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ORIGINAL ARTICLE

TLR activation inhibits the osteogenic potential of human periodontal ligament stem cells through Akt signaling in a Myd88- or TRIF-dependent manner

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Abstract

Background: This study investigated the effects of Toll-like receptors (TLRs) on human periodontal ligament stem cells (hPDLSCs) osteogenic differentiation and the associated mechanisms.

Methods: TLR1, TLR3, TLR4, and TLR6 expression in hPDLSCs was evaluated by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and flow cytometry, whereas their functional roles were assessed based on nuclear factor (NF)-κB activation and proinflammatory cytokine expression. The osteogenic effects of these TLRs were analyzed by alkaline phosphatase (ALP) staining, ALP activity, and alizarin red staining. The roles of Myd88, TRIF, and downstream molecules mitogen-activated protein kinases (MAPKs) and protein kinase B (Akt) in TLR-mediated impaired osteogenic differentiation were examined by real-time RT-PCR and western blotting using specific small interfering RNA siRNA and pharmacologic inhibitors. The involvement of Akt activation in restoring TLR1-, 4-, and 6-mediated osteogenic suppression was verified using the Akt activator SC-79.

Results: TLR1, TLR3, TLR4, and TLR6 were highly expressed functionally in hPDLSCs and high doses of TLR ligands inhibited osteogenic potential. Furthermore, blocking Myd88 partly rescued the decrease in osteogenesis mediated by TLR1, TLR4, and TLR6 activation by enhancing Akt phosphorylation; likewise, TRIF suppression partially rescued lipopolysaccharide (LPS)-mediated osteogenic inhibition through ERK and Akt activation. Moreover, Akt activation restored the TLR-mediated inhibition of hPDLSC osteogenic differentiation.

Conclusions: High doses of TLR1, TLR4, and TLR6 ligands suppress hPDLSC osteogenic differentiation by inhibiting Akt activation through Myd88- or TRIF-dependent signaling pathways. Blocking these adaptors or reactivating Akt could restore the TLR-mediated decrease in hPDLSC osteogenesis, and might be an ideal strategy for periodontitis treatment.

KEYWORDS

adaptor proteins, osteogenesis, periodontal ligament, proto-oncogene protein c-Akt, stem cells, Toll-like receptors

1 | INTRODUCTION

Periodontal disease, which is characterized by the destruction of alveolar bone and periodontal ligament by oral bacterial infection, is the major cause of tooth loss.[1] Periodontal ligament stem cells (PDLSCs) are derived from periodontal tissue and have been shown to harbor osteogenic potential and good regeneration capacity.[2–4] However, the bone regeneration capacity of PDLSCs might be affected by the immune microenvironment caused by elevated oral bacterial burden during periodontal disease.[5–7]

Toll-like receptors (TLRs) are pattern recognition receptors that recognize a variety of bacteria and nucleic acids from pathogens, in addition to sensing infection signals.[8] To date, 13 TLR analogues have been identified that have intracellular regions containing terminal inverted repeat (TIR) homology domains; this domain interacts with some adaptor molecules (with the key adaptor molecules being Myd88 and TRIF), which activate a cascade of events that results in the induction of different transcription factors.[8,9] Common signaling feature among all TLRs is the activation of the transcription factor nuclear factor (NF)-κB, which was shown to control the expression of inflammatory cytokines and cell maturation molecules.[8,9] Therefore, activation of NF-κB is regarded as a standard for evaluating the functionality of TLRs. In addition to NF-κB, other central molecules downstream of TLR signaling, such as mitogen-activated protein kinases (MAPKs) and protein kinase B (Akt), also play key roles in regulating cell proliferation and survival.[8] Moreover, some studies have shown that ERK1/2 and PI3K-Akt signaling can regulate the osteogenic differentiation of mesenchymal stem cells (MSCs).[10–15]

Some TLRs are expressed by MSCs and play important roles in regulating the differentiation of MSCs of diverse origins.[10,16–18] However, there have been some inconsistent, and even controversial findings regarding to the roles of TLRs in MSC osteogenic differentiation.[19–22] To date, there have been few studies that have characterized the expression of different TLRs and their effects on the osteogenic potential of hPDLSCs.[6,23,24] Therefore, in this study, we explored the expression profile of TLRs, with emphasis on TLR1, TLR3, TLR4, and TLR6, which showed higher expression in hPDLSCs. We also studied their effects on osteogenesis in these cells and speculated on the probable mechanism associated with this process. We showed here that high doses of TLR1, -4, and -6 ligands impaired the osteogenic differentiation of hPDLSCs through the downregulation of Akt activity, which occurred in a Myd88- or TRIF-dependent manner.

2 | MATERIALS AND METHODS

2.1 | Isolation of human PDLSCs, cell culture, and treatment

Healthy human premolars were extracted and collected from healthy adult patients (aged 20 to 30 years) undergoing orthodontic therapy in Peking University School of Stomatology with oral informed consent from all patients, and the study was approved by the Ethics Committee of Peking University (PKUSSIRB-201630098), which was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Human PDLSCs (hPDLSCs) were isolated and cultured as previously described.[2] In our assays, these cells were used between passage 3 and 5 and cultured with alpha modified Eagle's medium (α-MEM) containing 15% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin, 2 mM glutamine, and 100 mM L-ascorbate-2-phosphate,* and incubated at 37 °C with 5% CO_2.

PDLSCs were treated with or without various doses of different TLR ligands in osteogenic induction medium (α-MEM supplemented with 15% FBS, 100 nM dexamethasone, 5 mM β-glycerophosphate, and 50 μg/mL L-ascorbic acid) as follows: TLR4 ligand lipopolysaccharide† (LPS; 0.1 to 10 μg/mL); TLR3 ligand PolyI:C‡ (0.1 to 10 μg/mL); TLR1/2 ligand Pam3CSK4 (0.01 to 1 μg/mL); TLR2/6 ligand Pam2CGDPKHPKSF, a synthetic lipopeptide (FSL-1)§ (1 to 50 ng/mL). For pharmacological inhibition or activation of Akt, MK- 2206 (5 μM), and SC-79 (10μM)¶ were used to treat the cells. U0126# (10 μM) was used to inhibit ERK activation.

2.2 | Flow cytometric analysis

Flow cytometry was performed as previously described.[21] The primary antibodies against CD90,CD73,CD166,CD34 and CD45, as well as the isotype control mouse immunoglobulin 1 (IgG1), kappa light chain-Phycoerthrin (κ-PE), and mouse IgG1, kappa light chain-fluorescein isothiocyanate (κ-FITC)‖ were used to verify the identity of hPDLSCs. Anti-human TLR1-PE (clone GD2.F4), anti-human TLR3-PE (clone TLR3.7), anti-human TLR4-PE (clone HTA125),**

* HyClone, GE Healthcare Life Sciences, Pittsburgh, PA.

† Sigma–Aldrich, St. Louis, MO.

‡ Sigma–Aldrich.

§ InvivoGen, San Diego, CA.

¶ Apexbio Technology, Houston, TX.

Apexbio Technology.

‖ BD Biosciences, San Jose, CA.

** eBioscience, San Diego, CA.

anti-human TLR2-PE (clone TL2.1), and anti-humanTLR6-PE (clone TLR6.127),* as well as corresponding isotype control mouse IgG2a,κ -PE, and isotype control mouse IgG1, κ -PE† were used to analyze TLR expression.

2.3 | Real-time quantitative reverse transcription polymerase chain reaction

Total cellular RNA was extracted using Trizol reagent‡ according to the manufacturer's instructions. Reverse transcription§ and real-time polymerase chain reaction (PCR)¶ were performed to quantify all gene transcripts with the expression of *GAPDH* serving as an internal control. Primers for *TLR1–10* were synthesized as previously described.[25] Other primers and sequences are listed in Supplementary Table 1; all primers were commercially synthesized.#

2.4 | Transfection of small interfering RNA

Small interfering RNA (siRNA) targeting TRIF (siTRIF), Myd88 (siMyd88), and scrambled control (siNC)‖ were transfected into hPDLSCs using transfection reagent** according to the manufacturer's instructions. HPDLSCs were transfected with siRNA or siNC in complete medium; after 48 hours, cells were treated with or without different TLR ligands in osteogenic medium for 72 hours.

2.5 | In vitro osteogenic assay

HPDLSCs were cultured in osteogenic medium for 7 days with different treatments. The medium was changed every 3 days. On day 7, alkaline phosphatase staining†† and alkaline phosphatase activity assays‡‡ were performed according to the manufacturers' instructions. On days 14 to 21, osteogenic differentiation was assessed by performing alizarin red S staining.§§ For alizarin red quantification, lysis buffer (acetate:glycerin = 5:1 v/v) was added to each well and the absorbance was measured at 405 nm.

