteogenesis is influenced by several environmental factors, including inflammatory factors produced by bacteria.[12,13] Lipopolysaccharide (LPS) is the most common inflammatory factor, which is continuously liberated from Gram-negative bacteria colonizing the periodontal tissues, and can cause inflammatory diseases, such as periodontitis.[14] LPS activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway and induces inflammatory responses.[15,16] Several studies have reported that LPS-induced NF-κB activity in PDLSCs can be inhibited, enabling undisrupted osteogenesis.[12,13] However, in other types of MSCs, such as BMMSCs, LPS induces NF-κB activity, but does not alter osteogenic differentiation.[12] In addition, in adipose-derived mesenchymal stem cells (AdMSCs), LPS induced NF-κB activity and stimulated osteogenic differentiation.[17] Therefore, NF-κB inhibition affects the osteogenic potency of different types of MSCs differently. The aim this study was to evaluate the effect of LPS-induced NF-κB activity, and its inhibition using a specific inhibitor, Bay 11-7082, in DPSCs and PDLSCs.

## Methodology

### Cells Thawing and Culture

Cryopreserved passage five DPSCs and PDLSCs reported in previous research[6,11] were thawed and cultured in MesenCult MSC Basal Medium (StemCell Technologies, Vancouver, Canada) supplemented with MesenCult MSC Stimulatory Supplement (StemCell Technologies), 200 U/mL penicillin, 200 $\mu$g/mL streptomycin, and 0.5 $\mu$g/mL amphotericin (Gibco). DPSCs and PDLSCs were harvested after reaching confluency and used in this study. This study was conducted in accordance with the Declaration of Helsinki. Approval was obtained from the Ethics Committee of xxx (No. xxx). Written informed consent was obtained for the collection of human samples.

### Flow Cytometric Analysis

Flow cytometric analysis was conducted using a BD Stemflow hMSC Analysis Kit (BD Biosciences, Franklin Lakes, NJ, USA) to confirm whether DPSCs and PDLSCs had MSC markers as previously described.[11] DPSCs ($1 \times 10^7$ cells) and PDLSCs ($1 \times 10^7$ cells) were incubated with/without marker-specific antibodies as well as their isotypes for positive (CD90, CD105, and CD73) and negative (CD45, CD34, CD11b, CD19, and HLA-DR) markers. FACSCanto II flow cytometer (BD Biosciences) was used to analyze labeled DPSCs and PDLSCs using the FACSDiva software (BD Biosciences). The characteristics of DPSCs and PDLSCs were confirmed using the minimal surface marker criteria for defining MSCs, proposed by the International Society for Cellular Therapy (ISCT).[18]

### *In vitro* Osteogenic Functional Assay

*In vitro* osteogenic functional assay was performed as previously described.[6] DPSCs ($8 \times 10^4$ cells) and PDLSCs ($8 \times 10^4$ cells) were cultured using osteogenic medium containing 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich), and 50 μg/mL L-ascorbic acid (Sigma-Aldrich) on a 6-well plate. DPSCs and PDLSCs were pretreated with/without 100 μM NF-κB inhibitor Bay 11-7082 (Sigma-Aldrich) for 30 min and supplemented with/without 10 μg/mL *Porphyromonas gingivalis* LPS (Wako, Osaka, Japan) for 1, 2, or 3 weeks. After removing the medium, the plates were washed twice with PBS and fixed for 2 min in 4% paraformaldehyde (Wako) in phosphate buffer solution (PBS). This was followed by treatment with glycerol (Bio-Rad, Hercules, CA, USA) at room temperature for 5 min. The cells were washed thrice with distilled water after removal of the fixative. The cells were then stained with 2% alizarin red solution (Sigma-Aldrich) for 20 min. After removing the alizarin red stain, the plates were washed thrice with distilled water. The cells were finally observed and documented under an inverted light microscope (Zeiss, Jena, Germany). The experiment was performed twice in triplicate.

### NF-κB Activity Assay

After pretreatment with Bay 11-7082 for 30 min and LPS supplementation for three weeks, NF-κB activity in DPSCs ($2 \times 10^6$ cells) and PDLSCs ($2 \times 10^6$ cells) was determined using NF-κB p65 Transcription Factor Assay Kit (Abcam, Cambridge, UK) in accordance with the manufacturer's protocol. Nuclear extraction of the treated DPSCs and PDLSCs was performed using the Nuclear Extraction Kit (Abcam) in accordance with the manufacturer's instructions, before determining NF-κB activity. The nuclear extracts containing NF-κB were loaded into 96-well plates containing dsDNA with NF-κB response element sequence, followed by the sequential addition of

rabbit anti-NF-κB primary antibody and HRP-linked goat antirabbit IgG secondary antibody. Results were measured at $OD_{450}$ nm using a spectrophotometer (Bio-Rad). The experiment was performed twice in triplicate.

### Alkaline Phosphatase (ALP) Activity Assay

Following pretreatment with Bay 11-7082 for 30 min and LPS supplementation with/without Bay 11-7082 for three weeks, ALP activity in DPSCs and PDLSCs was evaluated using the colorimetric Alkaline Phosphatase Assay Kit (Abcam) in accordance with the manufacturer's protocol. Briefly, homogenized DPSCs or PDLSCs ($1 \times 10^5$ cells) and *p*-nitrophenyl phosphate (pNPP) were loaded into 96-well plates. The plates were incubated in the dark. This was followed by the addition of the stopping solution, and measurement at $OD_{405}$ nm using a spectrophotometer (Bio-Rad). The activity of ALP (U/L) was calculated. The experiment was performed twice in triplicate.

