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Toll-like receptor-4-dependence of the lipopolysaccharide-mediated inhibition of osteoblast differentiation

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ABSTRACT. Bone fractures or bones subjected to open conduction and internal fixation are easily infected by bacteria; bacterial lipopolysaccharide (LPS) has been recognized as an important

pathogenic factor affecting bone fracture healing. Therefore, the effect of LPS on bone metabolism is relevant for bone healing. In this study, we investigated the effect of LPS on the expression of Toll-like receptor (TLR)-4 (an LPS receptor) by using real-time quantitative PCR and western blotting. We also examined the regulatory role of LPS in osteoblast differentiation by measuring the ALP activity, matrix mineralization, and *ALP*, *OCN*, and *Runx2* mRNA (essential factors affecting osteoblast differentiation) expression in LPS-treated mouse osteoblast MC3T3-E1 cells. We also evaluated the effect of TLR-4 on LPS-mediated inhibition of osteoblast differentiation using RNA interference. LPS promotes TLR-4 mRNA and protein expression in MC3T3-E1 cells ($P < 0.05$, $P < 0.01$ or $P < 0.001$), and inhibits osteoblast differentiation by downregulating matrix mineralization and ALP activity ($P < 0.05$, $P < 0.01$ or $P < 0.001$), and suppressing the expression *ALP*, *OCN*, and *Runx2* mRNA in MC3T3-E1 cells ($P < 0.05$ or $P < 0.01$). Conversely, RNAi-mediated TLR-4 knockdown abrogates the LPS-mediated inhibition of osteoblast differentiation ($P < 0.05$ or $P < 0.01$). In summary, LPS was shown to inhibit osteoblast differentiation by suppressing the expression of *ALP*, *OCN*, and *Runx2* in a TLR-4-dependent manner. The results of this study may provide insights into the signal pathway of LPS-induced bone loss or delayed bone fracture healing.

Key words: Toll-like receptor (TLR)-4; Lipopolysaccharide; Osteoblast differentiation

INTRODUCTION

Bone fractures commonly develop infections after open conduction and internal fixation (Petty et al., 1985; Nair et al., 1996; Darouiche, 2004; Berkes et al., 2010). On the other hand, the stability provided by implant retention has also been shown to reduce the likelihood of infection after fixation (Merritt and Dowd, 1987; Worlock et al., 1994; Schmidt and Swiontkowski, 2000). Implant retention in the presence of an underlying infection and in the absence of antibiotic treatment leads to a weaker callus and impedes callus maturation in a rat model, compared to non-infected controls (Bilgili et al., 2015). Lipopolysaccharide (LPS) is an important pathogenic factor affecting bone fracture healing. Experimental and clinical studies have indicated that the LPS endotoxin from localized infections, or those that translocate from the gut (Hiki et al., 1995; Buttenschoen et al., 2000), potentially trigger inflammatory response cascades (Tobias et al., 1997) and inhibit bone fracture healing (Nair et al., 1996). Therefore, the effect of LPS on bone metabolism is a clinically relevant factor of bone healing in cases of trauma resulting from endotoxinemia or sepsis after fractures.

LPS promotes the production of multiple-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and receptor activator of nuclear factor-kappa B ligand (RANKL) in osteoblasts (Ishimi et al., 1990; Keeting et al., 1991; Kikuchi et al., 2001), which in turn induces osteoclast maturation and activation. Several studies have demonstrated that LPS could inhibit the

osteogenic differentiation of osteoblasts (Kadono et al., 1999; Tomomatsu et al., 2009). However, little is known about the intracellular signals of LPS that mediate the inhibitory effect on osteoblast differentiation. The matrix mineralization and alkaline phosphatase (ALP) activities of osteoblasts are significantly inhibited by LPS in the presence of myeloid differentiation factor 88 (MyD88) (Bandow et al., 2010). Moreover, mRNA expression of ALP, runt-related transcription factor 2 (Runx2), and osteocalcin (OCN) are also downregulated by LPS treatment (Bandow et al., 2010; Wang et al., 2014).