2.6 | Protein isolation and Western blotting

Total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail according to the manufacturer's instructions.¶¶ Protein concentration was determined following the manufacturer's instructions.## Western blotting analysis was performed as previously described. Primary antibodies|||| included those targeting TRIF, Myd88, phosphorylated Akt, Akt, phosphorylated P38, P38, phosphorylated ERK, ERK, phosphorylated c-Jun N terminal kinase (JNK), JNK, phosphorylated NF-κB inhibitor alpha (IκB-α), IκB-α, and runt related transcription factor 2 (Runx2) were used in this study.

2.7 | Statistical analysis

All data were statistically analyzed using software. All experiments were repeated at least three times and data are presented as mean ± SD. One-way ANOVA followed by the Turkey test for multigroup comparisons was used to determine the statistical significance. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | TLR1, -3, -4, and -6 are functionally expressed at high levels in hPDLSCs

HPDLSCs were verified by positive staining of MSC markers CD90, CD73, and CD166, as well as by negative staining of hematopoietic markers CD45 and CD34 (see supplementary Figure 1 in online *Journal of Periodontology*). The following experiments were performed with these identified cells. The mRNA expression of *TLR1–10* was examined by real-time RT-PCR and the results are presented in Figure 1. HPDLSCs expressed *TLR1*, *-3*, *-4*, and *-6* at high levels; however, *TLR7*, *-8*, and *-10* were expressed at very low levels. Based on these results, we analyzed the protein expression of TLR1, -3, -4, and -6 by flow cytometry, as shown in Figure 1. The protein expression of TLRs was determined as the percentage of positive cells in the relative population determined by forward scatter and side scatter characteristics (Figure 1B, C). Since TLR1, -2, and -6 commonly cooperate with each other to induce the activation of downstream signaling, TLR2 expression was also tested. Although flow cytometry results showed low levels of constitutive TLR1, TLR2, TLR3, TLR4, and TLR6 protein expression, trends in their protein expression were consistent with mRNA expression.

To evaluate the functional status of TLR1, -3, -4, and -6 in hPDLSCs, as previously mentioned,[8,9] NF-κB activation

* Biolegend, San Diego, CA.

† eBioscience.

‡ Thermo Fisher Scientific, Waltham, MA.

§ Thermo Fisher Scientific.

¶ FastStart Universal SYBR Green Master, Roche Diagnostics, Indianapolis, IN.

Sangon Biotech, Shanghai, China.

‖ Santa Cruz Biotechnology, San Diego, CA.

** Lipofactamine RNAiMAX Transfection Reagent, Thermo Fisher Scientific.

†† CWBIO Biotech, Beijing, China.

‡‡ Wako Chemicals USA, Richmond, VA.

§§ Sigma–Aldrich.

¶¶ Thermo Fisher Scientific.

Pierce BCA Protein Assay Kit, Thermo Fisher Scientific.

|||| Cell Signaling Technology, Boston, MA.

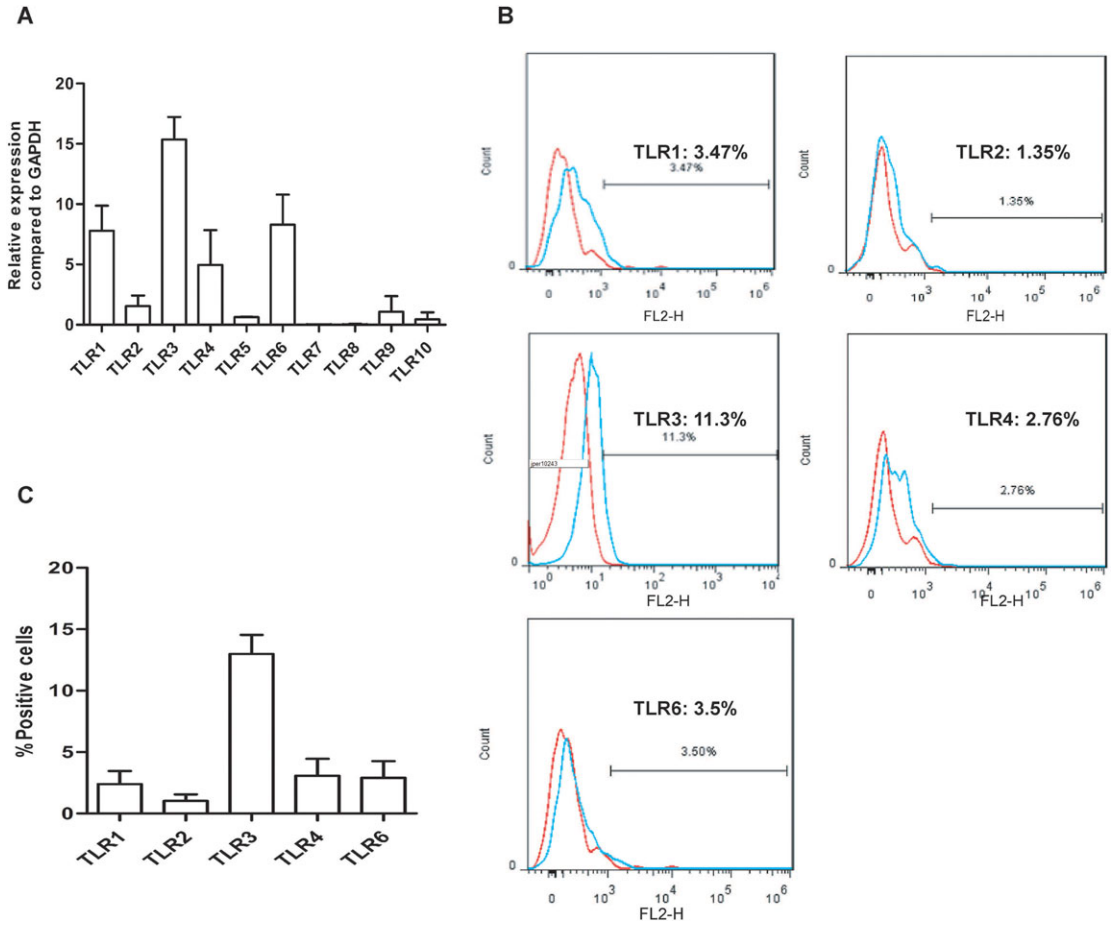

FIGURE 1 Toll-like receptor (TLR)-1, -3, -4, and -6 are highly expressed in human periodontal ligament stem cells (hPDLSCs). **A**, Relative mRNA expression of *TLR1–10* was determined by real-time RT-PCR in hPDLSCs. *GAPDH* served as the internal control. **B**, Protein expression of TLR1, TLR2, TLR3, TLR4, and TLR6 was analyzed by flow cytometry. Isotype antibody controls are shown as red lines; the area under the blue line represents the expression of TLRs. All analyses were performed in triplicate. Shown is the representative data from three independent experiments. **C**, Histogram of positive cell percentages derived from three independent experiments. Data are presented as mean ± SD

and the expression of several proinflammatory cytokines were analyzed in response to specific ligands (see Supplementary Figure 2A, B in online *Journal of Periodontology*). Stimulation with PolyI:C (10 μg/mL); LPS (10 μg/mL), Pam3CSK4 (1 μg/mL), and FSL (50 ng/mL) led to the upregulation of phosphorylated IκBα, which further resulted in potent NF-κB activation and increased IL-6, IL-8, and tumor necrosis factor-α (TNF-α) expression in hPDLSCs. These results proved that TLR1, -3, -4, and -6 are functionally expressed at high levels.

3.2 | High doses of TLR ligands inhibit the osteogenic potential of hPDLSCs

It has been reported that TLRs modulate the osteogenic differentiation of MSCs.[6,19,22,26] To determine the effect of TLRs on the osteogenic capacity of hPDLSCs, various doses of TLR1, TLR3, TLR4, and TLR6 ligands were used to treat hPDLSCs cultured in osteogenic medium for 7 days; after this, cells were subjected to alkaline phosphatase (ALP) determination: ALP staining assays and ALP activity measurements were performed to examine hPDLSC osteogenesis . As shown in Figure 2A and B, Pam3CSK4, and LPS had no significant effect on the ALP staining and ALP activity in hPDLSCs at lower doses (0.01 and 0.1 μg/mL, respectively); FSL-1 (1 ng/mL) had a marginal suppressive effect on ALP activity in hPDLSCs, as compared with activity in untreated control, whereas a lower dose of PolyI:C (0.1 μg/mL) induced the osteogenic ability of hPDLSCs significantly. In contrast, compared with that in the untreated control, we observed that the presence of high doses of TLR ligands, specifically PolyI:C (10 μg/mL), LPS (10 μg/mL), Pam3CSK4 (1 μg/mL), and FSL-1(50 ng/mL) significantly inhibited hPDLSC osteogenesis. These discrepant effects on osteogenic differentiation in hPDLSCs, depending on TLR ligand dose, were further confirmed by alizarin red staining and quantitative calcium measurements, which demonstrated mineralization after 14 days of osteogenic induction (Figure 2C, D). Therefore, these results indicated that high doses of TLR ligands inhibit the osteogenic differentiation of hPDLSCs.

