TLR2 and TLR4 Differentially Regulate the Osteogenic Capacity of Human Periodontal Ligament Fibroblasts

Sujiwan Seubbuk,[1,2,3] Rudee Surarit,[4] Danielle Stephens,[2] Hatice Hasturk,[2] Thomas E. Van Dyke[2] and Alpdogan Kantarci[2]

[1]Molecular Medicine Program, Faculty of Science, Mahidol University, Ratchthewi, Bangkok Thailand; [2]Department of Applied Oral Sciences, The Forsyth Institute, Cambridge, MA, USA; [3]Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, Ratchthewi, Bangkok Thailand; [4]Department of Oral Biology, Faculty of Dentistry, Mahidol University, Ratchthewi, Bangkok Thailand

Abstract

Aims: To test that the osteogenic capacity of periodontal ligament (PDL) fibroblasts can be mediated by TLR2 and TLR4 activation.

Materials and Methods: Human PDL fibroblasts were cultured in osteogenic medium and treated with TLR2 and TLR4 agonists (Pam3CSK4 and monophosphoryl Lipid A (MPLA), respectively). Cell proliferation was measured by MTT and BrdU incorporation. Osteogenic differentiation was measured by alkaline phosphatase (ALP) activity. Nodule formation was measured for osteoblast function. The expression of markers of potential signaling pathways (*RUNX2, OCN, BSP and Osterix)* was evaluated by quantitative PCR.

Results: PDL fibroblasts grew at the same rate during the first 5 days in response to both Pam3CSK5 and MPLA. On day 7, cells cultured in the presence of Pam3CSK4 had a significantly higher rate of DNA replication, while cells in MPLA group had a significantly lower DNA replication rate (one-third) compared to the control *($p<0.05$)*. Pam3CSK4 induced significantly higher ALP activity and larger calcified nodules. TLR4 activation significantly reduced the expression of *RUNX2* and *osterix* and enhanced *OCN*. Neither TLR2 nor TLR4 affected *BSP* expression.

Conclusion: These data suggest that the activation of TLR2 and TLR4 differentially and perhaps antagonistically modulate osteogenesis by human PDL fibroblasts and have a direct role of TLR-mediated PDL function during periodontal regeneration as a potential target for therapeutics.

Keywords: Periodontal fibroblasts, toll-like receptor, osteogenesis

Introduction

Periodontitis is a chronic inflammatory disease induced by microorganisms (Van Dyke and van Winkelhoff, 2013). Periodontal bacteria such as *Porphyromonas gingivalis* (Mysak *et al*., 2014) stimulate the innate immune response through specific toll-like receptors (TLRs). TLRs expressed on immune cells initiate the primary response, play an important role in several cellular functions that are critical for the host defense, and induce secretion of inflammatory cytokines including IL-6, IL-8, IL-1β, and TNF-α (Van Dyke *and* van Winkelhoff, 2013). For example, the activation of macrophages by *P. gingivalis* induces TNF-α production through TLR2 and TLR4 (Papadopoulos *et al*., 2013). Signaling through TLR2 and TLR4 is differentially regulated by cytokine production at different stages of periodontal disease progression. TLR2 mediates TNF-α activation and IL-10 inhibition in response to *P. gingivalis* infection at a later stage and this process is associated with alveolar bone loss (Lin *et al*., 2014). TLR2 deficiency was further shown to prevent bone loss when TLR2-deficient mice were treated with *P. gingivalis* (Burns *et al*., 2006). While the role of

Correspondence to: Alpdogan Kantarci, Department of Applied Oral Sciences, the Forsyth Institute, 245 First Street, Cambridge, MA 02142; Tel.: +1 617 892 8530; Fax number: +1 617 892 4711; E-mail address: akantarci@forsyth.org

TLR signaling in the innate immune response during periodontal disease initiation and progression is well established, it is not clear how TLRs expressed on other cells of the host regulate cell function. Studies have suggested that TLR3 and TLR4 impact the differentiation of bone marrow stem cells (BMSCs) to osteoblasts and it was demonstrated that other cells of the host that are responsible for structural integrity and repair also sense *P. gingivalis* and its components through different TLRs (Qi *et al.*, 2014).