### Statistical Analysis

IBM SPSS Statistics version 26.0 was used to conduct the statistical analyses (SPSS IBM, Armonk, NY, USA). The Shapiro–Wilk test was used as a normality test. Comparison of NF-κB and ALP activities of DPSCs and PDLSCs in different treatment groups was accomplished using two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD). $p$-values $< 0.05$ were considered statistically significant.

## Results

### Phenotypic Characterization of DPSCs and PDLSCs

High expression of CD90, CD105, and CD73 (>95%) was exhibited by DPSCs and PDLSCs, whereas expression of negative markers were <2% (Figure 1, Figure 2). The characteristics of these surface biomarkers matched the standard criteria defining MSCs proposed by the International Society for Cell and Gene Therapy (ISCT), suggesting that the cultured DPSCs and PDLSCs had the properties of MSCs.

### LPS Inhibited Osteogenic Differentiation of DPSCs and PDLSCs

Bone nodules, in the form of alizarin positive-red mineralized deposits, were observed in DPSCs on the third-week culture and in PDLSCs on the second-week culture under an inverted light microscope. No bone nodules were observed in 10 $\mu$g/mL LPS-supplemented DPSCs and PDLSCs after 1, 2, and 3 weeks (Figure 3).

### LPS-Induced NF-κB Activity in DPSCs and PDLSCs

NF-κB activities of untreated DPSCs and PDLSCs were 0.236 ± 0.005 AU and 0.253 ± 0.008 AU, respectively. Following three weeks of LPS supplementation, NF-κB activities of DPSCs and PDLSCs were 0.580 ± 0.029 AU and 0.667 ± 0.051 AU. NF-κB activities of LPS-supplemented DPSCs and PDLSCs following pretreatment with Bay 11-7082 were 0.349 ± 0.037 and 0.420 ± 0.022 AU (Figure 4).

No significant interaction between the types of stem cells and treatments on NF-κB activity was indicated by two-way ANOVA ($p$ = 0.148). NF-κB activity significantly differed in different treatment groups ($p$ = 0.000). The 3-week-LPS-supplemented NF-κB activities of DPSCs and PDLSCs were significantly higher than those of untreated DPSCs and PDLSCs ($p$ = 0.000) as well as those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p$ = 0.000). The NF-κB activities of untreated

DPSCs and PDLSCs were significantly lower than those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p = 0.000$). These results demonstrated that LPS-induced NF-κB activation in DPSCs and PDLSCs, and that Bay 11-7082 partially inhibited the LPS-induced NF-κB pathway.

**LPS Reduced ALP Activity and Inhibited Bone Nodule Formation in DPSCs and PDLSCs**

Two-way ANOVA did not indicate a significant interaction between stem cells and treatments on ALP activity ($p = 0.148$). Significant differences in ALP activity were observed in different treatment groups ($p = 0.000$). ALP activities of untreated DPSCs and PDLSCs were 60.893 ± 6.516 U/mL and 70.637 ± 4.902 U/mL, respectively. The ALP activities of DPSCs (5.333 ± 0.323 U/mL) and PDLSCs (6.277 ± 2.026 U/mL) were significantly lower than those of untreated DPSCs and PDLSCs after three weeks of LPS supplementation ($p = 0.000$) (Figure 5). Lower ALP activity was associated with the absence of bone nodule formation in LPS-supplemented DPSCs and PDLSCs (Figure 6). Pretreatment with Bay 11-7082 resulted in significantly higher ALP activities of LPS-supplemented DPSCs (44.677 ± 5.193 U/mL) and PDLSCs (55.530 ± 4.478 U/mL) compared with those supplemented with LPS ($p = 0.000$), but significantly lower than those of untreated ($p = 0.000$). These results showed that Bay 11-7082 was responsible for the partial maintenance of ALP activity in DPSCs and PDLSCs (Figure 5). Moreover, pretreatment with Bay 11-7082 partially maintained the osteogenic potency of LPS-supplemented DPSCs and PDLSCs (Figure 6).

**Discussion**

LPS-induced NF-κB activation, was reported to play an important role in inflammatory responses and bone loss in periodontitis.[12] This study demonstrated that

*P. gingivalis*-derived LPS not only induced NF-κB activity but also inhibited bone nodule formation in DPSCs and PDLSCs. These findings are consistent with a previously conducted study that demonstrated that LPS-induced NF-κB activity impaired the osteogenic potency of GMSCs.[19] LPS supplementation also inhibited osteogenic differentiation in dental follicle stem cells (DFSCs).[20]

The activated NF-κB targeted the κB site and inhibit Smad in regulating *Runx2*[21], thereby inhibiting ALP production.[22] In this study, bone nodule formation was clearly observed after 3 weeks of culturing with DPSCs and PDLSCs. In addition, ALP activity, which was observed in the 3-week culture, was reduced by LPS supplementation. Thus, NF-κB activity, which was induced by LPS, could reduce ALP activity in DPSCs and PDLSCs, leading to inhibition of bone nodule formation. This finding corroborates a previous study that revealed that LPS-induced NF-κB activity downregulated ALP mRNA and protein expressions in GMSCs.[19] Furthermore, ALP activity was reported to be reduced by LPS in DFSCs.[20]

NF-κB signaling can be blocked by several substances and natural products[23,24], one of which is Bay 11-7082, which inhibits NF-κB activity in various types of stem cells, including BMMSCs[25,26], AdMSCs[26], and neural stem cells (NSCs)[27]. This study highlighted the role of Bay 11-7082 and its mechanism in maintaining osteogenic differentiation in LPS-stimulated DPSCs and PDLSCs. Bay 11-7082 supplementation led to the suppression of NF-κB activity, which was partially responsible for maintaining ALP activity and osteogenic potency in DPSCs and PDLSCs.