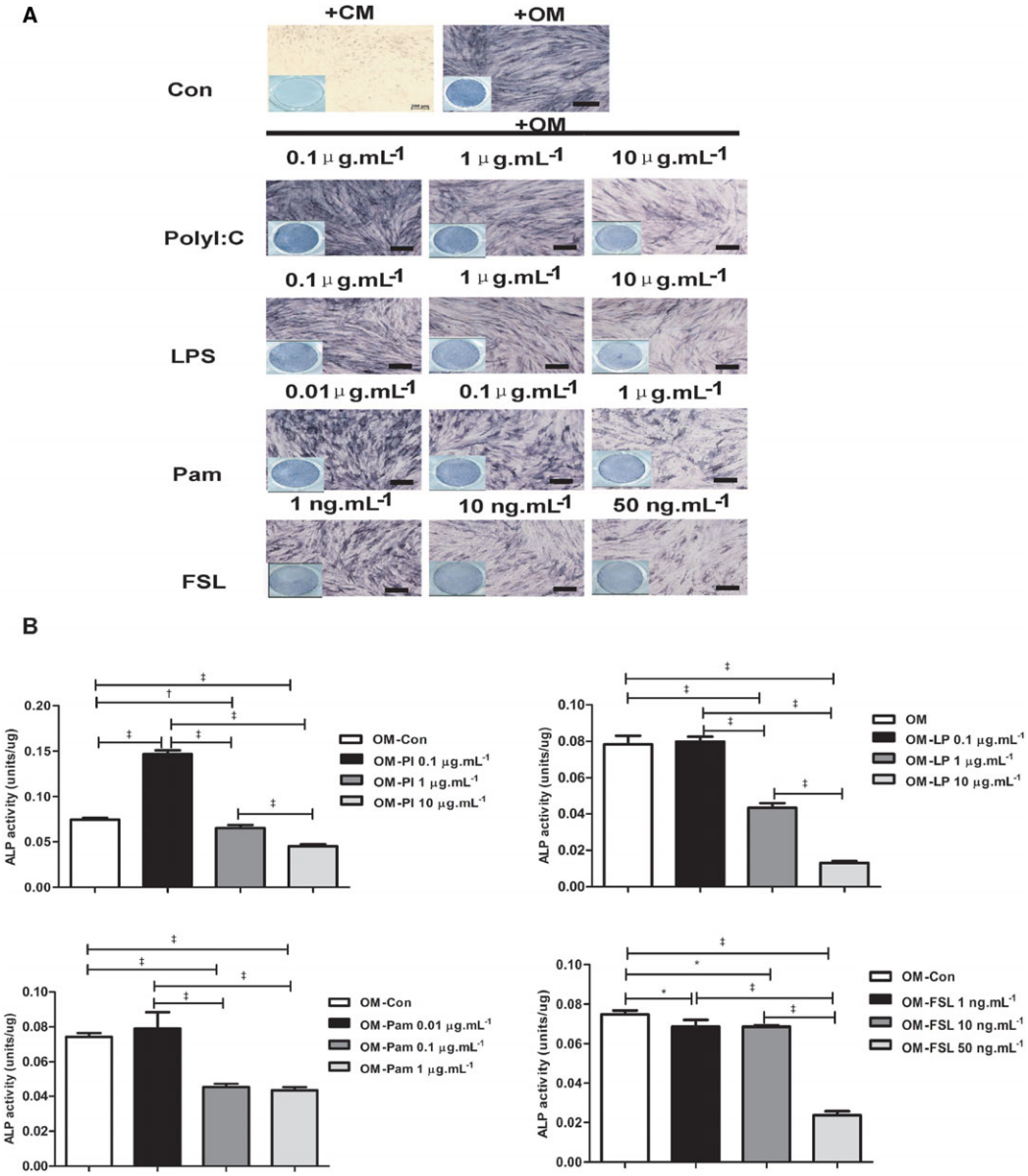

FIGURE 2 High doses of Toll-like receptor (TLR)-1, -3, -4, and -6 ligands suppress the osteogenic potential of human periodontal ligament stem cells (hPDLSCs). HPDLSCs were cultured in osteogenic medium for 7 or 14 days and treated with different doses of TLR ligands. **A**, Osteogenesis was determined by alkaline phosphatase (ALP) staining at day 7; original magnification, ×100; bar = 200 μm; **B**, Measurement of ALP activity was performed by ELISA at 405 nm. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA. **C**, Osteogenesis was also examined by alizarin red staining at day 14 after treatment. Original magnification, ×100; bar = 200 μm. **D**, Alizarin red staining in different groups was extracted and qualified by measuring the optical density (OD) at 405 nm. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA. The results are presented as mean ± SD from three different individual experiments. CM = culture medium; OM = osteogenic medium; Con = control; PI = PolyI:C; LP = LPS; Pam = Pam3CSK4; FSL = FSL-1

3.3 | Knockdown of Myd88 partially rescues high dose TLR-ligand–mediated decreased osteogenic capacity and upregulates Akt activity

Because most TLR signaling pathways are myeloid differentiation factor 88 (Myd88)-dependent, except TLR3,[8,9,27] to ascertain the inhibitory role of TLRs during the osteogenic differentiation of hPDLSCs, we analyzed the osteogenic potential of hPDLSCs after suppression of Myd88 in the presence or absence of high doses of LPS (10 μg/mL), Pam3CSK4 (1 μg/mL), and FSL-1 (50 ng/mL). Real-time RT-PCR data are presented in Figure 3A; results show that *Myd88* expression was effectively suppressed by the specific siRNA. With respect to osteogenic differentiation, the TLR-mediated reduction in *ALP* mRNA expression was significantly reversed by Myd88 suppression. Consistent with this finding, Runx2 expression, which was inhibited by those

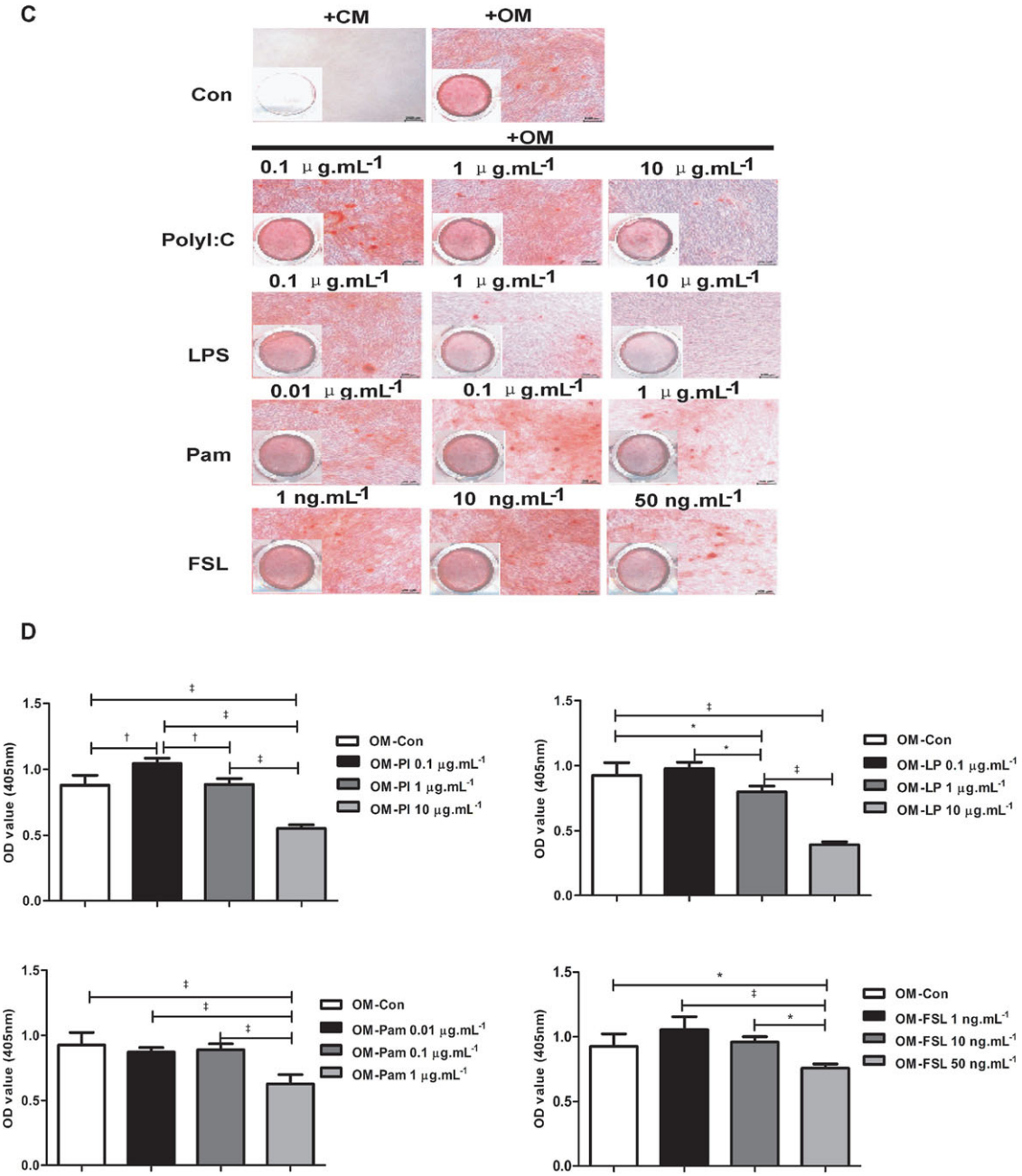

FIGURE 2 Continued

TLR ligands, was also reversed, although the change was not as remarkable as that with Myd88 knockdown.