The periodontal ligament (PDL) is a connective tissue that links teeth and alveolar bone and supports the masticatory system (Beertsen *et al.*, 1997). While their major function is to regenerate the damaged periodontal attachment, PDL fibroblasts can respond to bacterial stimuli during disease progression (Benatti *et al.*, 2007). PDL contains multipotent stem cells that can differentiate into osteoblasts, chondrocytes, and adipocytes (Kim *et al.*, 2012). Cells from human inflamed PDL tissues express mesenchymal stem cell markers and exhibit the capacity to form a mineralized matrix *in vitro* and *in vivo* (Liao *et al.*, 2011; Park *et al.*, 2011). Periodontal ligament fibroblasts express higher level of osteopontin, osteocalcin, BMP-2, and BMP-4 compared to gingival fibroblasts (Ivanovski *et al.*, 2001).

Human PDL fibroblasts constitutively express TLR1, TLR2, TLR4 and TLR7 (Scheres *et al.*, 2011). PDL fibroblasts from people with periodontitis express higher TLR1, TLR4, and TLR7 than those from healthy control subjects and also express higher TLR2 than gingival, skin, and lung fibroblasts (Scheres *et al.*, 2011). Anti-TLR4 and anti-TLR-2 treatment of PDL fibroblasts blocks IL-8 production suggesting that PDL fibroblast-induced recruitment of phagocytes during periodontal inflammation may be actively regulated by the TLR signaling (Hatakeyama *et al.*, 2003). It is not known, however, how TLR-induced PDL fibroblast function is associated with new bone formation independent of the activation of TLR by the bacterial sources. Therefore, the aim of this study was to test the hypothesis that TLR2 and TLR4 activation will mediate the osteogenic capacity of the PDL fibroblasts.

Materials and Methods

Cell Culture

Human periodontal ligament (PDL) fibroblasts were purchased from Lonza (Walkersville, MD, USA). Cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Corporation, Grand Island, NY, USA). The medium was supplemented with 10% fetal bovine serum (FBS; Hyclone UK Ltd., Cramlington, Northumberland, UK), and antibiotic/antimycotic solution containing 100 U/ml penicillin G, 100 μg/ml Streptomycin, 0.025 μg/ml Amphotericin B (Life Technologies Corporation, Grand Island, NY, USA). PDL fibroblasts were maintained in a humidified atmosphere at 5% CO_2 at 37 ˚C. Cells were subcultured after reaching confluence with medium changed every 2-3 days. PDL fibroblasts from passages 5 to 9 were used.

To induce osteogenic differentiation, PDL fibroblasts were further cultured in osteogenic medium, which contained 50 μg/ml of ascorbic acid, 10 mM of β-glycerophosphate and 100 nM Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). Pam3CSK4 (1 μM; InvivoGen, San Diego, CA, USA) was used as a TLR2 ligand. To activate TLR4, monophosphoryl Lipid A (MPLA, 1 μM, InvivoGen, San Diego, CA, USA) was used. Osteogenic medium alone was used in the control group. Each experiment was performed in triplicate.

PDL fibroblast proliferation in response to TLR2 and TLR4 agonist

To test the proliferation and cell viability of PDL fibroblasts, the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay, which is a quantitative colorimetric used to estimate cell viability and cell proliferation, was performed. The assay is based on the metabolization of MTT (a yellow tetrazole) by mitochondrial succinate dehydrogenase in living cells to a purple formazan product. PDL fibroblasts were seeded in 96-well plates (Costar®, Corning Incorporated, Corning, NY, USA). Cell density was adjusted at 4×10^3 cells/well. After incubation for 24 hours, the medium was changed to osteogenic medium (see above). On days 1, 3, 5 and 7, cells were incubated with 100 μl of 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours. The formazan product was dissolved in 50 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 37 ˚C for 10 minutes. The absorbance was measured at 540 nm.