In this study, we investigated the effect of LPS on the expression of the LPS receptor, Toll-like receptor (TLR)-4; additionally, we attempted to quantify the effect of LPS on osteoblast differentiation by measuring the ALP activity, matrix mineralization, and ALP, OCN, and Runx2 expressions in LPS-treated MC3T3-E1 cells. We also evaluated the TLR-4-dependence of LPS activity using RNA interference (RNAi).

MATERIAL AND METHODS

Reagents, cell culture, and treatment

MC3T3-E, a murine osteoblastic cell line, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and was cultured in α-minimal essential medium (α-MEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (CSPC Pharmaceutical Group Limited, Shijiazhuang, China) at 37°C in a 5% CO_2 incubator. Phenol-extracted LPS from *Escherichia coli* 0111:B4, purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in α-MEM supplemented with 2% FBS. Confluent MC3T3-E1 cells (>85%) were treated with 0, 50, 100, 200, or 400 ng/mL LPS for 0, 4, 8, 12, or 24 h. TLR-4 expression in MC3T3-E1 cells was abrogated by transfecting MC3T3-E1 cells with 30 or 60 nM siRNA-TLR-4 or control siRNA (GenePharma Technology, Shanghai, China), using Lipofectamine 2000 (Invitrogen).

Quantitative analysis of *TLR-4*, *ALP*, *OCN*, and *Runx2* mRNA

Cellular mRNA, isolated from MC3T3-E1 cells using a standard mRNA Isolation and Purification kit (Takara & Clontech, Tokyo, Japan), was quantified by real-time quantitative PCR (RT-qPCR) using one-step SYBR Green PCR kits (TaKaRa, Tokyo, Japan) and paired primers for *TLR-4*, *ALP*, *OCN*, and *Runx2*. RT-qPCR was performed on a LightCycler (v.2.0; Roche Diagnostics, Mannheim, Germany). The paired primers for each marker were synthesized by Shanghai Sangon Company (Sangon, Shanghai, China). All data are reported as the fold-change over the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was calculated using the $^{\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

Western blotting for TLR-4

Cytosolic proteins were isolated using a standard cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA), and supplemented with a protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). Protein samples were separated on a gel (12%)

using sodium dodecyl sulfate polyacrylamide gel electrophoresis; these were transferred to a polyvinylidene difluoride hydrophobic membrane (Millipore, Bedford, MA, USA). Non-specific binding sites on the membrane were blocked overnight with 5% skimmed milk (Solarbio, Beijing, China) at 4°C; the membrane was then inoculated with a rabbit polyclonal antibody against TLR-4 (Abcam, Cambridge, UK) or GAPDH (Sinobio, Beijing, China) at room temperature for 2 h; subsequently, the membrane was stained with a peroxidase-conjugated secondary antibody against rabbit IgG (Pierce, Rockford, IL, USA) at room temperature for 1 h. Finally, specific binding was visualized by chemiluminescence by using the enhanced chemiluminescence western blotting detection reagent (Amersham, Uppsala, Sweden). TLR-4 expression (gray color) was determined relative to that of GAPDH.

Von Kossa staining and ALP activity assay

The effect of LPS on osteoblast differentiation was determined by culturing MC3T3-E1 cells in α-MEM with 10% FBS and 0, 50, 200, or 400 ng/mL LPS. Matrix mineralization in the MC3T3-E1 cells was assayed by Von Kossa staining as previously described (Chan et al., 2008). For the ALP assay, MC3T3-E1 cells were lysed post-treatment; the cell lysate (with a protein concentration of 2 mg) was added to the assay buffer (1 mM $MgCl_2$, 50 mM Tris-HCl, pH 9.2) containing 2 mM p-nitrophenol phosphate, and incubated for 10 min at 37°C. The reaction was terminated using 0.45 M NaOH, and the absorbance of p-nitrophenol liberated in the reaction solution was read at 420 nm. The ALP activity was determined relative to that of the control group.