Based on studies mentioned previously,[10–15] to explore if TLRs could inhibit the osteogenic potential of hPDLSCs by altering the activation of MAPKs and Akt in a Myd88-dependent manner, we analyzed Runx2 expression and the phosphorylation of MAPKs and Akt in Myd88-silenced and negative control cells in the presence or absence of different TLR ligands by western blotting. The data presented in Figure 3B and C showed that Myd88 silencing enhanced Runx2 expression, as compared with that in the negative control; moreover, Myd88 silencing partially rescued the decreased expression of Runx2 driven by TLR ligands. In addition, as shown in Figure 3D, the decline in ALP activity driven by TLR activation was also restored by Myd88 silencing. Therefore, we suspected that TLR-mediated impaired osteogenic differentiation is Myd88 dependent. We also found that the phosphorylation of ERK1/2, P38, and JNK1/2 was downregulated with or without TLR ligand treatment after Myd88 suppression (Figure 3B, C). Intriguingly, Akt phosphorylation was upregulated with Myd88 suppressing. Therefore, to further test whether Myd88-regulated Akt phosphorylation has roles in hPDLSC osteogenic differentiation, cells were treated with an Akt inhibitor (MK-2206) for 3 days after Myd88 suppression. The results presented in Figure 3E and F showed that MK-2206 significantly

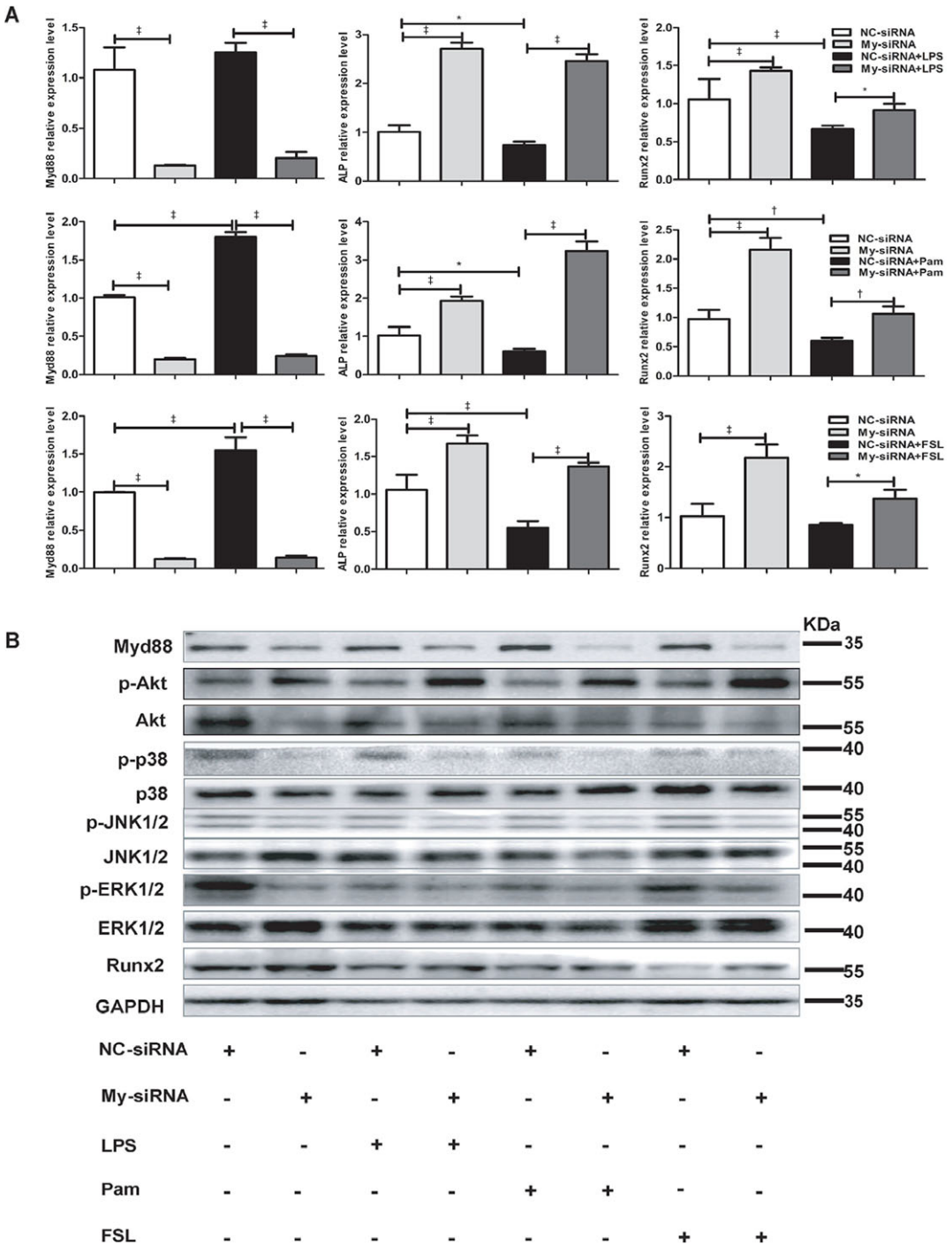

FIGURE 3 Myd88 silencing partially rescues the suppression of osteogenic differentiation caused by TLR activation and regulates Akt phosphorylation. Human periodontal ligament stem cells (hPDLSCs) were transfected with Myd88 siRNA or negative control siRNA; 48 hours later, the cells were cultured in osteogenic medium in the absence or presence of high doses of TLR ligands (LPS: 10 μg/mL; Pam3CSK4: 1 μg/mL; FSL-1: 50 ng/mL) for 3 days. The cells were harvested for real-time RT-PCR and western blotting analysis. **A**, The mRNA expression of *Myd88*, *Runx2*, and alkaline phosphatase (*ALP*) was measured by real-time RT-PCR. **B**, The protein expression of Myd88, Runx2, phospho-ERK1/2, ERK1/2, phospho-P38, P38, phospho-JNK1/2, JNK1/2, phospho-Akt, and Akt was measured by western blotting. GAPDH was used as the internal control. The data are representative of three independent experiments. **C**, Relative intensity of the tested protein was quantitatively analyzed using software. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA. **D**, ALP activity in different treatment groups was analyzed as previously mentioned. $^{\ddagger}P < 0.001$, one-way ANOVA. **E**, Knockdown of Myd88 induced Runx2 expression, which was inhibited by MK-2206 (5 μM). Runx2 expression and Akt phosphorylation were determined by western blotting. The data are representative of three independent experiments. **F**, Relative intensity of the tested protein was quantitatively analyzed using software. $^{\ddagger}P < 0.001$, $^{*}P < 0.05$, one-way ANOVA. All data are presented as mean ± SD from three individual experiments. NC-siRNA = negative control siRNA; My-siRNA = Myd88 siRNA; Pam = Pam3CSK4; MK = MK-2206