To further assess cell proliferation, the BrdU incorporation assay was performed. PDL fibroblasts were seeded in 96-well plates (Costar®, Corning Incorporated, Corning, NY, USA) at a density of 4×10^3 cells/well at 5% CO_2 at 37 °C. Cells were treated as above. DNA replication was detected using the BrdU Cell Proliferation Assay (Abcam®, Cambridge, MA, USA) on days 1, 3, 5 and 7. One day before measurement of cell proliferation, the BrdU reagent was added to each well for 24 hours. On days 1, 3, 5 and 7, the BrdU reagent was removed and 100 μl of fixing solution was added. Cells were incubated at room temperature for 30 minutes for permeabilization and washed 3 times with a wash buffer. 100 μl of pre-diluted BrdU detection antibody was incubated in each well at room temperature for 1 hour. 100 μl goat anti-mouse IgG was added and incubated at room temperature for additional 30 minutes. The cells were further washed 3 times. One hundred μl of the

substrate was added; cells were further incubated in the dark for 30 minutes at room temperature and 100 µl stop solution was added. Absorbance was read at 450/550 nm on a Beckman visible light spectrophotometer.

Alkaline phosphatase activity in response to TLR2 and TLR4 activation

To measure the osteogenic activity of PDL fibroblasts, cells were cultured in an osteogenic medium and differentiated into an osteoblastic lineage for 7, 14, and 21 days. The osteogenic medium contained DMEM with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone. Cells were then washed twice with 1× PBS and lysed with 200 µl of lysis buffer containing proteinase inhibitor in CelLytic M (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature. Lysate was removed from each well by scraping and transferred into individual microtubes. The lysed cells were centrifuged at 13,000 × g at 4 °C for 3 minutes. The supernatant was collected and stored at -80 °C until subsequent use. Alkaline phosphatase (ALP) concentration was measured using a colorimetric assay (Abcam Inc., Cambridge, MA, USA). For standard wells, 1 mM of the p-nitrophenol phosphate (pNPP) and assay buffer was added. For sample wells, 40 µl of cell lysate was added followed by 40 µl of assay buffer. Ten µl of ALP was added into every standard well; 50 µl of 5 mM pNPP was added into the sample wells as a substrate for the enzyme from cells. The reaction was stopped by addition of 20 µl of stop solution. Absorbance was measured at 405 nm.

Protein concentration was determined using Novagen BCA Protein Assay Kit (Novagen®, USA). In brief, 25 µl of each BSA standard or protein sample was pipetted into each well of a 96-well plate. 200 µl BCA working reagent (50 parts BCA solution and 1 part 4% Cupric Sulfate) was added to each well. The reaction was incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm on a plate reader. The ALP activity was calculated as nM of p-nitrophenol/mg of protein/minute as described (Sabokbar, *et al*., 1994).

Nodule Formation by PDL fibroblasts after osteogenic induction

To detect the calcified nodule formation by PDL fibroblasts, cells were incubated with osteogenic medium for 28 days. All groups of induced cells were washed twice with 1× PBS. After fixing the cells with cold absolute methanol at room temperature for 10 minutes, calcified nodules were stained with 1% Alizarin Red S (pH 4.1-4.3) (Sigma-Aldrich, St. Louis, MO, USA). After 30 minutes, the excess dye was removed and plates rinsed with distilled deionized water. Nodules were observed under the light microscope (Virtanen and Isotupa, 1980).

Quantitative polymerase chain reaction (qPCR) for osteogenic gene expression

To evaluate the expression of genes, the PDL fibroblasts were induced with osteogenic medium for 3 days. Cells were washed with 1× PBS twice and kept in TRIzol® (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). qPCR was performed using TaqMan® Fast master mix (Thermo Fisher Scientific, Carlsbad, CA, USA). The expression of target genes was analyzed using *RUNX2, OCN, BSP* and *Osterix* assay probes Hs01047973_m1, Hs01587814_g1, Hs00195432_m1 and Hs01866874_s1 (Thermo Fisher Scientific, Carlsbad, CA, USA). Comparative cycle threshold (C_T) was further analyzed for gene expression. *β-actin* expression was used as an endogenous control.

Data analysis

The data were analyzed for the differences among groups using one-way ANOVA with Bonferroni's correction for multiple comparisons. Statistical significance was determined at $p<0.05$.