Statistical analysis

Statistical differences between the two groups were analyzed using the Student *t*-test with SPSS v.18.0 (IBM SPSS, Armonk, NY, USA). A P value <0.05 was considered to be significant.

RESULTS

LPS promotes TLR-4 in mouse osteoblast MC3T3-E1 cells

LPS signaling in osteoblast cells was examined by analyzing the TLR-4 expression in LPS-treated MC3T3-E1 cells. Treatment with 100, 200, and 400 ng/mL LPS significantly upregulated *TLR-4* mRNA in the MC3T3-E1 cells ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) in a dose-dependent manner ($P < 0.05$; Figure 1A). The time-dependence of LPS-mediated TLR-4 upregulation was then evaluated. *TLR-4* mRNA was significantly upregulated 4 h post-treatment (HPT) with 200 ng/mL LPS; this upregulation lasted up to 8 HPT, with a significant difference being observed between 4 and 6 HPT ($P < 0.05$; Figure 1B). LPS-mediated TLR-4 protein upregulation was then analyzed in LPS-treated MC3T3-E1 cells. LPS treatment (100, 200, or 400 ng/mL) resulted in a significant upregulation in TLR-4 protein expression ($P < 0.01$ or $P < 0.001$) in a dose-dependent manner ($P < 0.01$; Figure 1C). The time-dependence ($P < 0.01$) of LPS-mediated TLR-4 protein upregulation was also analyzed ($P < 0.01$ or $P < 0.001$; Figure 1D). Taken together, we confirmed that LPS treatment resulted in an upregulation in TLR-4 expression in mouse osteoblast MC3T3-E1 cells.