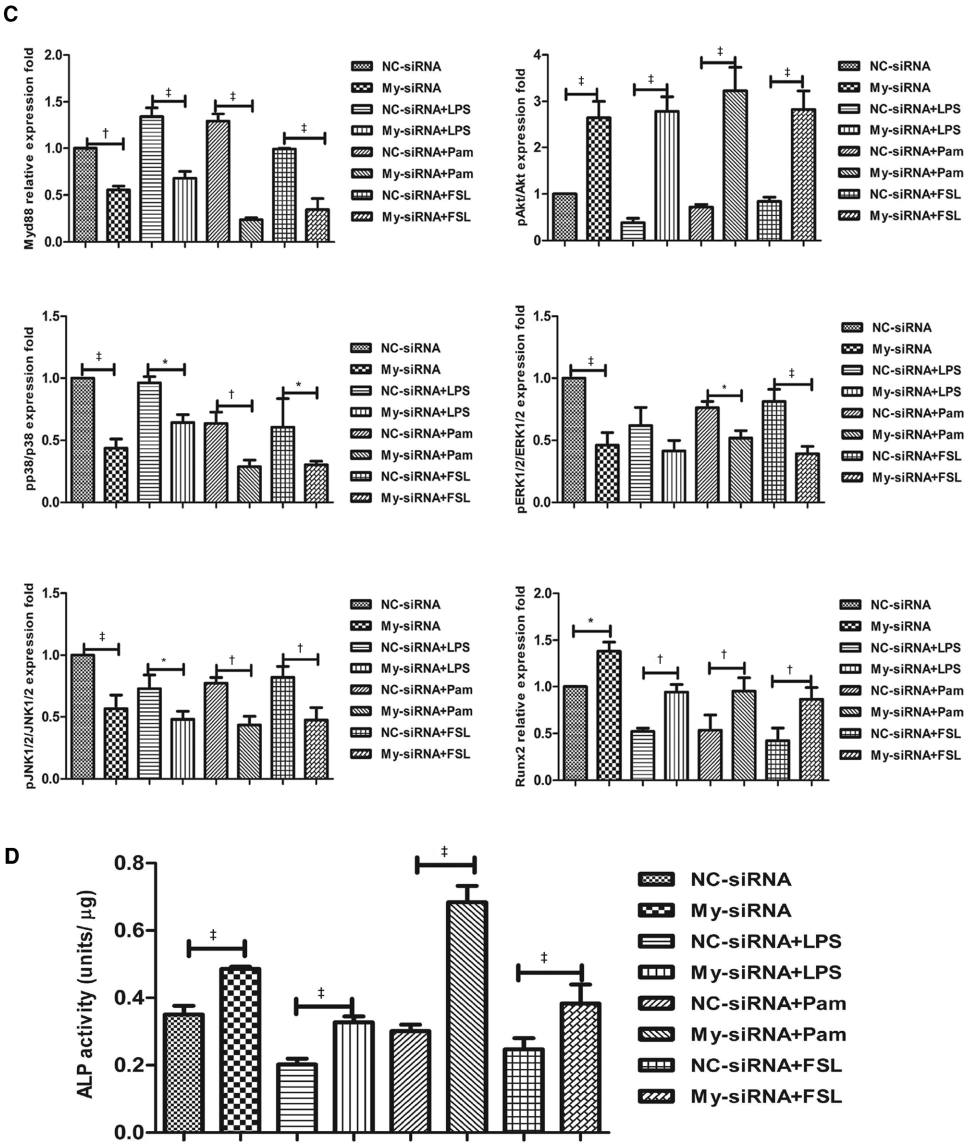

FIGURE 3 Continued

attenuated the induction of Runx2 expression mediated by Myd88 silencing. As previously mentioned, Myd88 is a key adaptor in the TLR1, TLR4, and TLR6 signaling pathway.[9] These results seemed to indicate that the inhibitory effect of TLR activation on hPDLSC osteogenic differentiation occur partially through Myd88-dependent Akt signaling.

3.4 | Knockdown of TRIF partially rescues the suppression of osteogenic capacity mediated by high doses of LPS via ERK and Akt signaling

As described by previous studies, only TLR3 and TLR4 signaling depend on the adaptor molecule TIR-domain containing adapter-inducing interferon-β (TRIF) to transmit active signals.[8,9] To determine if the inhibition of osteogenic potential mediated by TLR3 and TLR4 is TRIF-dependent, we analyzed the osteogenic potential of hPDLSCs after silencing TRIF in the presence or absence of LPS or PolyI:C at high concentrations. Real-time RT-PCR data, presented in Figure 4A, shows that TRIF expression was reduced effectively by siRNA compared with that in the negative control. TRIF silencing rescued the LPS-induced down-regulation of Runx2 and ALP expression significantly, but had no effect on the reduced expression of Runx2 and ALP driven by PolyI:C. Next, to further investigate if ERK1/2 and Akt activation down-stream of TRIF would be affected, we analyzed Runx2 expression and the phosphorylation of ERK1/2 or Akt in TRIF-silenced and negative control cells with or without LPS or PolyI:C treatment by western blotting. As shown in Figure 4B and C, Runx2 expression was significantly induced in TRIF-silenced cells, as compared with that in negative controls; furthermore, the LPS-induced downregulation of Runx2 expression was partially restored by TRIF silencing, but this had no effect on the PolyI:C-mediated

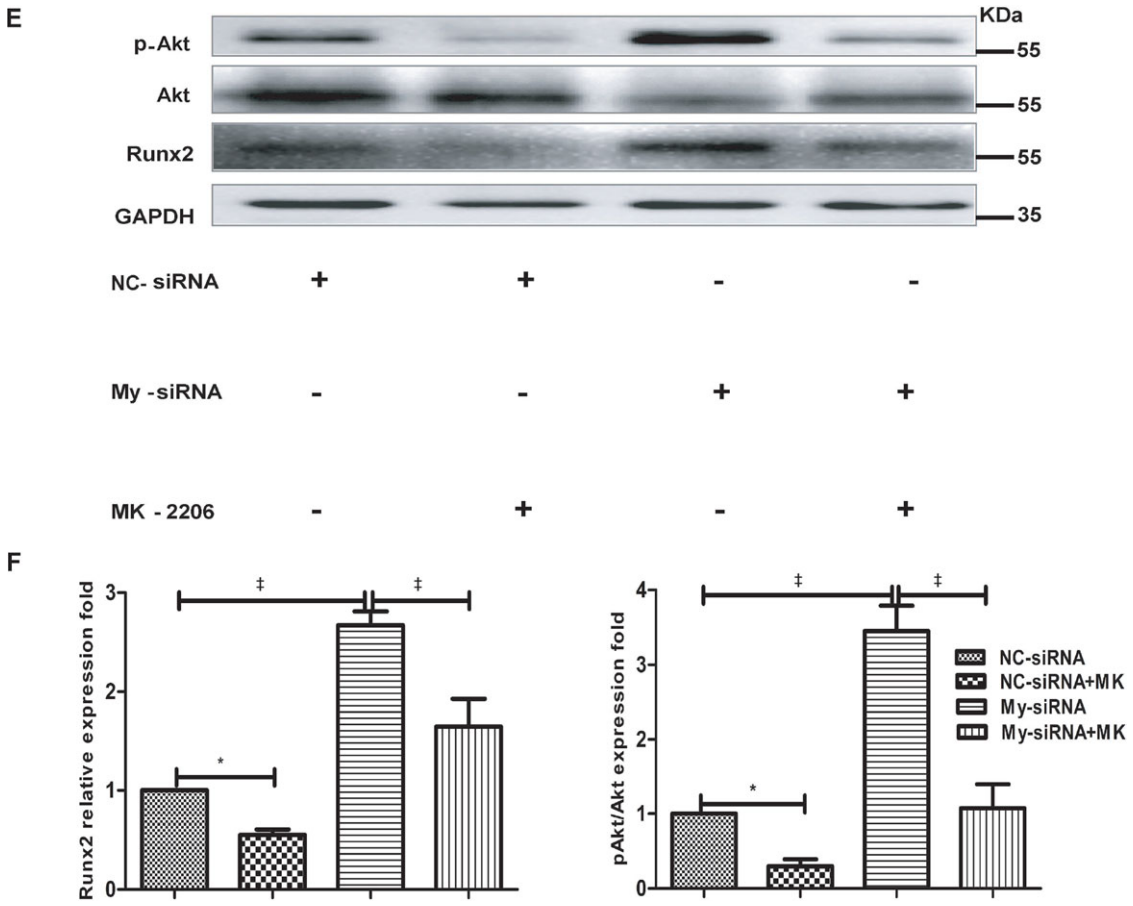

FIGURE 3 Continued

inhibition of Runx2 expression. Similar results were also found with ALP activity (Figure 4D). We also found that TRIF silencing promoted the phosphorylation of ERK1/2 and Akt, which was attenuated by LPS treatment (Figure 4B and C). To further ascertain whether alterations in ERK or Akt phosphorylation contribute to the osteogenic differentiation of hPDLSCs in a TRIF-dependent manner, the TRIF-silenced or negative control cells were treated with an ERK (U0126) or Akt inhibitor (MK-2206) and then subjected to real-time RT-PCR assay or western blotting. The results presented in Figure 4E showed that increased Runx2 and ALP expression driven by TRIF suppression was attenuated by ERK or Akt inhibition. These results demonstrated that the LPS-mediated suppression of osteogenic potential in hPDLSCs occurs at least partially through TRIF-dependent ERK or Akt signaling.