Results

PDL fibroblast proliferation in response to TLR2 and TLR4 activation

When PDL fibroblasts were cultured in osteogenic medium, they grew at the same rate in response to both Pam3CSK5 and MPLA with no loss of viability demonstrating that TLR2 or TLR4 activation does not interfere with cell viability over the assay period (Figure 1A). BrdU results (Figure 1B) showed that the proliferation capacity of PDL fibroblasts in all groups declined by day 5. On day 7, the TLR2 agonist (Pam3CSK4) sustained a higher rate of DNA replication of PDL fibroblasts (2-fold) compared to the control ($p<0.05$), while the TLR4 agonist (MPLA) induced a significantly lower DNA replication rate (one-third) compared to the control ($p<0.05$) suggesting a differential proliferative response to different TLR agonist activation by the PDL fibroblasts.

Osteogenic capacity of PDL fibroblasts in response to TLR2 and TLR4 activation

Next, we measured whether TLR2 and TLR4 activation was associated with the osteogenic capacity of the PDL fibroblasts. When the cells were cultured in osteogenic medium for 14 days, the TLR2 agonist significantly induced ALP activity by approximately 0.6 nM pNP/mg of protein/min when compared to the control and the TLR4 agonist (Figure 2A). In parallel, PDL fibroblasts cultured with TLR2 agonist produced