LPS could induce an inflammatory signaling pathway via NF-κB and other molecules, such as AP-1.[28] Therefore, Bay 11-7082 was only able to partially suppress the inflammatory signaling pathway via NF-κB; however, AP-1 could still inhibit the

osteogenic differentiation of DPSCs and PDLSCs. Consequently, further investigation of other inhibitors is necessary to enable complete suppression of the LPS-induced inflammatory signaling pathway, so that osteogenic differentiation of DPSCs and PDLSCs could be undisrupted.

## Conclusion

Inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.

## Declaration of Interest

The authors certify the absence of commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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**Figure Legends**

**Figure 1:** Flow cytometry results of DPSCs. DPSCs were harvested and labeled with specific antibodies for MSC markers as described in the Methodology. (A) Granularity and size of DPSCs. (B) A Dot plot for a negative cocktail (CD45, CD34, CD11b, CD19, and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibodies. (D) Histogram for CD90. (E) Histogram for the negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin 5.5 area.

**Figure 2:** Flow cytometry results of PDLSCs. PDLSCs were harvested and labeled with specific antibodies for MSC markers as described in the Methodology. (A) Granularity and size of PDLSCs. (B) A Dot plot for a negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for the negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin5.5 area.

**Figure 3:** LPS inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium and treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in the methodology. Black bar: 100 $\mu$m.

**Figure 4:** LPS induced NF-κB activity in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 $\mu$M Bay 11-7082 for 30 min, and treated with/without 10 $\mu$g/mL LPS for 3 weeks. NF-κB activity was measured as described in the methodology. The data are expressed as mean ± standard deviation (n = 6). $^{*}p < 0.05$, Tukey's HSD.

**Figure 5:** Bay 11-7082 prevented LPS-decreased ALP activity of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 $\mu$M Bay 11-7082 for 30 min, and treated with/without 10 $\mu$g/mL LPS for 3 weeks. ALP activity was measured as described in the methodology. The data are expressed as mean ± standard deviation (n = 6). $^{*}p < 0.05$, Tukey's HSD.

**Figure 6:** Bay 11-7082 prevented LPS-inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 $\mu$M Bay 11-7082 for 30 min, and treated with/without 10 $\mu$g/mL LPS and for 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in the methodology. Black bar: 100 $\mu$m.

**4. Bukti proofreading terhadap *layout design*/*format editing* dari jurnal Brazilian Oral Research**
**(22 Maret 2024)**

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Ferry Sandra <ferry@trisakti.ac.id>

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To: "ferry@trisakti.ac.id" <ferry@trisakti.ac.id>

Dear Ferry Sandra

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Your manuscript was recently accepted to the Brazilian Oral Research.

Your paper has undergone some format editing. Please review the attached file(s) and then contact me with your approval or questions. My contact information is below.

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If there is no reply until this date, we will consider the PDF file as approved by the author.

We appreciate your time and await your reply.

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# RE: Brazilian Oral Research - BOR-2022-0680.R1 - Inquiry Regarding PDF File for the Proofreading Process

**Ferry Sandra** <ferry@trisakti.ac.id> Mon, Mar 25, 2024 at 11:44 AM
To: Secretaria BOR <office.bor@ingroup.srv.br>

Dear Mrs. Cristina Fleury Leitão,

We sincerely apologize for the delay, however we have now completed the review of the proofread layout of the article, "**Inhibition of lipopolysaccharide-induced NF-κB maintains osteogenesis of dental pulp and periodontal ligament stem cells**," for *Brazilian Oral Research*, Herewith we attached the commented PDF file. Parts that need to be revised are highlighted in yellow, and the corrections are written in the comments. Hopefully, you can find the PDF file well.

Thank you.

Best Regards,
Dr. Ferry Sandra, PhD
[Quoted text hidden]
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Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

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Original Research
Pulp Biology

# Inhibition of lipopolysaccharide-induced NF-κB maintains osteogenesis of dental pulp and periodontal ligament stem cells

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**Declaration of Interests:** The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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**Abstract:** Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) can differentiate into osteoblasts, indicating that both are potential candidates for bone tissue engineering. Osteogenesis is influenced by many environmental factors, one of which is lipopolysaccharide (LPS). LPS-induced NF-κB activity affects the osteogenic potencies of different types of MSCs differently. This study evaluated the effect of LPS-induced NF-κB activity and its inhibition in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without NF-κB inhibitor Bay 11-7082, and treated with/without LPS. Alizarin red staining was performed to assess bone nodule formation, which was observed under an inverted light microscope. NF-κB and alkaline phosphatase (ALP) activities were measured to examine the effect of Bay 11-7082 pretreatment and LPS supplementation on osteogenic differentiation of DPSCs and PDLSCs. LPS significantly induced NF-κB activity ($p = 0.000$) and reduced ALP activity ($p = 0.000$), which inhibited bone nodule formation in DPSCs and PDLSCs. Bay 11-7082 inhibited LPS-induced NF-κB activity, and partially maintained ALP activity and osteogenic potency of LPS-supplemented DPSCs and PDLSCs. Thus, inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.