3.5 | Activation of Akt by SC-79 reverses the decrease in hPDLSC osteogenic potential caused by TLR activation

Based on the results in this study, the inhibitory effect of TLR1, -4, and -6 on hPDLSC osteogenesis appeared to be dependent on reduced Akt activity down-stream of Myd88 or TRIF. To further clarify the role of Akt activation in this process, we treated hPDLSCs with SC-79 (Akt activator) with or without LPS (TRIF- or Myd88-dependent), Pam3CSK4, and FSL-1(Myd88-dependent) for 7 days, and then analyzed Runx2 expression and ALP activity. As shown in Figure 5A, we found that co-administration of SC-79 obviously restored the decreased Akt phosphorylation and Runx2 expression mediated by TLR activation in hPDLSCs. Furthermore, as shown in Figure 5B, Akt activation, via SC-79 treatment, reversed the inhibition of ALP activity mediated by TLR activation. These data further proved that the suppression of Akt activity has important roles in the TLR-dependent impaired osteogenic differentiation of hPDLSCs.

4 | DISCUSSION

PDLSCs derived from periodontium have been shown to have the capacity to differentiate into multiple cell types and are considered to be a promising tool for periodontal regeneration and alveolar bone regeneration.[2] Therefore, understanding the mechanisms through which the immune environment regulates the osteogenic ability of PDLSCs is crucial for future therapeutic use. In this study, functional TLR1, -3, -4, and -6

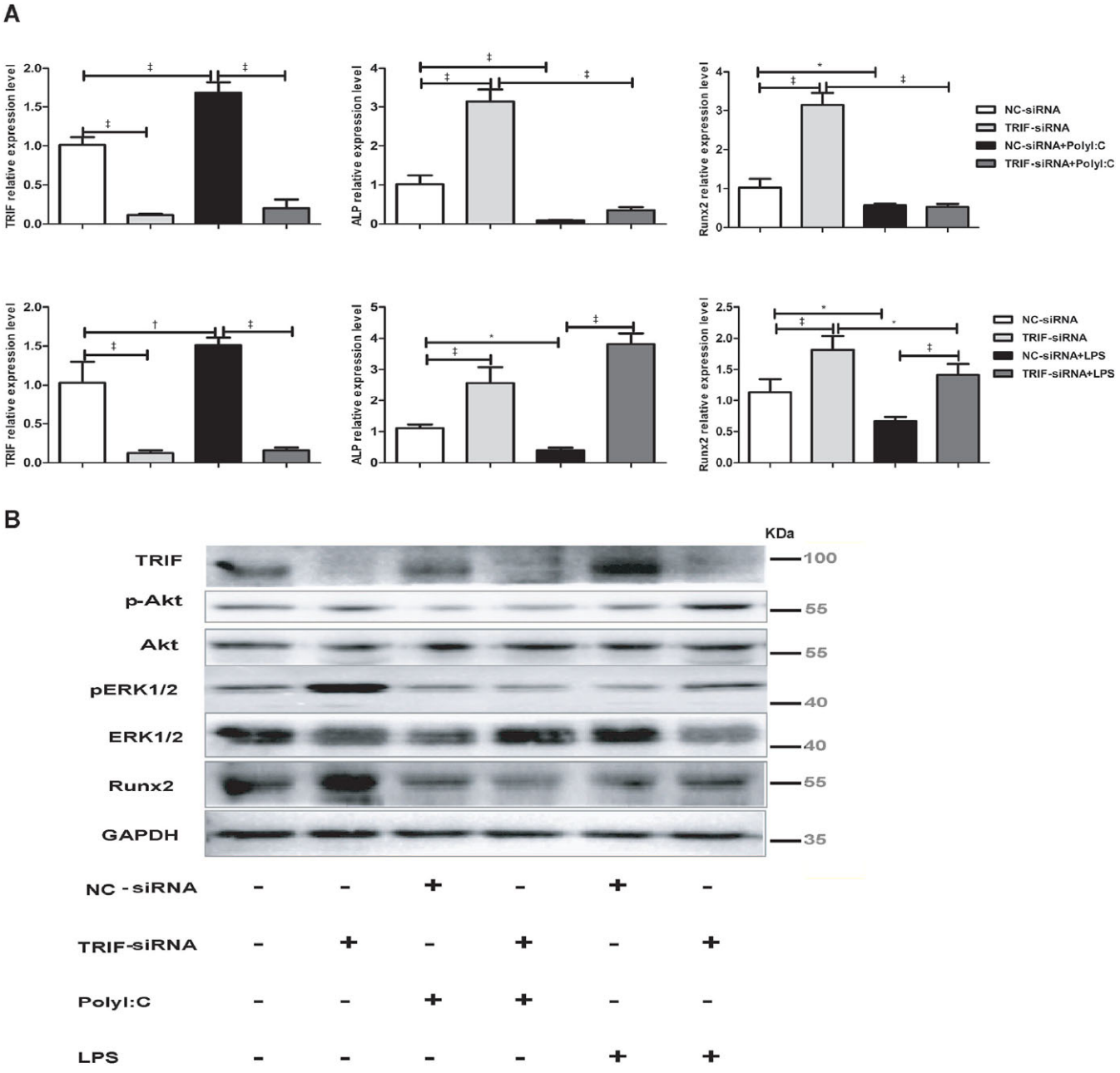

FIGURE 4 TRIF silencing partially rescues the suppression of osteogenic potential caused by high-dose LPS by increasing ERK and Akt activity. Human periodontal ligament stem cell (hPDLSCs) were transfected with TRIF siRNA or negative control siRNA; 48 hours later, cells were cultured in osteogenic medium with or without PolyI:C (10 μg/mL) or LPS (10 μg/mL) treatment for 3 days, and then harvested for real time RT-PCR or western blotting analysis. **A**, The mRNA expression of *TRIF*, *Runx2*, and alkaline phosphatase (*ALP*) was measured by real time RT-PCR. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA. **B**, The protein expression of TRIF, Runx2, phospho-ERK1/2, ERK1/2, phospho-Akt, and Akt were measured by western blotting. GAPDH served as the internal control. The data are representative of three independent experiments. **C**, Relative intensity of the tested proteins was quantitatively analyzed using software. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA. **D**, ALP activity with different treatments was analyzed as previously described. $^{\ddagger}P < 0.001$, $^{\dagger}P < 0.01$, one-way ANOVA. **E**, TRIF silencing induced the expression of Runx2 and ALP, which was inhibited by U0126 (10 μM). The mRNA expression of *Runx2*, *ALP*, and *TRIF* was performed by real time RT-PCR. $^{\ddagger}P < .001$, $^{*}P < 0.05$, one-way ANOVA. **F**, TRIF silencing enhanced Runx2 expression, which was suppressed by MK-2206 (5 μM); Runx2 expression and Akt phosphorylation were determined by western blotting. GAPDH was used as the internal control. The data are representative of three independent experiments. **G**, Relative intensity of the tested proteins was quantitatively analyzed using software. $^{\ddagger}P < 0.001$, one-way ANOVA. All data are presented as mean ± SD from three independent experiments. NC-siRNA = negative control siRNA; MK = MK-2206

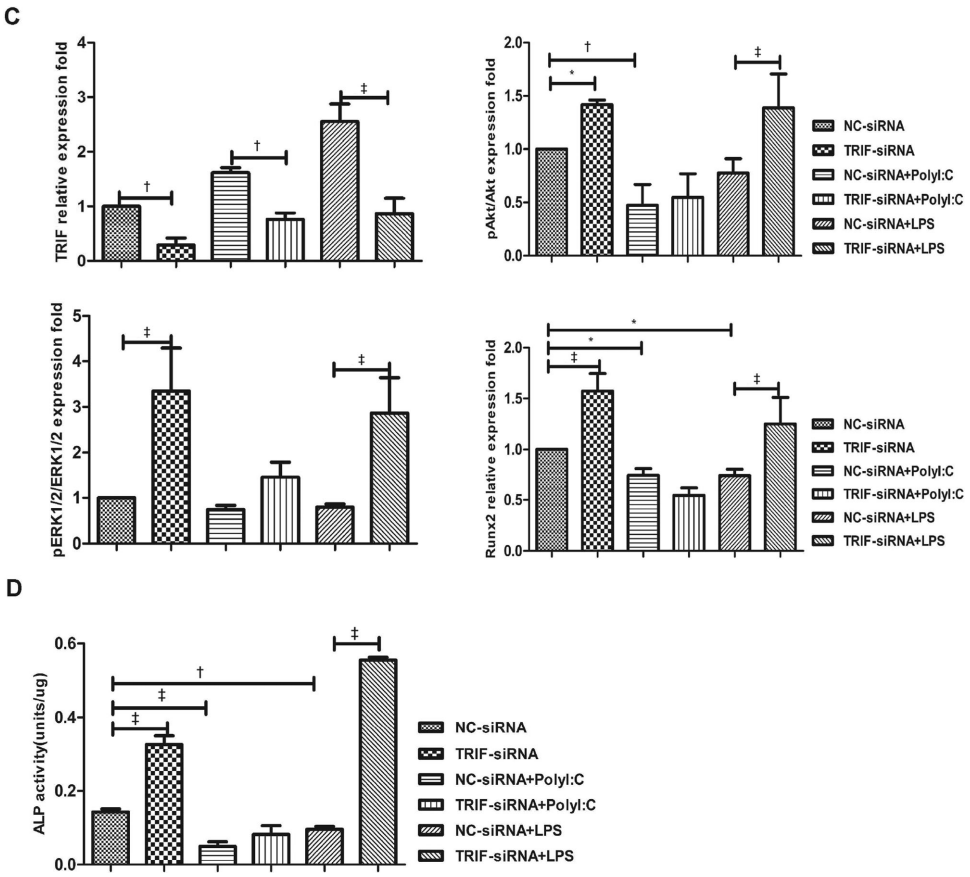

FIGURE 4 Continued

were found to be highly expressed in hPDLSCs . Moreover, high doses of TLR1, -4, and -6 ligands were found to inhibit the osteogenic ability of hPDLSCs by inhibiting Akt activation in a Myd88- or TRIF-dependent manner.