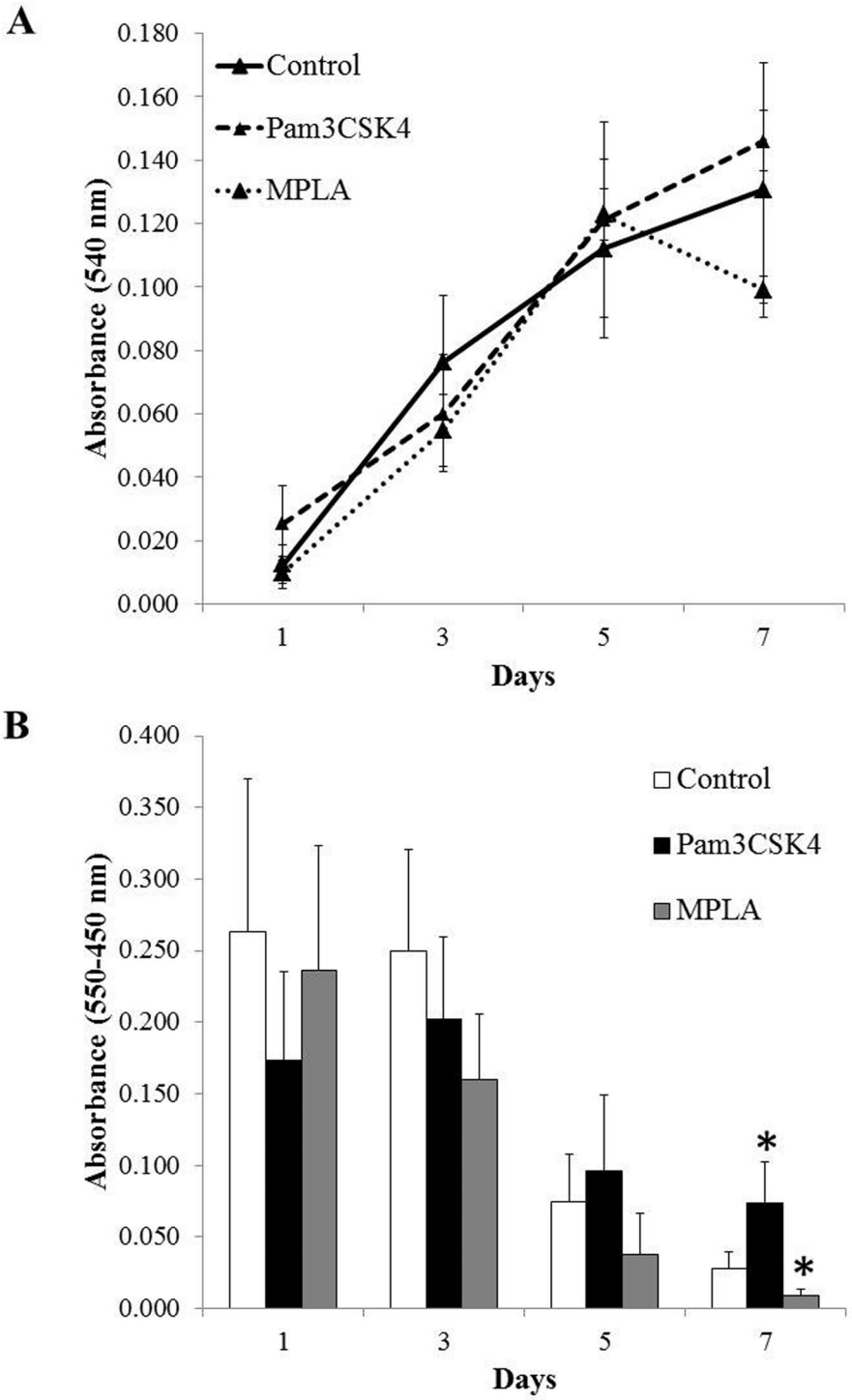

Figure 1. (A) MTT assay shows the relative number of PDL fibroblasts under various conditions and (B) BrdU assay shows the relative DNA replication of PDL fibroblasts when cultured in osteogenic medium alone, plus TLR2 agonist (Pam3CSK4) or plus TLR4 agonist (MPLA). *Statistically significant difference ($p<0.05$) when compared to control on the same day.

larger calcified nodules than those in the control and TLR4 activation groups (Figure 2B). While there was no statistical significance among groups, TLR4 activation tended to induce a higher number of calcified nodules than TLR2 activation without affecting the size of the nodules (Figure 2C).

Osteogenic gene expression by the PDL fibroblasts in response to TLR2 and TLR4 activation

We focused on the expression of the osteogenic genes in the PDL fibroblasts when the cells were treated with TLR2 and TLR4 agonists. Activation of TLR4 significantly reduced the expression of *RUNX2* and *Osterix*