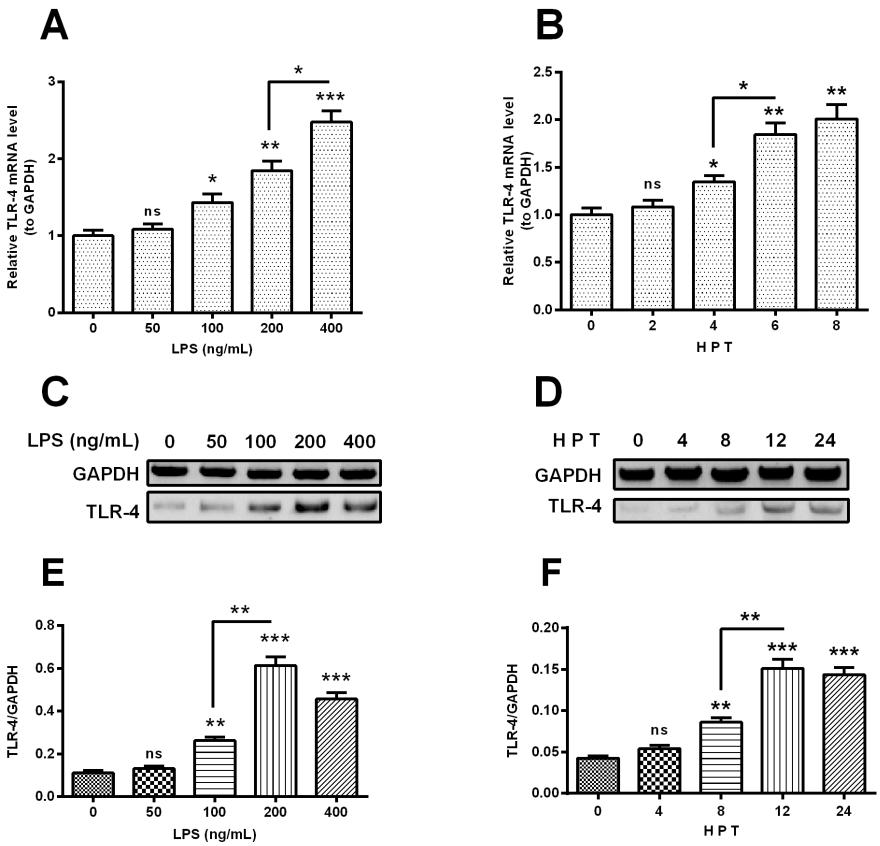

Figure 1. TLR-4 expression in LPS-treated mouse osteoblast MC3T3-E1 cells. **A.***TLR-4* mRNA levels in MC3T3-E1 cells treated with different concentrations (0, 50, 100, 200, or 400 ng/mL) of LPS for 6 h. **B.***TLR-4* mRNA levels in MC3T3-E1 cells treated with >200 ng/mL LPS for 0, 2, 4, 6, or 8 h. **C.** and **D.** Western blot analysis of TLR-4 protein expression in LPS-treated MC3T3-E1 cells for 12 h using GAPDH as a control. **E.** and **F.** TLR-4 protein level in MC3T3-E1 cells treated with > 200 ng/mL LPS for 0, 4, 8, 12, or 24 h. HPT: hours post-treatment. All data are reported as means ± standard deviation (SD) of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, or ns: no significance.

LPS inhibits matrix mineralization and ALP activity in MC3T3-E1 cells

The effect of LPS on osteoblast differentiation was then examined by analyzing the matrix mineralization and ALP activity in LPS-treated MC3T3-E1 cells. We observed a marked decrease in ALP activity in MC3T3-E1 cells treated with 100, 200, and 400 ng/mL LPS ($P < 0.05$, $P < 0.01$, or $P < 0.001$), in a dose-dependent manner ($P < 0.05$; Figure 2A). Matrix mineralization was measured in MC3T3-E1 cells by examining the mineralization dots. Treatment of MC3T3-E1 cells with 200 or 400 ng/mL LPS resulted in the formation of a significantly lower number of mineralization dots ($P < 0.01$, 200 ng/mL LPS; $P < 0.001$, 400 ng/mL LPS; Figure 2B and C) in a dose-dependent manner ($P < 0.01$).

