LPS-Stimulated Inflammatory Environment Inhibits BMP-2-Induced Osteoblastic Differentiation Through Crosstalk Between TLR4/MyD88/NF- κ B and BMP/Smad Signaling

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Abstract

Bone morphogenetic protein-2 (BMP-2) is a novel differentiation factor that is capable of inducing osteoblast differentiation and bone formation, making it an attractive option in treatment of bone defects, fractures, and spine fusions. Inflammation, which was a common situation during bone healing, is recognized to inhibit osteogenic differentiation and bone formation. However, the effect of inflammation on BMP-2-induced osteoblastic differentiation remains ambiguous. In this study, we showed that an inflammatory environment triggered by lipopolysaccharide (LPS) *in vitro* would suppress BMP-2-induced osteogenic differentiation of bone marrow mesenchymal stem cells, which represented by decreased alkaline phosphatase (ALPase) activity and down-regulated osteogenic genes. In addition, LPS activated nuclear factor- κ B (NF- κ B) via a TLR4/MyD88-dependent manner and inhibited BMP-2-induced phosphorylation and nuclear translocation of Smad1/5/8. The blocking of NF- κ B signaling by pretreatment with specific inhibitors such as BAY-11-7082, TPCK and PDTC, or by transfection with plasmids encoding p65 siRNA or I κ B α siRNA could significantly reverse the inhibitory effect of LPS on BMP-2-induced BMP/Smad signaling and osteogenic differentiation. By contrast, even without stimulation of LPS, overexpression of p65 gene showed obvious inhibitory effects on BMP-2-induced BMP/Smad signaling and ALPase activity. These data indicate that the LPS-mediated inflammatory environment inhibits BMP-2-induced osteogenic differentiation, and that the crosstalk between TLR4/MyD88/NF- κ B and BMP/Smad signaling negatively modulates the osteoinductive capacity of BMP-2.

Introduction

NOWADAYS, BONE MORPHOGENETIC PROTEIN-2 (BMP-2) is widely-accepted as a novel differentiation factor that is capable of inducing entire sequence of bone formation, making it an attractive option in bone fracture and bone nonunion application to stimulate new bone growth [1]. Human recombinant BMP-2 has been approved by the United States Food and Drug Administration (FDA) to use in anterior lumbar interbody fusions (2002), open tibial fractures (2004), and oral maxillofacial reconstructions (2007) [2,3]. The current clinical and experimental applications of BMP-2 come typically with

some biodegradable materials such as a collagen carrier to retain BMP-2 at the wound site and permit its slow release into the extracellular milieu. However, the inflammation response induced by application of exogenous BMP-2, degradation of biomaterials, surgical trauma and other environmental stress factors diminished the efficacy of BMP-2 in clinical settings [4–9]. Inhibition of local inflammation by specific drugs remarkably enhanced the osteoinductive efficacy of BMP-2 [7,10,11]. These evidences imply that there is a tight connection between inflammation and the osteoinductive capacity of BMP-2.

A robust body of recent literature supports the notion that inflammatory cytokines and mediators released after infection

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or environmental stress have profound effects on mesenchymal stem cells (MSCs) osteogenic differentiation and bone regeneration [12,13]. Although it is recognized that a proper inflammatory response is required to recruit MSCs from the surrounding environment to the injury site, and then initiate the differentiation processes of these cells, it ultimately promotes endochondral and intramembranous bone formation [14,15]. On other hand, persistent or excessive inflammation biologically modifies several subsequent signaling molecules and, consequently, inhibits or delays bone regeneration via inflammatory cytokines or mediators [13,16]. For instance, TNF- α [17,18], IL-6 [19] and IL-1 β [20] inhibit osteogenic differentiation in a variety of stem and progenitor cells. However, even though the influence of inflammation on osteogenic differentiation of MSCs is well understood, little is currently known about the precise role inflammation plays in the osteoinductive capacity of BMP-2.