**Keywords:** Stem Cells; Dental Pulp; Periodontal Ligament; Lipopolysaccharides; NF-kappa B.

## Introduction

Several studies have reported that mesenchymal stem cells (MSCs) have potential uses in tissue engineering and regenerative medicine,[1-3] including the field of dentistry.[4] Dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) are oral tissue-derived stem cells that possess the properties of MSCs.[4-6] Under specific culture conditions, DPSCs and PDLSCs can be differentiated into mesenchymal lineages, including osteoblasts.[7-9] DPSCs and PDLSCs have higher growth potential compared with bone marrow mesenchymal stem

cells (BMMSCs),[10] and possess immunomodulatory activity.[2,3,11] Hence, DPSCs and PDLSCs are potential candidates for bone tissue engineering and regeneration applications, such as alveolar bone repair.[4]

The process of osteogenesis is influenced by several environmental factors, including inflammatory factors produced by bacteria.[12,13] Lipopolysaccharide (LPS) is the most common inflammatory factor, which is continuously liberated from Gram-negative bacteria colonizing the periodontal tissues, and can cause inflammatory diseases, such as periodontitis.[14] LPS activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway and induces inflammatory responses.[15,16] Several studies have reported that LPS-induced NF-κB activity in PDLSCs can be inhibited, enabling undisrupted osteogenesis.[12,13] However, in other types of MSCs, such as BMMSCs, LPS induces NF-κB activity, but does not alter osteogenic differentiation.[12] In addition, in adipose-derived mesenchymal stem cells (AdMSCs), LPS induced NF-κB activity and stimulated osteogenic differentiation.[17] Therefore, NF-κB inhibition affects the osteogenic potency of different types of MSCs differently. The aim this study was to evaluate the effect of LPS-induced NF-κB activity, and its inhibition using a specific inhibitor, Bay 11-7082, in DPSCs and PDLSCs.

## Methodology

### Cells Thawing and Culture

Cryopreserved passage five DPSCs and PDLSCs reported in previous research[6,11] were thawed and cultured in MesenCult MSC Basal Medium (StemCell Technologies, Vancouver, Canada) supplemented with MesenCult MSC Stimulatory Supplement (StemCell Technologies), 200 U/mL penicillin, 200 µg/mL streptomycin, and 0.5 µg/mL amphotericin (Gibco). DPSCs and PDLSCs were harvested after reaching confluency and used in this study. This study was conducted in accordance with the Declaration of Helsinki. Approval was obtained from the Ethics Committee of Faculty of Dentistry Universitas Trisakti, Indonesia (No. #167/KE/FKG/11/2014). Written informed consent was obtained for the collection of human samples.

### Flow Cytometric Analysis

Flow cytometric analysis was conducted using a BD Stemflow hMSC Analysis Kit (BD Biosciences, Franklin Lakes, USA) to confirm whether DPSCs and PDLSCs had MSC markers as previously described.[11] DPSCs ($1 \times 10^7$ cells) and PDLSCs ($1 \times 10^7$ cells) were incubated with/without marker-specific antibodies as well as their isotypes for positive (CD90, CD105, and CD73) and negative (CD45, CD34, CD11b, CD19, and HLA-DR) markers. FACSCanto II flow cytometer (BD Biosciences) was used to analyze labeled DPSCs and PDLSCs using the FACSDiva software (BD Biosciences). The characteristics of DPSCs and PDLSCs were confirmed using the minimal surface marker criteria for defining MSCs, proposed by the International Society for Cellular Therapy (ISCT).[18]

### *In vitro* Osteogenic Functional Assay

*In vitro* osteogenic functional assay was performed as previously described.[6] DPSCs ($8 \times 10^4$ cells) and PDLSCs ($8 \times 10^4$ cells) were cultured using osteogenic medium containing 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, USA), 100 nM dexamethasone (Sigma-Aldrich), and 50 µg/mL L-ascorbic acid (Sigma-Aldrich) on a 6-well plate. DPSCs and PDLSCs were pretreated with/without 100 µM NF-κB inhibitor Bay 11-7082 (Sigma-Aldrich) for 30 min and supplemented with/without 10 µg/mL *Porphyromonas gingivalis* LPS (Wako, Osaka, Japan) for 1, 2, or 3 weeks. After removing the medium, the plates were washed twice with PBS and fixed for 2 min in 4% paraformaldehyde (Wako) in phosphate buffer solution (PBS). This was followed by treatment with glycerol (Bio-Rad, Hercules, USA) at room temperature for 5 min. The cells were washed thrice with distilled water after removal of the fixative. The cells were then stained with 2%

alizarin red solution (Sigma-Aldrich) for 20 min. After removing the alizarin red stain, the plates were washed thrice with distilled water. The cells were finally observed and documented under an inverted light microscope (Zeiss, Jena, Germany). The experiment was performed twice in triplicate.