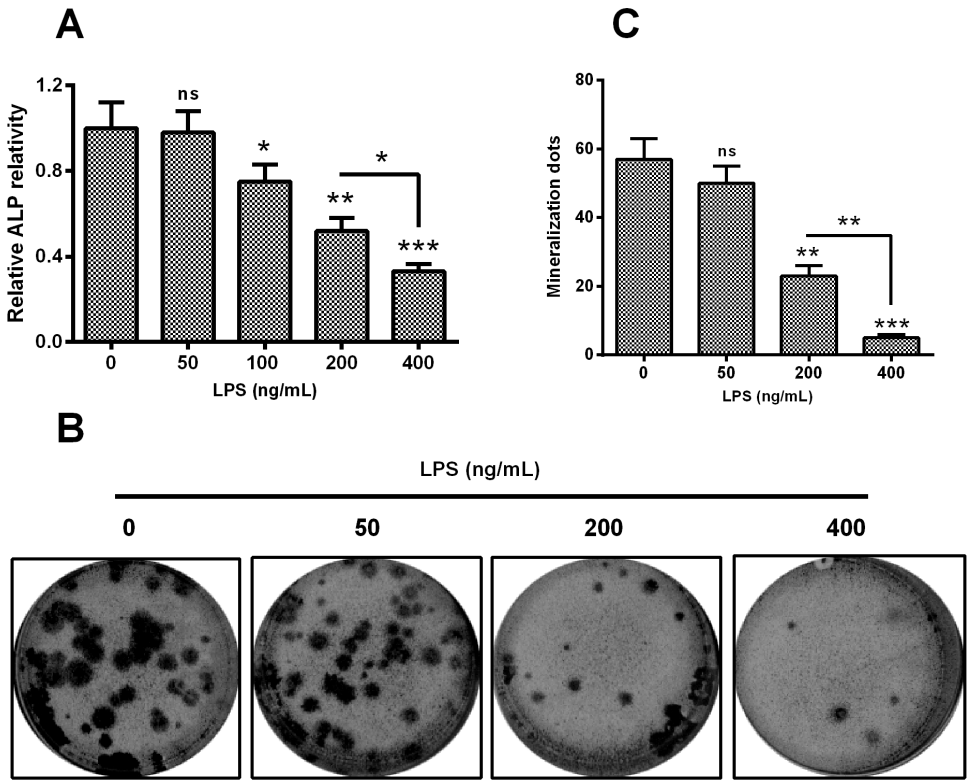

Figure 2. Matrix mineralization and ALP activity in LPS-treated MC3T3-E1 cells. **A.** ALP activity in MC3T3-E1 cells treated with 0, 50, 100, 200, or 400 ng/mL LPS for 8 h. **B.** Matrix mineralization examined in MC3T3-E1 cells treated with different concentrations of LPS (0, 50, 200, or 400 ng/mL) for 24 h by Von Kossa staining. **C.** Quantification of matrix mineralization in MC3T3-E1 cells post-LPS treatment. The experiments were performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, or ns: no significance.

LPS suppresses osteogenic marker expression in MC3T3-E1 cells

Osteoblast differentiation is characterized by an upregulated expression of multiple well-known osteogenic markers, such as ALP, OCN, and Runx2 (Bandow et al., 2010). Therefore, we analyzed the effect of LPS on the expression of *ALP*, *OCN*, and *Runx2* mRNA in osteoblasts by RT-qPCR. Treatment with 100, 200, or 400 ng/mL LPS led to a significant downregulation in *ALP* mRNA expression ($P < 0.05$, 100 ng/mL; $P < 0.01$, 200 or 400 ng/mL; Figure 3A), with a significant difference being observed between the cells treated with 100 and 400 ng/mL LPS. *OCN* (Figure 3B) and *Runx2* (Figure 3C) mRNA levels were also significantly downregulated as a result of treatment with >100 ng/mL LPS ($P < 0.05$, 100 ng/mL for *OCN* or *Runx2*; $P < 0.01$, 200 or 400 ng/mL for both markers) in a dose-dependent manner. This confirmed that osteoblast differentiation was inhibited by LPS treatment.