Lipopolysaccharide (LPS) is derived from the cell wall of Gram-negative bacteria after elimination of pathogenic bacteria with antibiotics [21]. To date, there is much evidences supporting the fact that activation of cells though LPS is mediated by its transmembrane receptor toll-like receptor 4 (TLR4) and other molecules [22]. Downstream signals of TLR4 have been divided into myeloid differentiating factor 88 (MyD88)-dependent pathways and MyD88-independent pathways [23,24]. However, only initiation of MyD88-dependent pathway could lead to activation of nuclear factor- κ B (NF- κ B) and transcription of several proinflammatory genes such as TNF- α , IL-1 β , IL-6 and NO [25–27].

NF- κ B is a multifunctional transcription factor that is associated with inflammatory response and bone metabolism. It is considered an important signal transduction pathway in modulations of cell differentiation, proliferation, and tumorigenesis in multiple cell types [28–30]. Growing evidences suggest that activation of NF- κ B signaling by LPS, TNF- α , or inflammatory mediators would inhibit osteoblastic differentiation in many cell types [17,31,32]. Specific inhibition of NF- κ B signaling by overexpression of a dominant-negative form of I κ B α (I κ B α DN) or by NF- κ B signaling inhibitors would promote induction of osteoblastic differentiation and postnatal bone formation [33,34]. In contrast to NF- κ B, BMP-2-induced BMP/Smad signaling provides robust differentiation signals to osteoblasts as well as to other tissues [35]. Furthermore, BMP-2, acting through both Smad-dependent and -independent mechanisms, inhibits cell cycle and increases apoptosis by regulating pro-apoptotic proteins [36]. There seems to be antagonistic effects between BMP/Smad and NF- κ B signaling systems on cell growth, apoptosis, and differentiation. However, the possible involvement of NF- κ B in BMP-2-induced osteogenic differentiation, especially the crosstalk between NF- κ B and BMP/Smad signaling, remains to be conclusively resolved.

In the present work, we hypothesize that inflammatory environment inhibits the osteoinductive capacity of BMP-2. We focus our work on the signal transduction from LPS to NF- κ B and the possible molecular mechanism of the inhibitory effect of NF- κ B on BMP/Smad signaling. Here, we identify that LPS-mediated inflammatory environment inhibits BMP-2-induced osteogenic differentiation through crosstalk between TLR4/MyD88/NF- κ B and BMP/Smad signaling, which may provide new insights regarding the clinical application of BMP-2 to enhance bone regeneration.

Materials and Methods

Reagents, plasmids, and animals

Purified recombinant human BMP-2 was provided by PeproTech, Inc. (Rocky Hill). LPS, PDTC and TPCK were purchased from Sigma-Aldrich. Anti-phospho-Smad1/5/8, anti-phospho-NF- κ B-p65, anti-total NF- κ B-p65, anti-I κ B α , anti-MyD88, anti-Runx2 and anti-GAPDH were obtained from Cell Signaling Technology, Inc. NF- κ B p65 siRNA, I κ B α siRNA, TLR4 neutralization antibody and isotype IgG control antibody were obtained from Santa Cruz Biotechnology. MyD88 inhibitory peptide, MyD88 control peptide, TPCK and BAY-11-7082 were purchased from InvivoGen. Transfection reagent, pNF- κ B- and pSBE-Luciferase reporter plasmids were provided by Promega Corporation (Promega). TLR4^{lps-del} (C57BL/10ScNJ) mice and MyD88-knock out (MyD88^{-/-}) mice were purchased from the Model Animal Research Centre of Nanjing University, and all animal procedures were approved under the guidelines of the Laboratory Animal Research Committee.