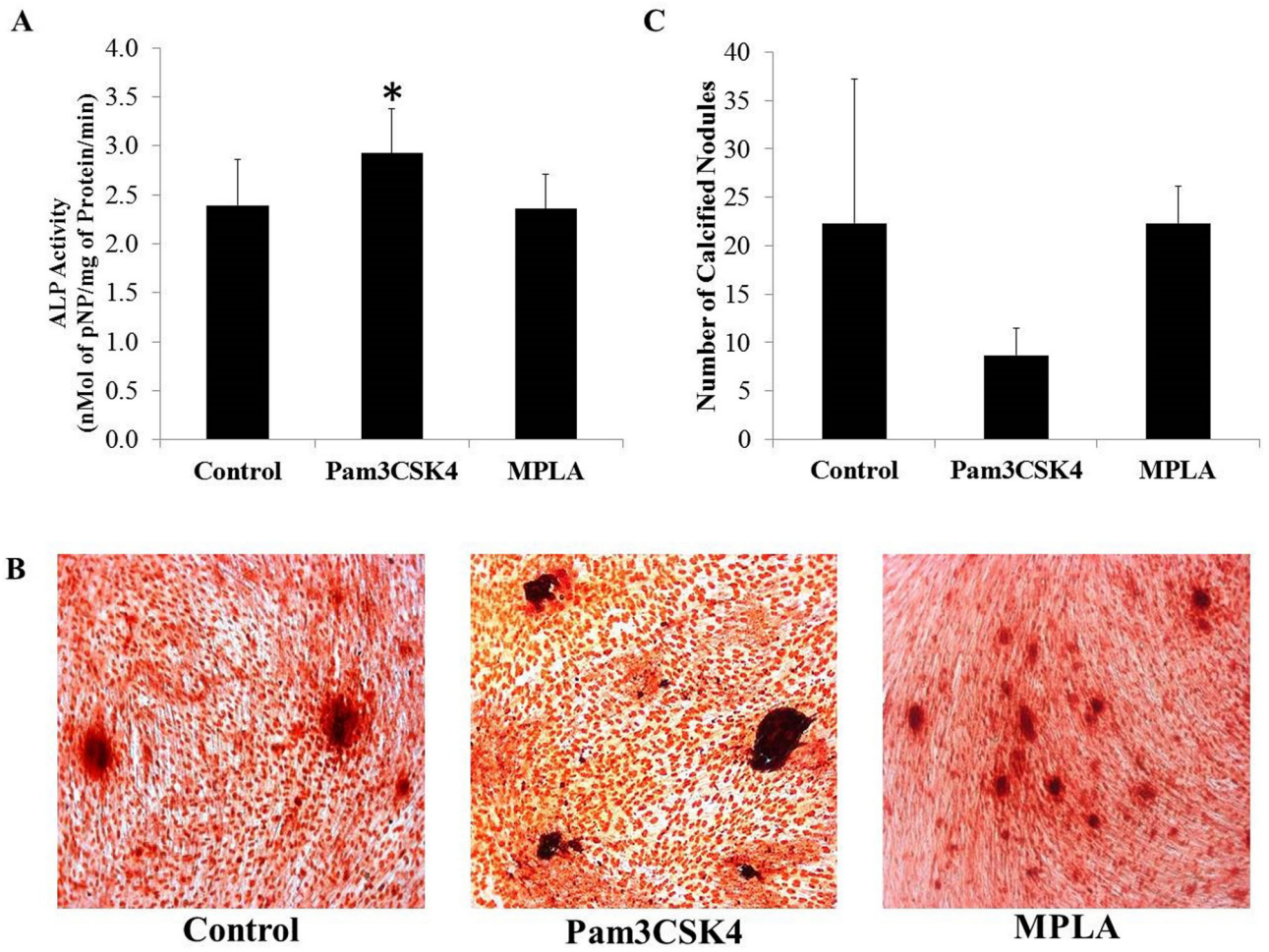

Figure 2. (A) Alkaline phosphatase activity of PDL fibroblasts after culture in osteogenic medium alone, plus TLR2 agonist (Pam3CSK4) or plus TLR4 agonist (MPLA) for 14 days. (B) Calcified nodules and (C) the number of calcified nodules after PDL fibroblasts were cultured in osteogenic medium alone, plus TLR2 agonist (Pam3CSK4) or plus TLR4 agonist (MPLA) for 28 days. *Statistically significant difference ($p<0.05$) when compared to control.

(Figure 3). In contrast, the activation of TLR2 did not affect the levels of *RUNX2* and *Osterix* expression. TLR4 activation significantly induced *OCN* expression ($p<0.05$), while TLR2 activation did not have an impact. *BSP* expression was not affected by either TLR2 or TLR4 activation (Figure 4).

Discussion

Toll-like receptors are expressed on various cells of the mammals and are involved in a wide array of functions during infection (Akira *et al*., 2001). A previous study demonstrated the expression of TLR2 and TLR4 on human PDL fibroblasts and showed that the TLRs were induced by *P. gingivalis* and its LPS leading to a stimulation of cytokine production (Sun *et al*., 2010). While TLR expression has been shown on non-immune cells of the periodontium such as the PDL fibroblasts, the impact of the TLRs on PDL function is not clear. Our work is the first study to evaluate a direct activation of TLR2 and TLR4 activation on the PDL fibroblast proliferation and osteogenic capacity. The data demonstrate that DNA replication and ALP activity of PDL fibroblasts was increased by TLR2 agonist together with induction of larger mineralized nodules. The results may also suggest that the TLR2-agonist-induced cell proliferation reach a particular density at which these cells are ready to differentiate but cannot proliferate further. Conversely, the expression of *RUNX2* and *Osterix* was decreased by the TLR4 agonist while the TLR4 agonist significantly upregulated the expression of *OCN*.

RUNX2 is expressed on osteoblast lineage cells and hypertrophic chondrocytes, odontoblasts, and ameloblasts (Liu *and* Lee, 2013). During bone formation, RUNX2 is an important transcription factor for osteoblast differentiation, matrix production, and mineralization (Liu *and* Lee, 2013). It induces the differentiation of mesenchymal cells into osteoblast-like cells which direct the formation of osteoid material leading to bone formation (Komori, 2010). Osterix, the downstream gene of RUNX2, is an early marker when mesenchymal cells enter the osteoblast lineage, and its signal becomes stronger as osteoblast differentiation occurs (Tang *et al*., 2011). It has been shown that the TLR4 expression