TLRs are type-I glycoproteins expressed by many cell types that recognize a variety of pathogen-associated molecular patterns.[27] Increasing evidence suggests that TLR1–6 are expressed in human MSCs from different origins.[17,28] However, reports on TLR7–10 are inconsistent,[6,21,25,29] and to date, there are few studies reporting TLR expression in hPDLSCs.[6,23,24] In this study, we found that TLR1, TLR3, TLR4, and TLR6 are functionally expressed at high levels, whereas the expression of TLR7, -8, and -10 is very low in hPDLSCs. However, the expression of TLR2 and TLR5 was not remarkable in hPDLSCs, which is inconsistent with a recent report.[6] These discrepancies might be because of variations in donors, cell isolation methods, or culture conditions.

Previous studies have shown the functional expression of TLRs in adult MSCs and that their activation, upon stimulation by specific ligands, can regulate MSC function.[21,25] To date, numerous studies have reported that TLRs regulate the osteogenic differentiation of MSCs from different origins.[6,19,21,25,30–32] However, contradictory results exist among those studies. One previous study found that TLR activation had no effect on osteogenic differentiation in human bone marrow-derived MSCs (BMMSCs).[21] However, other studies reported that TLR2 and TLR4 activation reduced osteogenic differentiation of BMMSCs or PDLSCs.[6,25] Furthermore, studies have reported greater osteogenic differentiation of MSCs upon LPS, Pam3CSK4, PGN, and PolyI:C activation.[18,19,22,31] In our study, high doses of TLR ligands obviously inhibited the osteogenic potential of hPDLSCs, whereas low doses of TLR1, TLR4, and TLR6 ligands had little effect, with the exception of 0.1 μg/mL PolyI:C, which significantly induced osteogenic differentiation. In previous reports, different doses of LPS led to different osteogenic effects in BMMSCs.[20,33–35] From those previous findings, we speculated that the dose of TLR ligand might be a key factor for the osteogenic capacity of hPDLSCs. In addition, the origins of MSCs are also important with respect to their osteogenic differentiation.[6,20]

As one previous report described, TLR activation triggers MyD88- and TRIF-dependent downstream signaling cascades that lead to the nuclear translocation of NF-κB and the activation of several signaling pathways including MAPKs and PI3K-Akt, as well as many related genes.[8] Some studies have shown that inflammation or TLR stimulation in human MSCs leads to the inhibition of osteogenic differentiation through the activation of NF-κB.[6,33] In addition to

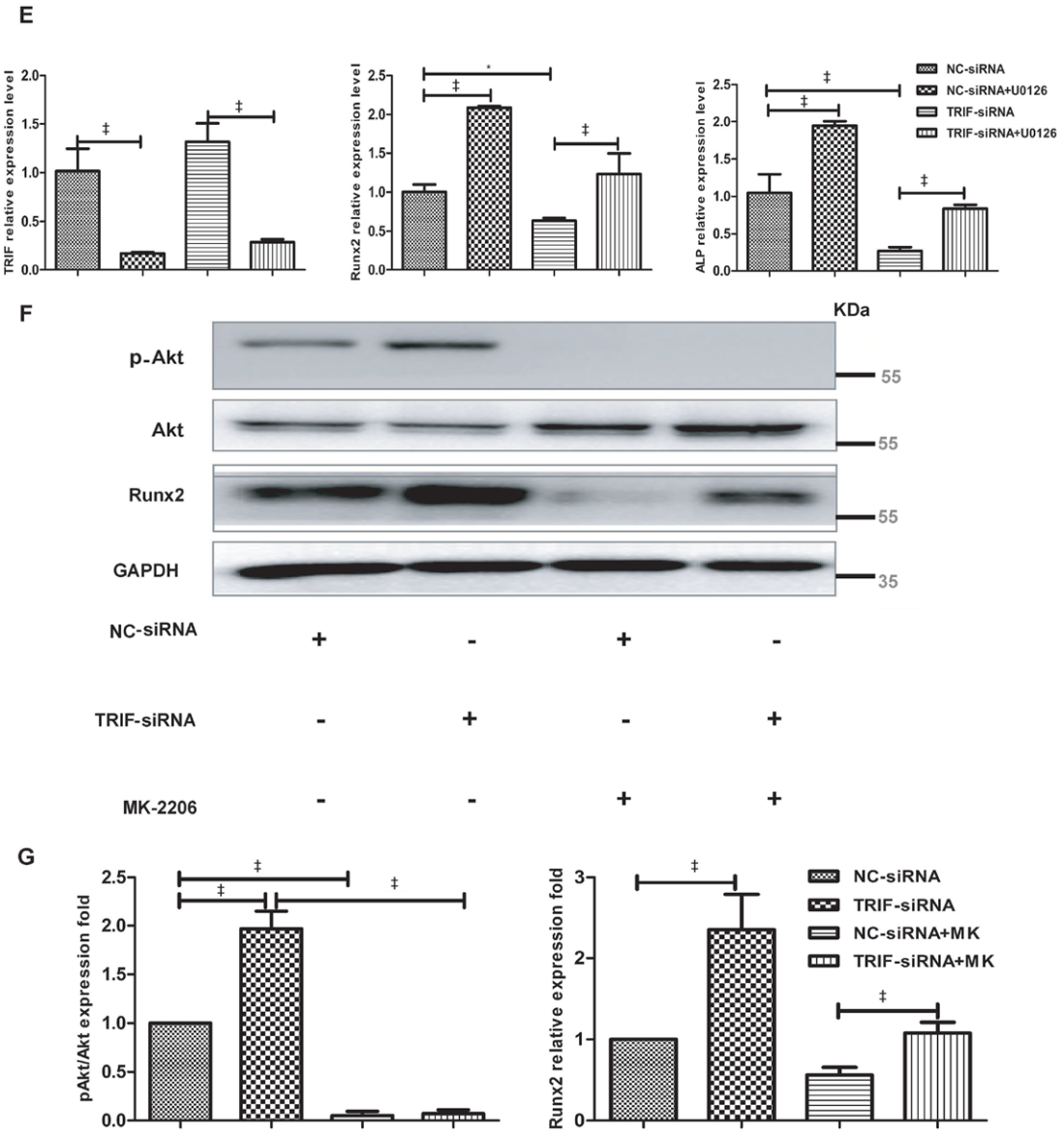

FIGURE 4 Continued

NF-κB, other downstream signaling pathways of TLRs including MAPKs and PI3K-Akt have also been reported to be essential for osteogenic differentiation.[5,11,14,31,36,37] Therefore, we next examined whether TLRs influence the osteogenesis of PDLSCs through these downstream pathways, focusing on the key associated adaptors Myd88 and TRIF.

One previous work found that MSCs derived from MyD88-deficient mice lack the capacity to differentiate into osteogenic cells.[25] Further, LPS and PolyI:C induce secretion of pro-inflammatory cytokines in a TRIF-dependent manner in MSCs.[38] These findings showed that Myd88 and TRIF have important roles in regulating MSC functions. In this study, we found that Myd88 is essential for high-dose TLR ligand-induced osteogenesis, which is consistent with the previous study focusing on the molecular mechanism associated with LPS and osteoblast differentiation.[39]

Our subsequent study revealed that Myd88-mediated negative regulation of Akt and Akt is indispensable for Runx2 upregulation induced by Myd88 silencing. A recent study proved that interleukin 1 (IL-1)R1/MyD88 signaling impairs MSC differentiation by inhibiting the Akt pathway in mice.[40] Since most TLRs share the common adaptor Myd88 with IL-1R, and because Akt activity has a critical role in MSC osteogenic differentiation,[40,41] our findings suggest that TLR1, TLR4, and TLR6 ligand stimulation could inhibit the osteogenic differentiation of hPDLSCs by downregulating the phosphorylation of Akt through Myd88.