Isolation of bone marrow mesenchymal stem cells and cell cultures

Primary bone marrow mesenchymal stem cells (BMSCs) were isolated from the bone marrow of 4- to 6-week-old TLR4^{lps-del} mice, MyD88-knockout mice and their matched wild-type mice (C57BL/10J and C57BL/6J respectively). The murine multipotent mesenchymal progenitor cell line, C2C12, was purchased from American Type Culture Collection (ATCC). All cells were cultured in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco). The cultured medium was changed every 3 days. Only BMSCs from early passages (2 and 3) were utilized for this experiment. The immunologic phenotypes of mice BMSCs were positive for CD29 (99.78%), CD44 (77.51%) and CD90 (98.69%), but negative for CD11b (1.18%), CD34 (0.5 evidence 2%) and CD45 (0.44%) (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/scd). We also demonstrated that the BMSCs derived from wild-type mice bone marrow could differentiate into osteoblasts and adipocytes (Supplementary Fig. S1B).

Measurements of cytokines

BMSCs were treated with LPS at different concentration for 48 h. The concentration of TNF- α , IL-1 β and IL-6 in the supernatant were analyzed by using ELISA kits (R&D).

Flow cytometry analysis

BMSCs were washed in PBS and resuspended in PBS. The cell aliquots were incubated with anti-TLR4-PE (eBiosciences). Subsequently, cells were analyzed on a flow cytometer using CellQuest PROTM software (BD Biosciences).

Cell proliferation assays

The cell counting kit-8 (CCK-8) (Dojindo) was used to determine cell proliferation of wild-type (WT) BMSCs. Cells were seeded in 96-well plates at an initial density of 4000 cells per well. After every 12 h of LPS treatment, the CCK-8 reagent was added for an incubation period of 2 h at 37°C.

The absorption rate at 450 nm and the reference wavelength at 600 nm were measured.

Alkaline phosphatase and alizarin red staining

Before staining, WT BMSCs were washed with PBS and fixed with 4% paraformaldehyde for 30 min. For alkaline phosphatase (ALPase) staining, cells were stained with naphthol AS-BI alkaline solution for 45 min to visualize ALPase activity. For alizarin red, cells were stained with 40 mM alizarin red S (Sigma) solution (pH 4.1) for 10 min to visualize matrix calcium deposition.

Measurements of ALPase activity

WT BMSCs were exposed to BMP-2 and LPS at indicated concentrations and times. Cells were lysed and cellular ALPase activity was measured by using an alkaline phosphatase detection kit (Nanjing Jiancheng Bioengineering Institute). The amount of ALP in the cells was normalized against total protein content.

RNA extraction, reverse transcriptase-polymerase chain reaction, and quantitative real-time polymerase chain reaction analysis

Total RNAs were extracted by using Trizol reagent (Invitrogen), and 2 µg of total RNA were used for reverse transcription. The product was then used for reverse transcriptase-polymerase chain reaction (RT-PCR) or real-time PCR. RT-PCR was performed using an RT-PCR Kit (Takara) according to the manufacturer's instruction. The quantification levels of TLR4 and osteogenic genes were analyzed by ABI 7500 Real-Time PCR System. PCR primer pairs were selected from different exons of the corresponding genes as shown in Supplementary Table S1.

Western immunoblot analysis

Proteins were extracted with RIPA lysis buffer containing 1 mM PMSF. Cytosolic and nuclear fractions were prepared by using a nuclear and cytoplasmic protein extraction kit (Sangon biotech). Samples were subjected to immunoblotting to examine phosphorylation of NF-κB and Smad1/5/8, and expression of MyD88, Runx2 and IκBα.

Immunofluorescence staining

After applied treatments, WT BMSCs were fixed in 4% paraformaldehyde and then blocked in 5% goat serum; next, the cells were immunostained with the indicated primary antibodies followed by goat anti-rabbit AlexaFluor-555-conjugated secondary antibody (Invitrogen). The cells were covered with Anti-Fade Reagent (Cell Signaling).

Transient transfection and dual-luciferase assay

All transient transfections were performed using FuGENE[®]HD Transfection Reagent. Total amounts of transfected plasmids in each group were equalized by the addition of an empty vector. For each transfection, C2C12 cells were separately co-transfected with the control vector or the plasmid DNA containing p65, p65 siRNA or IκBαDN. Luciferase assay was performed by using the Dual Luciferase Reporter Assay

kit. BMP signaling was monitored by using the pSBE-Luciferase reporter plasmid, and NF-κB signaling was monitored by using the pNF-κB-Luciferase reporter plasmid.