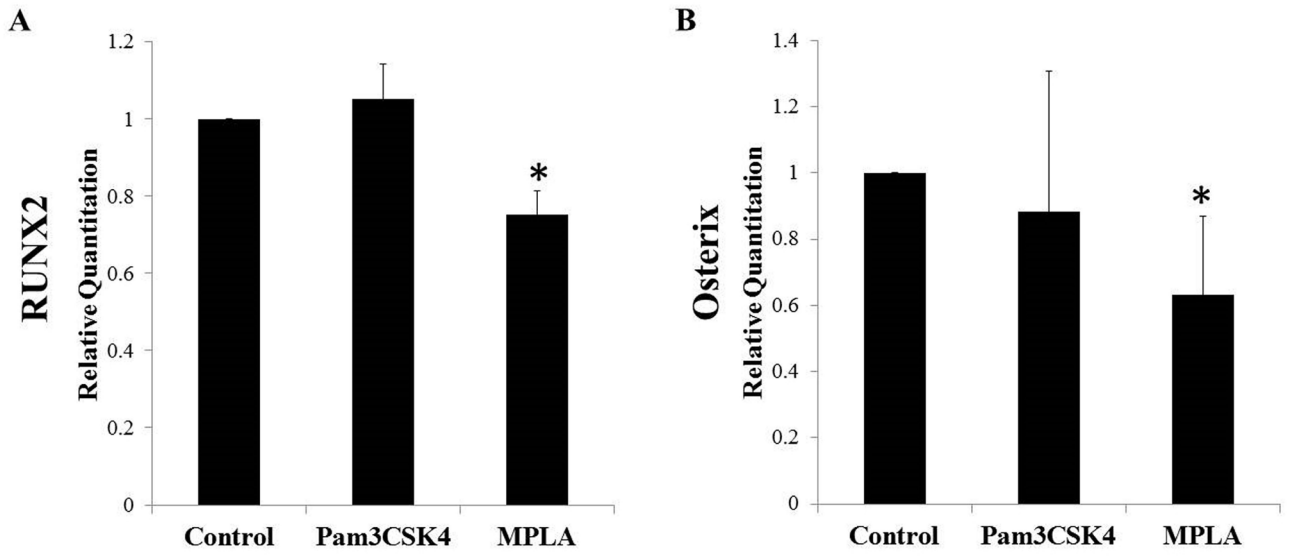

Figure 3. mRNA expression of RUNX2 (A) and Osterix (B) by qRT-PCR in PDL fibroblasts after they were cultured in osteogenic medium alone, plus TLR2 agonist (Pam3CSK4) or plus TLR4 agonist (MPLA) for 3 days. * Statistically significant difference (p<0.05) when compared to control.