Downstream signals of TLR3 or TLR4 are mediated by TRIF through the activation of MAPK, NF-κB, and interferon regulatory factor 3 (IRF3) pathways.[9] Furthermore, Akt has recently been reported to be a downstream molecule of TRIF/TANK-binding kinase 1 (TBK1) and play an important

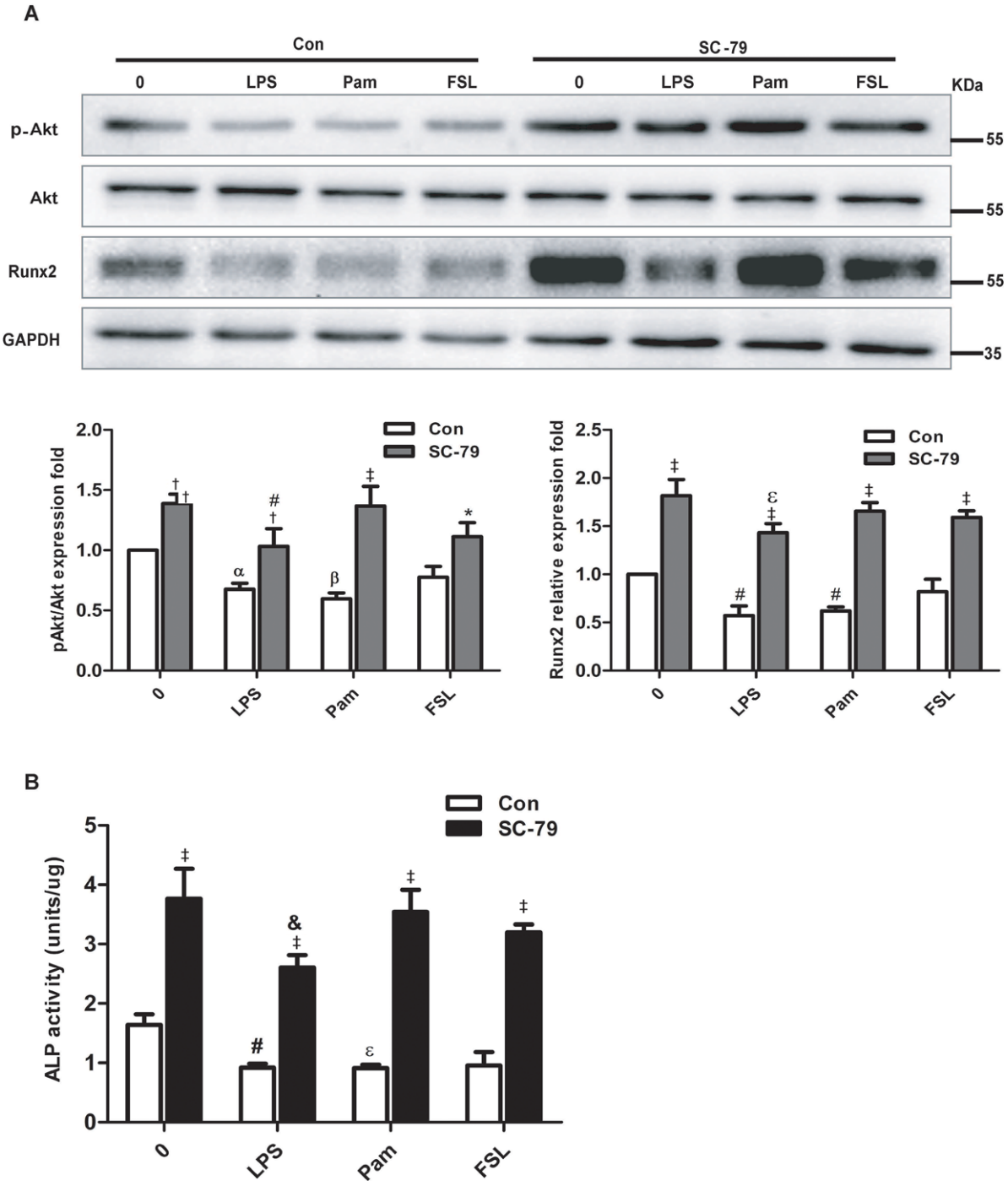

FIGURE 5 Activation of Akt reverses the impaired osteogenic differentiation caused by Toll-like receptor (TLR) activation. Human periodontal ligament stem cell (hPDLSCs) were treated with or without TLR1, -4, and -6 ligands at high doses (LPS: 10 μg/mL; Pam3CSK4: 1 μg/mL; FSL-1: 50 ng/mL) combined with or without SC-79 (Akt activator) in osteogenic medium for 7 days; the medium was changed after 3 days. The cells were collected for western blotting and ALP activity analysis. **A**, The protein expression of Runx2, phospho-Akt, and Akt was measured by western blotting; GAPDH was used as the internal control. The data are representative of three independent experiments (upper panel). Relative intensity of the tested protein was quantitatively analyzed using software (lower panel). $^{‡}P < 0.001$, $^{†}P < 0.01$, $^{*}P < 0.05$, SC-79 treatment versus SC-79 untreated control; $^{\#}P < 0.05$, TLR ligand treatment versus untreated control; $^{\varepsilon}P < 0.05$, SC-79 co-treatment with LPS versus SC-79 treatment control, one-way ANOVA. Data are presented as mean ± SD from three independent experiments. **B**, Alkaline phosphatase (ALP) activity in different treatment groups was applied to analyze osteogenic differentiation. $^{‡}P < 0.001$, SC-79 treatment versus SC-79 untreated control; $^{\#}P < 0.05$, LPS treatment versus untreated control; $^{\varepsilon}P < 0.05$, Pam3CSK4 treatment versus untreated control; $^{\&}P < 0.05$, SC-79 co-treatment with LPS versus SC-79 treatment control, one-way ANOVA. All data are presented as mean ± SD from three independent experiments. Con = control; Pam = Pam3csk4; FSL = FSL-1

role in the activation of IRF3 by TLR3 and TLR4 agonists.[41] In this study, we found that TRIF suppression could enhance the osteogenic potential of hPDLSCs, which was accompanied by increased ERK and Akt phosphorylation. Moreover, inhibition of ERK or Akt activity could suppress the upregulation of Runx2 caused by TRIF silencing; therefore, we speculated that LPS inhibits the osteogenic differentiation of hPDLSCs by downregulating ERK or AKT activity in a TRIF-dependent manner. Meanwhile, we noticed that ERK and Akt activity did not significantly change in TRIF-knockdown cells, compared with that in the negative control, in the presence of PolyI:C. The previous study showed that TLR3 and TLR4 have different roles during the regulation of inflammation, which led to different osteogenic mechanisms between TLR3 and TLR4 in BMMSCs.[42] Thus, we speculated that TLR3 and TLR4 might use different signaling pathways to regulate osteogenesis in hPDLSCs, which requires further study.

A previous report showed that high-dose LPS (10 μg/mL) treatment could decrease the phosphorylation of Akt or NF-κB.[43] In this study, we found that high doses of TLR1, TLR4, and TLR6 ligands downregulates Akt phosphorylation in hPDLSCs, which is in accordance with that report. Moreover, enhanced Akt activity suppressed the activated TLR-induced reduction in PDLSC-osteogenic potential. PI3K/Akt signaling was previously shown to be the key pathway required for human MSC osteogenesis; additionally, Runx2 is a downstream molecule of Akt.[10,14,32,44,45] In this study, we speculated that high doses of TLR1, -4, and -6 ligands could suppress hPDLSC osteogenic differentiation by downregulating Akt activity.

Since high bacterial burden is present in periodontitis,[7] this study focused on the effect of high doses of TLR ligand stimulation. However, the diverse effect associated with low-dose TLR ligand treatment should be further studied, to determine if this is mediated by a different mechanism. We found here that high doses of TLR1, TLR3, TLR4, and TLR6 ligands inhibit the osteogenic differentiation of hPDLSCs. Mechanistically, TLR1, -4, and -6 ligands could inhibit osteogenic differentiation at high concentrations via a Myd88/Akt-dependent pathway. Further, high doses of LPS could impair osteogenic differentiation by inhibiting ERK or Akt activation in a TRIF-dependent manner. However, the relationship between Akt and ERK or downstream signaling pathways of them have not been examined in this study; for example, Wnt or mTOR signaling has also been shown to be related to osteogenic differentiation.[46–48] Therefore, those signaling pathways or the crosstalk among them need to be studied in the future; this would help us to better control the osteogenic capacity of hPDLSCs at different levels in the immune microenvironment. Furthermore, our findings in this study should be tested in vivo.

In periodontal diseases, hPDLSCs employed for therapy could potentially be exposed to TLR ligands, which might modulate their therapeutic effect. Understanding the mechanism through which TLRs affect hPDLSCs seems to be important from a physiological or clinical point of view. This study focused on the inhibitory role of high doses of TLR ligands on the osteogenic potential of hPDLSCs and found that Myd88 or TRIF regulate Akt signaling, which plays an important role in this process; this might serve as a new therapeutic target for periodontal disease and alveolar bone regeneration.

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SUPPORTING INFORMATION

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