Statistical analysis

Statistical evaluations were performed using a Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

LPS treatment promotes BMSCs proliferation and triggers an inflammatory environment in vitro

To examine the effect of LPS on BMSCs proliferation, WT BMSCs were challenged with LPS at different concentrations. Our data have shown that a low concentration of LPS (0.25–2.5 µg/mL) is beneficial for WT BMSCs proliferation (Fig. 1A). To first establish an in vitro inflammation model, we challenged WT BMSCs with LPS and then examined the expression of inflammatory cytokines in cell culture supernatants. After 48-h stimulation of LPS, the expression of TNF-α, IL-6 and IL-1β were elevated at both transcriptional level and cytokine level in a dose- and time-dependent manner (Fig. 1B–G). On these grounds, we concluded that LPS treatment mimicked a permanent and effective inflammatory environment of BMSCs in vitro.

LPS-stimulated inflammation inhibits BMP-2-induced osteogenic differentiation

To investigate the effect of LPS-mediated inflammation on BMP-2-induced osteogenic differentiation of WT BMSCs, we first examined the influence of LPS on BMP-2-induced expression of ALPase, a typical early marker of osteoblast differentiation, and calcium deposition, a late marker of osteoblast differentiation. As demonstrated in Fig. 2A, BMP-2 treatment increased ALPase expression and calcium deposition in BMSCs, while the presence of LPS apparently abolished this osteogenic induction. Furthermore, the ALPase activity assay showed that LPS exerted an inhibitory effect on BMP-2-induced ALPase activity via a dose-dependent manner (Fig. 2B).

To further confirm the inhibitory effect of LPS-stimulated inflammatory environment on BMP-2-induced osteogenic differentiation, we investigated the transcriptional level of osteogenic genes such as ALP, Collagen type 1 (COL1A1), osteocalcin (OCN), osteopontin (OPN), Runx2 and Osterix. The expression patterns of these genes were similar to the ALPase activity: BMP-2 induction up-regulated the transcriptional level of these genes, and LPS treatment eliminated the positive effect of BMP-2 (Fig. 2C). These results provided strong evidences to support the conclusion that LPS-mediated inflammation inhibits BMP-2-induced osteoblastic differentiation of BMSCs.

LPS activates NF-κB signaling through TLR4 and MyD88

Previous studies have shown that LPS is sensed by its transmembrane receptor TLR4 [22]. We hypothesized that LPS inhibited BMP-2-induced osteogenic differentiation by binding to TLR4, resulting in activation of NF-κB and then suppression of BMP/Smad signaling. To confirm this hypothesis and clarify the role of TLR4 in activation of NF-κB, we employed

FIG. 1. Lipopolysaccharide (LPS) treatment triggers an inflammatory environment in vitro. Bone marrow mesenchymal stem cells (BMSCs) were treated with LPS in a dose-dependent and time-course experiment. **(A)** CCK-8 assay of cell proliferation. **(B–D)** Real-time polymerase chain reaction (PCR) and relative quantification of TNF- α **(B)**, IL-6 **(C)** and IL-1 β **(D)** mRNA expression, normalized with GAPDH expression. **(E–G)** ELISA assay of TNF- α **(E)**, IL-6 **(F)** and IL-1 β **(G)** protein accumulation in conditioned media. Each bar represents the mean $\pm$ SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated cells.