### NF-κB Activity Assay

After pretreatment with Bay 11-7082 for 30 min and LPS supplementation for three weeks, NF-κB activity in DPSCs ($2 \times 10^6$ cells) and PDLSCs ($2 \times 10^6$ cells) was determined using NF-κB p65 Transcription Factor Assay Kit (Abcam, Cambridge, UK) in accordance with the manufacturer's protocol. Nuclear extraction of the treated DPSCs and PDLSCs was performed using the Nuclear Extraction Kit (Abcam) in accordance with the manufacturer's instructions, before determining NF-κB activity. The nuclear extracts containing NF-κB were loaded into 96-well plates containing dsDNA with NF-κB response element sequence, followed by the sequential addition of rabbit anti-NF-κB primary antibody and HRP-linked goat antirabbit IgG secondary antibody. Results were measured at $OD_{450}$ nm using a spectrophotometer (Bio-Rad). The experiment was performed twice in triplicate.

### Alkaline Phosphatase (ALP) Activity Assay

Following pretreatment with Bay 11-7082 for 30 min and LPS supplementation with/without Bay 11-7082 for three weeks, ALP activity in DPSCs and PDLSCs was evaluated using the colorimetric Alkaline Phosphatase Assay Kit (Abcam) in accordance with the manufacturer's protocol. Briefly, homogenized DPSCs or PDLSCs ($1 \times 10^5$ cells) and *p*-nitrophenyl phosphate (pNPP) were loaded into 96-well plates. The plates were incubated in the dark. This was followed by the addition of the stopping solution, and measurement at $OD_{405}$ nm using a spectrophotometer (Bio-Rad). The activity of ALP (U/L) was calculated. The experiment was performed twice in triplicate.

### Statistical Analysis

IBM SPSS Statistics version 26.0 was used to conduct the statistical analyses (SPSS IBM, Armonk, USA). The Shapiro–Wilk test was used as a normality test. Comparison of NF-κB and ALP activities of DPSCs and PDLSCs in different treatment groups was accomplished using two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD). p-values $< 0.05$ were considered statistically significant.

## Results

### Phenotypic Characterization of DPSCs and PDLSCs

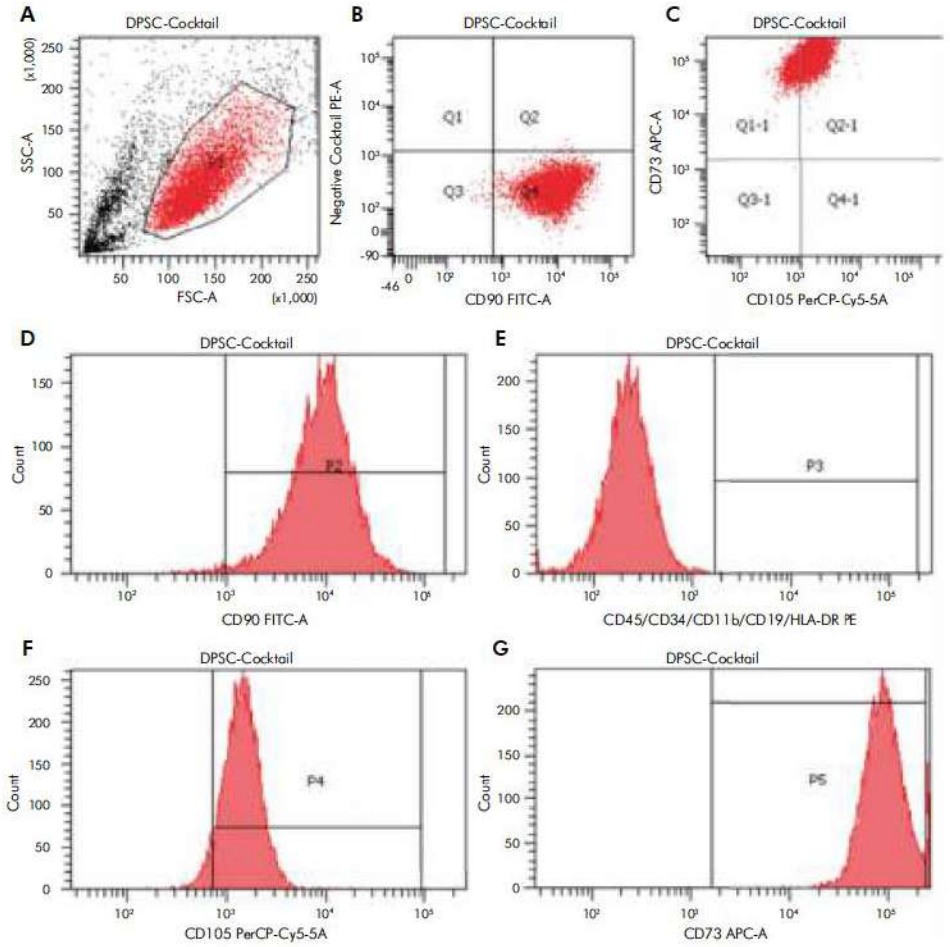
High expression of CD90, CD105, and CD73 (>95%) was exhibited by DPSCs and PDLSCs, whereas expression of negative markers were < 2% (Figures 1 and 2). The characteristics of these surface biomarkers matched the standard criteria defining MSCs proposed by the International Society for Cell and Gene Therapy (ISCT), suggesting that the cultured DPSCs and PDLSCs had the properties of MSCs.

### LPS Inhibited Osteogenic Differentiation of DPSCs and PDLSCs

Bone nodules, in the form of alizarin positive-red mineralized deposits, were observed in DPSCs on the third-week culture and in PDLSCs on the second-week culture under an inverted light microscope. No bone nodules were observed in 10 μg/mL LPS-supplemented DPSCs and PDLSCs after 1, 2, and 3 weeks (Figure 3).