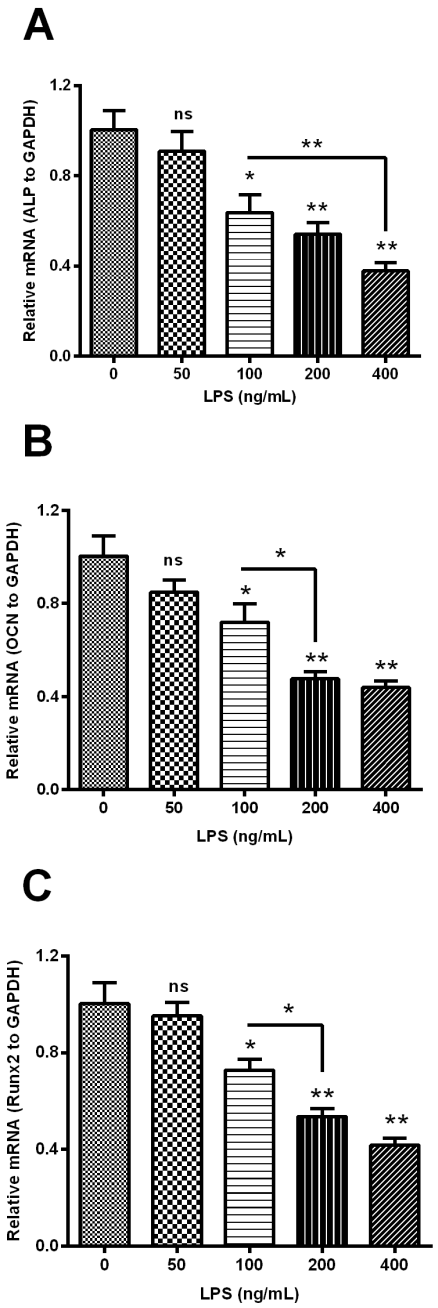

Figure 3. mRNA levels of osteogenic markers in LPS-treated MC3T3-E1 cells. mRNA was extracted from MC3T3-E1 cells treated with 0, 50, 100, 200, or 400 ng/mL LPS for 6 h. *ALP* (**A**), *OCN* (**B**), and *Runx2* (**C**) mRNA expression were analyzed by RT-qPCR. The results are reported as means ± SD of three independent experiments. *P < 0.05, **P < 0.01, or ns: no significance.

RNAi-mediated TLR-4 knockdown abrogates LPS-mediated inhibition of osteoblast differentiation

To further investigate the role of TLR-4 in the LPS-mediated inhibition of osteoblast differentiation, we abrogated the expression of TLR-4 in LPS-treated MC3T3-E1 cells and re-examined the matrix mineralization, ALP activity, and *ALP*, *OCN* and *Runx2* mRNA expression. *TLR-4* mRNA expression was significantly downregulated in MC3T3-E1 cells transfected with 30 or 60 nM TLR-4-specific siRNA (siRNA-TLR-4; $P < 0.001$; Figure 4A). Western blot analysis also confirmed that the TLR-4 protein level was significantly downregulated in MC3T3-E1 cells ($P < 0.001$; Figure 4B). Moreover, TLR-4 abrogation significantly ameliorated the inhibition of matrix mineralization ($P < 0.05$; Figure 4C and D). *ALP*, *OCN*, and *Runx2* mRNA expression was also significantly upregulated in 30 or 60 nM siRNA-TLR-4-transfected cells than in siRNA-control-transfected cells ($P < 0.05$ or $P < 0.01$). Thus, we confirmed that LPS-mediated inhibition of osteoblast differentiation was mediated by TLR-4 in MC3T3-E1 cells.