BMSCs derived from both wild-type and TLR4^{lps-del} mice to co-culture with BMP-2 and LPS. First, we confirmed that BMSCs from WT mice express TLR4 at mRNA (Fig. 3A) and receptor level (Fig. 3B). Interestingly, BMSCs treated with LPS increased the mRNA level of TLR4 in a dose-dependent

manner, while BMSCs treated with BMP-2 did not (Fig. 3D). Subsequently, we observed that LPS induced secretion of TNF- α (Fig. 3E) and phosphorylation of NF- κ B in WT BMSCs (Fig. 3C); while no similar phenomenon was observed in TLR4^{lps-del} BMSCs. To further verify the important role of

FIG. 2. LPS-mediated inflammation inhibits bone morphogenetic protein-2 (BMP-2)-induced osteoblast differentiation of BMSCs. **(A)** BMSCs were treated with BMP-2 in the presence or absence of LPS, ALP staining assay of alkaline phosphatase (ALPase) activity on day 7 (**A**, left), and Alizarin Red staining assay of calcium deposition on day 21 (**A**, right). **(B)** BMSCs were treated with BMP-2 in the presence or absence of LPS in a time-course experiment. Quantification assay of ALPase activity, normalized with total protein. **(C)** BMSCs were treated with BMP-2 in the presence or absence of LPS for 48 h, real-time PCR, and relative quantification of ALP, COL1A1, OCN, OPN, Runx2 and Osterix, normalized versus GAPDH. Each bar represents the mean $\pm$ SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated cells. Color images available online at www.liebertpub.com/scd

TLR4 in activation of NF- κ B, TLR4 antibody was utilized in C2C12 cells to neutralize the TLR4 receptor, and a luciferase reporter plasmid was used to monitor the activity of NF- κ B signaling. The data showed that the TLR4 antibody significantly decreased LPS-induced pNF- κ B-luciferase activity, and no obvious changes could be observed in C2C12 cells treated with the control IgG antibody (Fig. 3F).

Since the mammalian TLR4 signal transduces via the MyD88- or the TRIF/TRAM-dependent pathways, and only the former pathway results in activation of NF- κ B signaling and secretion of inflammatory cytokines, we investigated the role of MyD88 in the inhibitory effect of LPS on BMP-2-induced osteoblast differentiation. In this study, we observed that MyD88 was expressed in WT BMSCs (Fig. 4A) and the MyD88 gene-defective BMSCs were hypo-responsive to LPS stimulation, characterized by failing to induce TNF- α secre-

tion (Fig. 4C) and activate NF- κ B signaling (Fig. 4B). In addition, the blockade of the MyD88-dependent pathway by pretreating C2C12 cells with MyD88 inhibitory peptide significantly diminished LPS-induced pNF- κ B-luciferase (Fig. 4D). These results indicated that LPS activate NF- κ B signaling beginning with binding to TLR4 and then transmitting the signal into cells in an MyD88-dependent manner.

Activation of NF- κ B signaling inhibits phosphorylation and nuclear translocation of Smad1/5/8

To further study the influence of LPS-induced inflammation on BMP/Smad signaling, we detected the effect of NF- κ B activation on phosphorylation and nuclear translocation of Smad1/5/8, which acts as an early-stage activator of

FIG. 3. LPS activates NF- κ B signaling through TLR4. **(A–B)** Reverse transcriptase-PCR (RT-PCR) **(A)** and flow cytometry analysis **(B)** of TLR4 expression in TLR4^{Δps-del} and wild-type BMSCs, the mRNA level normalized with GAPDH. **(C)** TLR4^{Δps-del} and wild-type (WT) BMSCs were treated with LPS in different concentrations for 30 min, western blot (WB) analysis of phosphorylation of NF- κ B-p65, normalized versus total p65 and GAPDH. Results are representative of three independent experiments. **(D–E)** TLR4^{Δps-del} and WT BMSCs were treated with LPS in different concentration for 48 h, real-time PCR analysis of TLR4 expression **(D)**, and ELISA assay of TNF- α protein accumulation in culture supernatant **(E)**, the mRNA level normalized with GAPDH. **(F)** C2C12 cells were transiently transfected with pNF- κ B-Luc along with pRL-TK-Luc for 24 h. Subsequently, the cells were pretreated with TLR4 neutralization antibody or an IgG control antibody for 1 h and then challenged with LPS for another 48 h. Luciferase activity assay of the cell lysates, normalized with pRL-TK-Luciferase activity. Each bar represents the mean $\pm$ SD of three independent experiments. * P < 0.05 and ** P < 0.01 compared with the untreated cells or the indicated groups. Color images available online at www.liebertpub.com/scd