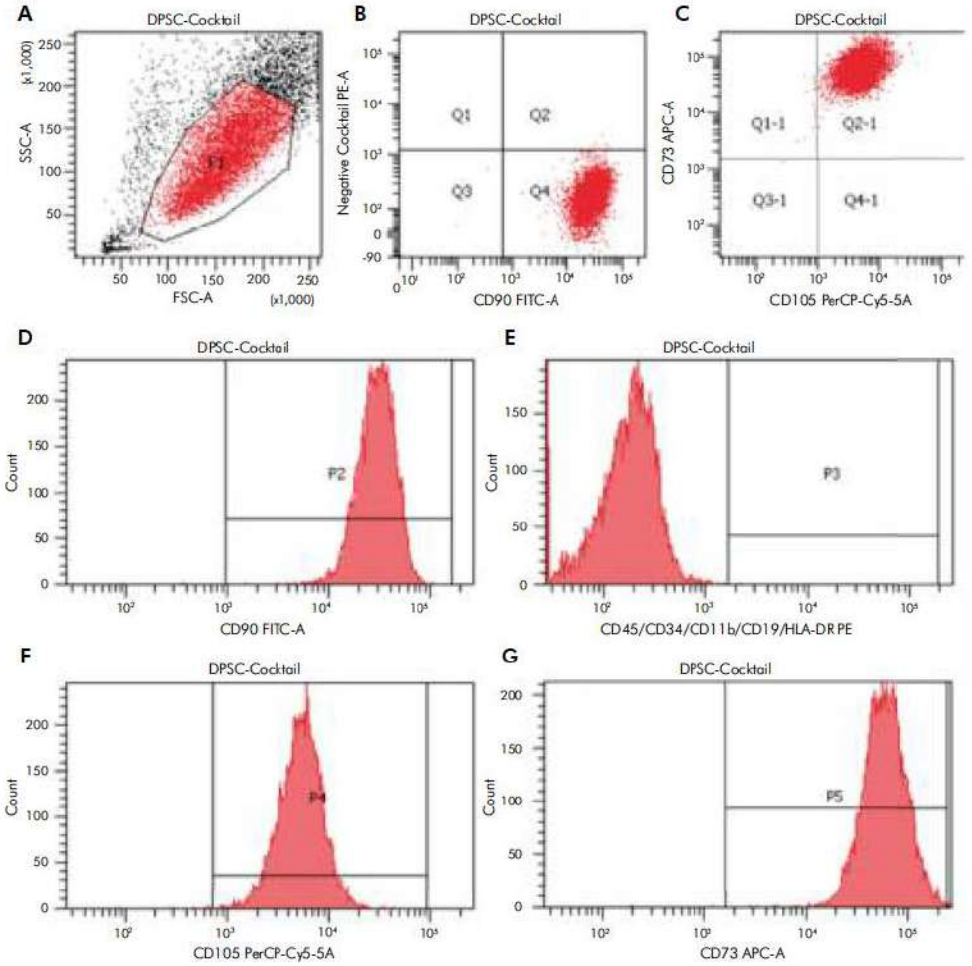
### LPS-Induced NF-κB Activity in DPSCs and PDLSCs

NF-κB activities of untreated DPSCs and PDLSCs were 0.236 ± 0.005 AU and 0.253 ± 0.008 AU, respectively. Following three weeks of LPS supplementation, NF-κB activities of DPSCs and PDLSCs were 0.580 ± 0.029 AU and 0.667 ± 0.051 AU. NF-κB activities of LPS-supplemented DPSCs and PDLSCs following pretreatment with Bay 11-7082 were 0.349 ± 0.037 and 0.420 ± 0.022 AU (Figure 4).




APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin 5.5 area.

**Figure 1.** Flow cytometry results of DPSCs. DPSCs were harvested and labeled with specific antibodies for MSC markers as described in the Methodology. (A) Granularity and size of DPSCs. (B) A Dot plot for a negative cocktail (CD45, CD34, CD11b, CD19, and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibodies. (D) Histogram for CD90. (E) Histogram for the negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.




APC-A: allophycocyanin area; FITC-A: fluorescein isothiocyanate area; FSC-A: forward scatter area; SSC-A: side scatter area; PE-A: phycoerythrin area; PerCP-Cy5-5-A: peridinin-chlorophyll-protein-cyanin5.5 area.

**Figure 2.** Flow cytometry results of PDLSCs. PDLSCs were harvested and labeled with specific antibodies for MSC markers as described in the Methodology. (A) Granularity and size of PDLSCs. (B) A Dot plot for a negative cocktail (CD45, CD34, CD11b, CD19 and HLA-DR) and CD90 antibody. (C) Dot plot for CD73 and CD105 antibody. (D) Histogram for CD90. (E) Histogram for the negative cocktail. (F) Histogram for CD105. (G) Histogram for CD73.