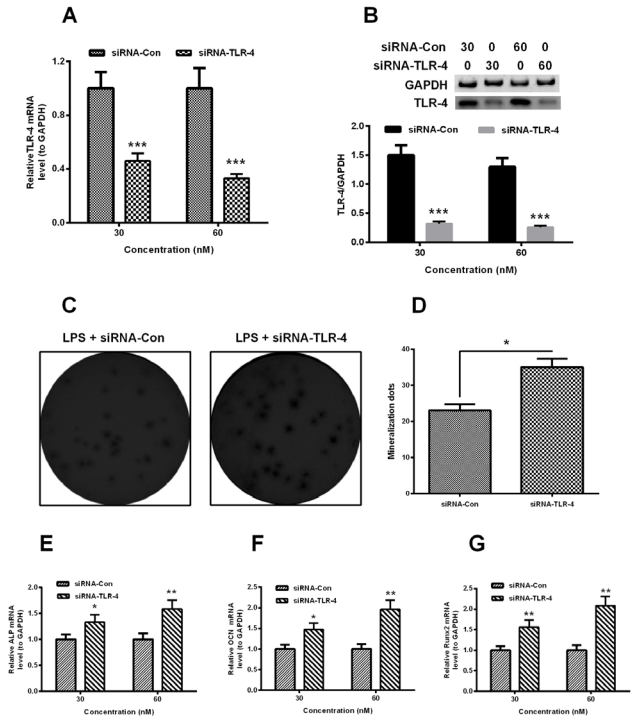

Figure 4. RNAi-mediated TLR-4 knockdown abrogates the inhibition of osteoblast differentiation by LPS. **A.***TLR-4* mRNA level in MC3T3-E1 cells transfected with 30 or 60 nM TLR-4-specific siRNA (siRNA-TLR-4) or control siRNA (siRNA-Con) for 12 h was determined by qRT-PCR, using GAPDH as the internal control. **B.** Western blotting to determine the level of TLR-4 protein expression in MC3T3-E1 cells, 24 h after transfection with siRNA-TLR-4 or siRNA-Con. **C.** and **D.** Quantification of matrix mineralization in MC3T3-E1 cells transfected with 60 nM siRNA-TLR-4 or siRNA-Con, in the presence of 200 ng/mL LPS; **E.-G.** *ALP* (E), *OCN* (F), and *Runx2* (G) mRNA levels in MC3T3-E1 cells transfected with 60 nM siRNA-TLR-4 or siRNA-Con, in the presence of 200 ng/mL LPS. All data are reported as means ± standard deviation (SD) of three independent experiments. *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$.

DISCUSSION

Bone mass is regulated by the balance between bone formation and resorption (Harada and Rodan, 2003). Osteoblasts have been recognized as key bone-forming cells, playing an essential role in bone mass acquisition (Karsenty and Wagner, 2002) during healing of bone fractures. The results of this study confirm that LPS suppressed osteoblast differentiation, as indicated by previous studies (Tomomatsu et al., 2009; Bandow et al., 2010). Our results demonstrate that LPS markedly inhibits the expression of *ALP*, *OCN*, and *Runx2* mRNA, all of which are essential markers in osteoblast differentiation (Komori et al., 1997; Nakashima et al., 2002; Komori, 2010; Bandow et al., 2010), and matrix mineralization of osteoblasts. In particular, since *Runx2*-deficient mice showed complete lack of bone formation (Komori et al., 1997), Runx2 is considered to be essential for the promotion of mesenchymal cells into osteoblast progenitors (Komori, 2010). Our data also showed that LPS strongly inhibits the expression of *Runx2* mRNA in mouse osteoblast MC3T3-E1 cells. These results are consistent with the results reported by previous studies (Azuma et al., 2004; Tomomatsu et al., 2009).

LPS is sensitized by TLR-4 in conjunction with other molecules such as LPS-binding protein and CD14 (Chow et al., 1999; Su et al., 2000). Sensitized TLR-4 then activates downstream signals, including the MyD88 and Toll-like receptor-IL-1 receptor domain containing adapter protein (TIRAP)/MyD88-adapter-like signaling (Rock et al., 1998; Fitzgerald et al., 2001; Yamamoto et al., 2002; Oshiumi et al., 2003). Furthermore, additional downstream signals, such as those resulting in the activation of nuclear transport of nuclear factor-kappa B, extracellular signal-regulated kinases, c-Jun N-terminal kinases, and p38 kinases (Zhang et al., 1999; Cario et al., 2000; Yang et al., 2000), are mediated by TLR-4 expression. However, little is known about the role of TLR-4 signaling in the LPS-mediated inhibition of osteoblast differentiation. In this study, we have shown that LPS treatment led to a significant upregulation in TLR-4 expression in MC3T3-E1 cells. Moreover, TLR-4-specific siRNA-mediated downregulation in TLR-4 expression inhibited the suppression of LPS-induced osteoblast differentiation by ameliorating matrix mineralization and reversing the reduction in *ALP*, *OCN*, and *Runx2* mRNA expression in the LPS-treated MC3T3-E1 cells. Thus, we confirmed that LPS-mediated osteoblast differentiation was dependent on TLR-4 expression.

In summary, LPS was shown to inhibit osteoblast differentiation by suppressing the expression of *ALP*, *OCN*, and *Runx2*, which are essential for osteoblast differentiation, in a TLR-4-dependent manner. This study may provide insights into the signal pathway of LPS-induced bone loss or delayed bone fracture healing.

Conflicts of interest

The authors declare no conflict of interest.

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