BMP/Smad signaling. As shown in Fig. 5A, 30-min-pretreatment of BMP-2 induced a high phosphorylation level of Smad1/5/8 (left panel, lanes 1, 0 min), but the presence of LPS decreased the phosphorylation level of Smad1/5/8 within 15 min (right panel, lanes 3) and thereafter. In addition, a dose-dependent inhibitory effect of LPS on BMP-2-induced phosphorylation of Smad1/5/8 and pSBE-luciferase activity was also observed (Fig. 5B) in C2C12 cells. Generally, in the canonical BMP/Smad pathway, the phosphorylated Smad1/5/8 assembles into complexes with Smad4 and translocates into the nucleus to activate Runx2, a master transcriptional factor in both endochondral and intramembranous bone formation. In our study, the results of immunoblotting and immunostaining showed that activation of NF- κ B inhibited Smad1 nuclear translocation (Fig. 5D, E) and expression of Runx2 (Fig. 5C).

Blockade of NF- κ B signaling by specific inhibitors reverses the inhibitory effect of LPS on BMP-2-induced osteogenic differentiation of BMSCs

Since NF- κ B plays a key role in regulation of various crucial genes which have important roles in inflammation and bone metabolism, we hypothesized that LPS inhibits BMP/Smad signaling through activation of NF- κ B. To verify this hypothesis, specific NF- κ B signaling inhibitors were used to inhibit activation of NF- κ B and observe the influence of BMP/Smad

signaling activation and ALPase activity. LPS induced a more than six-fold increase in pNF- κ B-luciferase activity (data not shown), and activated phosphorylation of p65 and degradation of I κ B α (Fig. 6C), which acts as a key point in regulation of the NF- κ B pathway and enables NF- κ B to translocate into the nucleus, where it can activate the target genes [37]. Meanwhile, LPS also decreased phosphorylation level of Smad1/5/8 (Fig. 6C) and pSBE-luciferase activity (Fig. 6B) in C2C12 cells. However, NF- κ B suppression by pretreatment with the I κ B α phosphorylation inhibitor BAY-11-7082, the I κ B protease inhibitor TPCK, and the NF- κ B inhibitor PDTC led to a recovery of the phosphorylation level of Smad1/5/8 and pSBE-luciferase activity to a certain degree (Fig. 6B, C). Similar results were observed in ALPase activity, presented as LPS, suppressed the BMP-2-induced ALPase activity, and NF- κ B signaling inhibitors made an effective turnover (Fig. 6A). Thus, NF- κ B appears to be required and acts as a pivotal mediator for the negative regulatory role of LPS on BMP-2-induced activation of BMP/Smad signaling and ALPase activity.

Overexpression of p65 inhibits BMP-2-induced ALPase activity and activation of BMP/Smad signaling

Next, to precisely determine the negative role of NF- κ B in BMP-2-induced BMP/Smad signaling and ALPase activity, C2C12 cells were transfected with plasmids expressing

FIG. 4. LPS activates NF- κ B signaling through MyD88. **(A)** RT-PCR and WB analysis of MyD88 expression in MyD88^{-/-} and WT BMSCs normalized versus GAPDH. **(B)** MyD88^{-/-} and WT BMSCs were exposed to LPS in differentiation concentrations for 15 min, WB analysis of phosphorylation of NF- κ B-p65, normalized versus total p65 and GAPDH. Results are representative of three independent experiments. **(C)** MyD88^{-/-} and WT BMSCs were exposed to LPS in differentiation concentration for 48 h, ELISA assay of TNF- α protein accumulation in conditioned media supernatant. **(D)** C2C12 cells were transiently transfected with pNF- κ B-Luc along with pRL-TK-Luc for 24 h. Then, the cells were pretreated with MyD88 inhibitory peptide or a control peptide for 12 h and treated with LPS for additional 48 h. Luciferase activity assay of the cell lysis, normalized with pRL-TK-Luciferase activity. Each bar represents the mean $\pm$ SD of three independent experiments. ** $P < 0.01$ compared with the untreated cells or the indicated groups.