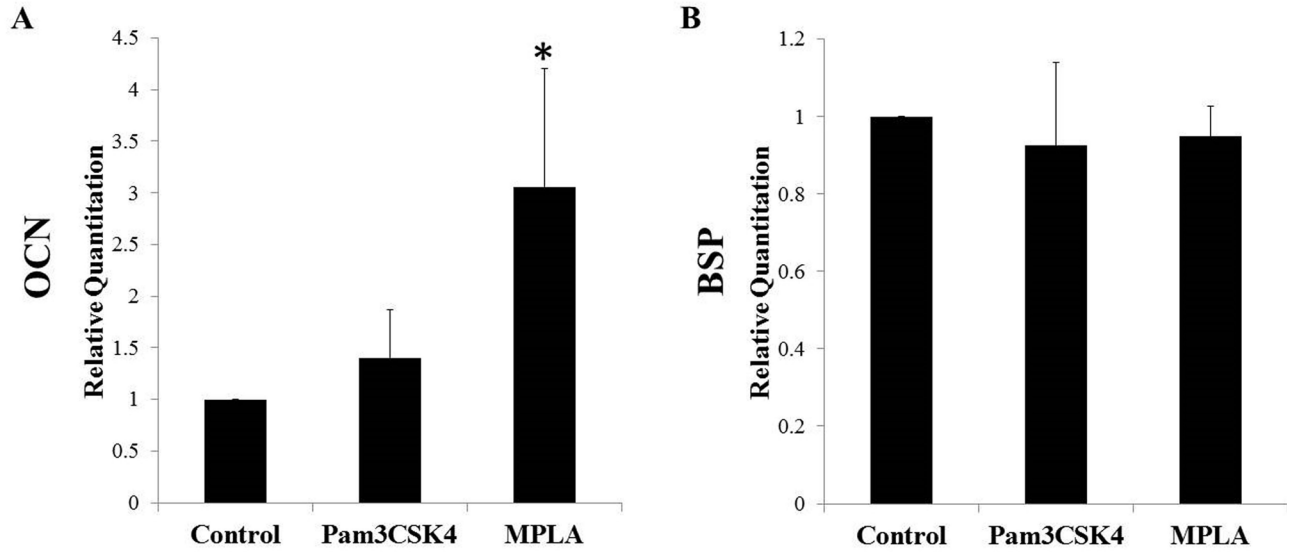

Figure 4. mRNA expression of OCN (A) and BSP (B) by qRT-PCR in PDL fibroblasts after they were cultured in osteogenic medium alone, plus TLR2 agonist (Pam3CSK4) or plus TLR4 agonist (MPLA) for 3 days. * Statistically significant difference (p<0.05) when compared to control.

on MC3T3-E1 cells promoted by LPS suppressed the expression of ALP, OCN, and RUNX2 mRNA resulting in an inhibition of the osteoblast differentiation (Liu *et al.*, 2016). The suppression of RUNX2 and osterix expression was demonstrated in osteoblasts by LPS (Bandow *et al.*, 2010), which corresponded to our result.

In contrast to the RUNX2, OCN is expressed in mature osteoblasts. In the diaphysis at 1 week of age and the metaphysis at 4 weeks of age, osteoblasts, which expressed RUNX2 and osteopontin strongly, weakly expressed OCN. In the diaphysis at 4 weeks of age, RUNX2 and osteopontin were barely detectable in osteoblasts, but osteocalcin was strongly expressed in the osteoblasts (Maruyama *et al.*, 2007). In addition, the study of Ducy *et al.* (1996) found the increase in bone formation without impairing bone resorption in osteocalcin-deficient mice suggesting OCN as a negative regulator of bone formation. These two studies, while performed on osteoblasts, support our results with TLR4 activation in that the upregulation of OCN with the reduction of RUNX2 affects the osteogenic differentiation of PDL fibroblasts. These data collectively suggest that PDL fibroblasts are actively involved in osteogenesis through TLR-mediated signaling.

When the actions of TLR2 and TLR4 agonists on PDL fibroblasts were compared, the TLR2 agonist showed a positive regulation of PDL fibroblast proliferation and osteogenesis, but the TLR4 agonist exhibited

negative regulation. TLR4 may impact the upstream level of RUNX2 at an early stage of osteogenesis, which leads to less differentiating preosteoblasts/osteoblasts. In the case of TLR4, *RUNX2* expression was reduced while *OCN* expression was increased without any impact on ALP. TLR4 activation did not block nodule formation. The data also suggested that TLR2 may impact at a later stage after the cells differentiate into osteoblasts possibly increasing their proliferation and ALP activity. Hence, bigger calcified nodules were seen in TLR2 activation. This difference in TLR 2 and TLR4 activation could be due to differential regulation of the PDL fibroblast function by the immune system of the host during microbial infection. When TLR4 was activated over TLR2, inflammation from pathogenic bacteria induced more damage of periodontal tissue and less osteogenesis (Chen *et al*., 2013). Our findings suggest a pathological involvement of the TLR-specific activation where a smaller size of calcified nodules was presented in response to TLR4 activation in line with these observations. Indeed, a more recent study in mice showed that the *P. gingivalis*-induced periodontal bone resorption is dependent on TLR4 (Lin *et al*., 2017) suggesting a differential role for TLR2 and TLR4 on periodontal ligament cells in periodontal diseases. This function is not limited to the PDL; epithelial cells from periodontitis patients express significantly higher TLR4 in health whereas those from health and periodontitis express equivalent level of TLR2 (AlQallaf *et al*., 2018) suggesting a more ubiquitous role for the TLRs. Collectively, our study was the first work suggesting a possible role of TLR2 and TLR4 expression on PDL fibroblasts and differential involvement in bone formation-a vital process in healing of periodontal defects. Further analyses are needed to validate the signaling pathways and post-receptor activation to elucidate their function in periodontal regeneration and in treatment of periodontitis.

Conclusion

The data collectively demonstrate that activation of TLR2 and TLR4 modulated osteogenesis by human periodontal ligament fibroblast differently at different stages of function.

Acknowledgement:

This work was supported by NIH/NIDCR grants DE020906, DE025020 and DE15566 and Mahidol Medical Scholars Program.

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