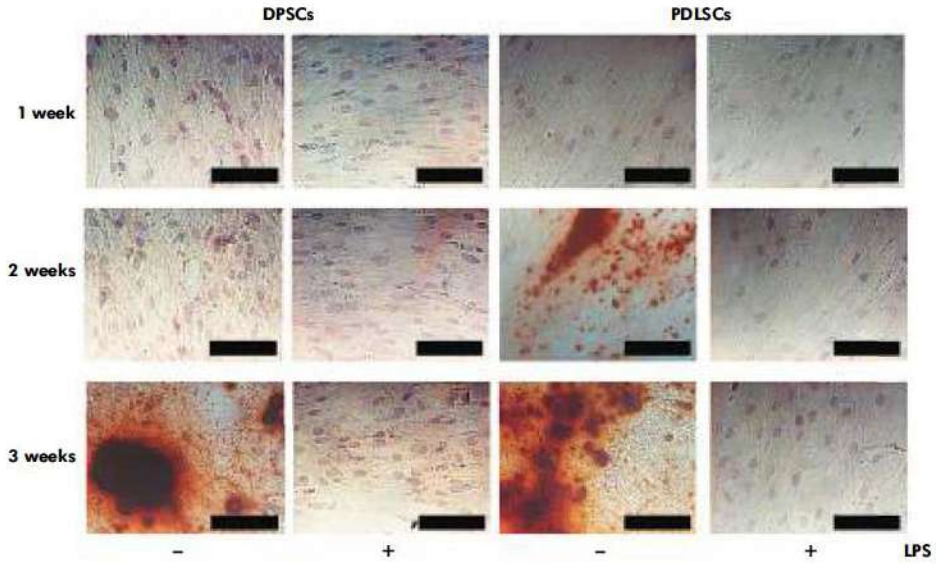



**Figure 3.** LPS inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium and treated with/without LPS for 1, 2, or 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in the methodology. Black bar: 100 μm.

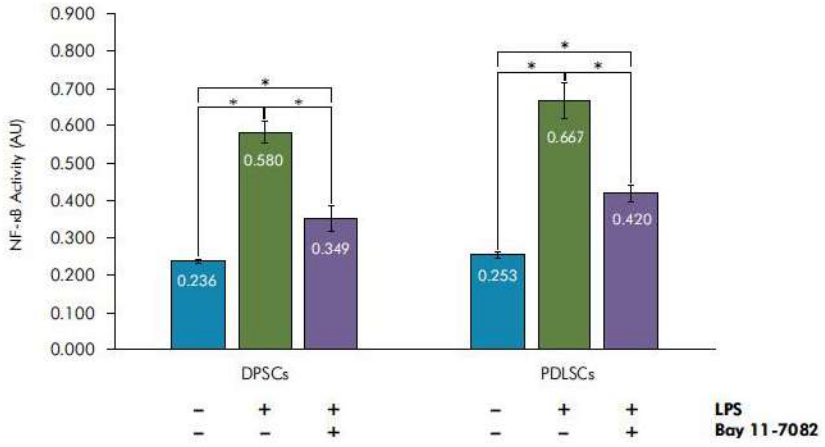



**Figure 4.** LPS induced NF-κB activity in DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 μM Bay 11-7082 for 30 min, and treated with/without 10 μg/mL LPS for 3 weeks. NF-κB activity was measured as described in the methodology. The data are expressed as mean ± standard deviation (n = 6). *$p < 0.05$, Tukey's HSD.

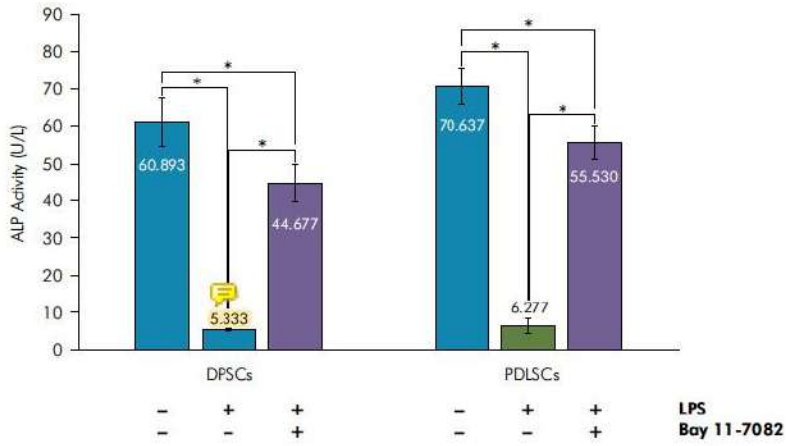



**Figure 5.** Bay 11-7082 prevented LPS-decreased ALP activity of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 μM Bay 11-7082 for 30 min, and treated with/without 10 μg/mL LPS for 3 weeks. ALP activity was measured as described in the methodology. The data are expressed as mean ± standard deviation (n = 6). *$p < 0.05$, Tukey's HSD.

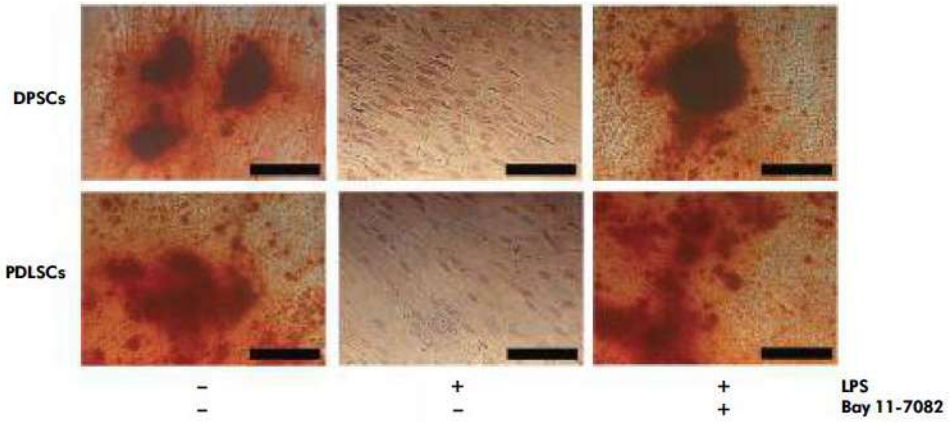



**Figure 6.** Bay 11-7082 prevented LPS-inhibited osteogenic differentiation of DPSCs and PDLSCs. DPSCs and PDLSCs were cultured in an osteogenic medium, pretreated with/without 100 μM Bay 11-7082 for 30 min, and treated with/without 10 μg/mL LPS and for 3 weeks. DPSCs and PDLSCs were stained with alizarin red as described in the methodology. Black bar: 100 μm.