exogenous p65 gene, p65 siRNA, and I κ B α siRNA before BMP-2 and LPS stimulation. If NF- κ B is the pivotal mediator for LPS-induced negative regulation of BMP/Smad signaling, ectopic expression of p65 should diminish BMP-2-induced activation of BMP/Smad signaling and after up-regulation of osteogenic markers. Indeed, overexpression of p65 was found to abrogate BMP-2-induced ALPase expression (Fig. 7A), pSBE-luciferase activity (Fig. 7B), and phosphorylation of Smad1/5/8 (Fig. 7C). By contrast, inhibition of NF- κ B by silencing p65 gene or I κ B α gene, which encoding an upstream protein of NF- κ B, abolished the inhibitory effect of LPS treatment, represented by increasing pSBE-luciferase activity and phosphorylation level of Smad1/5/8 (Fig. 7D, E) in C2C12 cells. These data collectively indicated that the activation of NF- κ B intercepted BMP/Smad pathway, and that they have crosstalk to regulate BMP-2-induced osteogenic differentiation of BMSCs in an inflammatory environment.

Discussion

This study examined the role of inflammation in the osteoinductive capacity of BMP-2 and the possible crosstalk between NF- κ B and BMP/Smad signaling in regulation of osteogenic differentiation. We mimicked an inflammatory environment by treating BMSCs with LPS and showed for the first time that: (1) LPS-mediated inflammation inhibits BMP-2-induced osteogenic differentiation of BMSCs; (2) LPS

exerts the inhibitory effect through a TLR4-MyD88-NF- κ B pathway; and (3) the crosstalk between NF- κ B and BMP/Smad signaling negatively modulates the osteoinductive capacity of BMP-2 (Fig. 8).

Bone regeneration is initiated with the local inflammation response, followed by the mobilization of hematopoietic stem cells and MSCs to form vascular networks, cartilage, and bone. Although the initial inflammatory responses are different under various conditions, they can be characterized by a common spectrum of intercellular signaling pathways and endogenous mediators involving in [14,38]. Since there were no negative influence on cell proliferation, LPS, at a concentration of 1 μ g/mL in the present study, was chosen as an agonist to trigger inflammatory response in vitro to activate several common intracellular pathways such as NF- κ B [24] and stimulate secretion of inflammatory cytokines [39,40]. Although the inflammatory environment in the patient who accepted BMP-2 treatment is hard to completely mimic by LPS treatment, the expression profile of inflammatory cytokines in our study is similar to that in bone fracture, which is characterized by elevation of TNF- α , IL-1, IL-6, and more during bone healing [12,16,41], and that indicating the inflammatory environment is similarly mimicked by LPS treatment.

Several published papers have demonstrated that inflammatory cytokines, such as TNF- α , suppress BMP-2-induced osteoblastic differentiation in vitro [32,42–44]. However, the cytokines secreted in the in vivo inflammatory

FIG. 5. Activation of NF- κ B by LPS inhibits phosphorylation and nuclear translocation of Smad1/5/8. **(A)** BMSCs were pretreated with BMP-2 for 30 min and then exposed to LPS or PBS for 5 to 240 min. WB analysis of phosphorylation of Smad1/5/8, normalized with GAPDH. **(B)** C2C12 cells were transiently transfected with pSBE-Luc along with pRL-TK-Luc for 24 h. Then, the cells were pretreated with BMP-2 in the presence or absence of LPS in different concentrations for 48 h. Luciferase activity assay of the cell lysis, normalized with pRL-TK-Luciferase activity. **(C)** BMSCs were pretreated with BMP-2 for 30 min and then exposed to LPS in different concentrations for 30 min. WB analysis of Smad1/5/8 phosphorylation and Runx2 expression, normalized to GAPDH. **(D)** BMSCs were prestimulated with LPS (1 μ g/mL) for 30 min and then incubated with BMP-2 (200 ng/mL) for another 30 min. Immunofluorescence analysis for Smad1 protein. Results are representative of three independent experiments, original magnification, 200 $\times$. **(E)** BMSCs were prestimulated with LPS for 30 min and incubated with BMP-2 for another 30 min. Cytosolic and nuclear fractions were prepared, WB analysis of nuclear translocation of Smad1, normalized with tubulin in cytosolic fraction. WB results are representative of three independent experiments. Each bar represents the mean $\pm$ SD of three independent experiments. * P < 0.05 and ** P < 0.01 compared with the untreated cells or the indicated groups. Color images available online at www.liebertpub.com/scd