No significant interaction between the types of stem cells and treatments on NF-κB activity was indicated by two-way ANOVA ($p = 0.148$). NF-κB activity significantly differed in different treatment groups ($p = 0.000$). The 3-week-LPS-supplemented NF-κB activities of DPSCs and PDLSCs were significantly higher than those of untreated DPSCs and PDLSCs ($p = 0.000$) as well as those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p = 0.000$). The NF-κB activities of

untreated DPSCs and PDLSCs were significantly lower than those of Bay 11-7082-pretreated LPS-supplemented DPSCs and PDLSCs ($p = 0.000$). These results demonstrated that LPS-induced NF-κB activation in DPSCs and PDLSCs, and that Bay 11-7082 partially inhibited the LPS-induced NF-κB pathway.

### LPS Reduced ALP Activity and Inhibited Bone Nodule Formation in DPSCs and PDLSCs

Two-way ANOVA did not indicate a significant interaction between stem cells and treatments on ALP activity ($p = 0.148$). Significant differences in ALP activity were observed in different treatment groups ($p = 0.000$). ALP activities of untreated DPSCs and PDLSCs were 60.893 ± 6.516 U/mL and 70.637 ± 4.902 U/mL, respectively. The ALP activities of DPSCs (5.333 ± 0.323 U/mL) and PDLSCs (6.277 ± 2.026 U/mL) were significantly lower than those of untreated DPSCs and PDLSCs after three weeks of LPS supplementation ($p = 0.000$) (Figure 5). Lower ALP activity was associated with the absence of bone nodule formation in LPS-supplemented DPSCs and PDLSCs (Figure 6). Pretreatment with Bay 11-7082 resulted in significantly higher ALP activities of LPS-supplemented DPSCs (44.677 ± 5.193 U/mL) and PDLSCs (55.530 ± 4.478 U/mL) compared with those supplemented with LPS ($p = 0.000$), but significantly lower than those of untreated ($p = 0.000$). These results showed that Bay 11-7082 was responsible for the partial maintenance of ALP activity in DPSCs and PDLSCs (Figure 5). Moreover, pretreatment with Bay 11-7082 partially maintained the osteogenic potency of LPS-supplemented DPSCs and PDLSCs (Figure 6).

## Discussion

LPS-induced NF-κB activation, was reported to play an important role in inflammatory responses and bone loss in periodontitis.[12] This study demonstrated that *P. gingivalis*-derived LPS not only induced NF-κB activity but also inhibited bone nodule formation in DPSCs and PDLSCs. These findings are consistent with a previously conducted study that demonstrated that LPS-induced NF-κB activity impaired the osteogenic potency of GMSCs.[19] LPS supplementation also inhibited osteogenic differentiation in dental follicle stem cells (DFSCs).[20]

The activated NF-κB targeted the κB site and inhibit Smad in regulating Runx2,[21] thereby inhibiting ALP production.[22] In this study, bone nodule formation was clearly observed after 3 weeks of culturing with DPSCs and PDLSCs. In addition, ALP activity, which was observed in the 3-week culture, was reduced by LPS supplementation. Thus, NF-κB activity, which was induced by LPS, could reduce ALP activity in DPSCs and PDLSCs, leading to inhibition of bone nodule formation. This finding corroborates a previous study that revealed that LPS-induced NF-κB activity downregulated ALP mRNA and protein expressions in GMSCs.[19] Furthermore, ALP activity was reported to be reduced by LPS in DFSCs.[20]

NF-κB signaling can be blocked by several substances and natural products,[23,24] one of which is Bay 11-7082, which inhibits NF-κB activity in various types of stem cells, including BMMSCs,[25,26] AdMSCs,[26] and neural stem cells (NSCs).[27] This study highlighted the role of Bay 11-7082 and its mechanism in maintaining osteogenic differentiation in LPS-stimulated DPSCs and PDLSCs. Bay 11-7082 supplementation led to the suppression of NF-κB activity, which was partially responsible for maintaining ALP activity and osteogenic potency in DPSCs and PDLSCs.

LPS could induce an inflammatory signaling pathway via NF-κB and other molecules, such as AP-1.[28] Therefore, Bay 11-7082 was only able to partially suppress the inflammatory signaling pathway via NF-κB; however, AP-1 could still inhibit the osteogenic differentiation of DPSCs and PDLSCs. Consequently, further investigation of other inhibitors is necessary to enable complete suppression of the LPS-induced inflammatory signaling pathway, so that osteogenic differentiation of DPSCs and PDLSCs could be undisrupted.

## Conclusion

Inhibition of LPS-induced NF-κB activity can maintain the osteogenic potency of DPSCs and PDLSCs.

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