environment are not only TNF- α , but also IL-1 β , IL-6, NO and even more, all of which may play different roles in osteoblastic differentiation of BMSCs [16,45–48]. Furthermore, it was reported that inhibition of local inflammation with special drugs, such as triptolide and bone morphogenetic protein-binding peptide, could significantly enhance the osteoinductive efficacy of BMP-2 in vivo [10–12]. However, direct evidence supporting the inhibitory effect of inflammation on BMP-2-induced osteogenic differentiation is currently lacking. We observed that BMP-2-induced osteogenic differentiation of BMSCs was strongly inhibited in an LPS-stimulated inflammatory environment, which manifested as decreased ALPase activity and up-regulation of osteogenic genes, including ALP, OCN, OPN, COL1A1, Runx2 and osterix. These findings imply that inflammation, which inevitably existed during bone

healing, may be the major cause of the low osteoinductive efficacy of exogenous BMP-2.

According to our study, NF- κ B is over-activated in the LPS-stimulated inflammatory environment. We consider that two mechanisms may be possibly related to NF- κ B activation in this in vitro inflammation model. First, LPS binds to TLR4, transducing the intracellular signals via an MyD88-dependent manner, and subsequently leading to activation of NF- κ B [24,49–51]. Second, the inflammatory cytokines secreted in LPS-induced inflammatory environment, such as TNF- α and IL-1 β , create a cytokines-NF- κ B loop to potentially stimulate NF- κ B activation [52,53]. TLR4 is reported to widely express in immune cells and BMSCs, and regarded as a prerequisite for cell activation by LPS [23,24]. We not only confirmed that TLR4 was expressed on the cell surface of

FIG. 6. Blockade of NF- κ B signaling by specific inhibitors reverses the inhibitory effect of LPS on BMP-2-induced osteogenic differentiation of BMSCs. **(A)** BMSCs were pretreated with BAY-11-7082, TPCK or PDTC for 1 h and then treated with BMP-2 in the presence or absence of LPS for another 7 days. Quantification assay of ALPase activity, normalized with total protein. **(B)** C2C12 cells were transiently transfected with pNF- κ B-Luc along with pRL-TK-Luc for 24 h. Then, the cells were pretreated with BAY-11-7082, TPCK or PDTC for 12 h and then treated with BMP-2 in the presence or absence of LPS for additional 48 h. Luciferase activity assay of cell lysis, normalized versus pRL-TK-Luciferase activity. **(C)** BMSCs were pretreated with BAY-11-7082, TPCK, or PDTC for 1 h, and then treated with LPS for 30 min and followed with BMP-2 for 60 min. WB analysis of p65, Smad1/5/8 phosphorylation and IkB α degradation, normalized to GAPDH. Results are representative of three independent experiments. Results are shown as mean $\pm$ SD. * P < 0.05 and ** P < 0.01 compared with the untreated cells or the indicated groups.

WTd BMSC, but also found that LPS treatment increased TLR4 transcription. An alternative possibility could be that the binding of LPS to TLR4 receptor leads to its degradation and then up-regulates TLR4 gene transcription, which then enhance the responsiveness of BMSCs to LPS stimulation [54]. In mammals, almost all TLRs signal via MyD88, as an adaptor protein for NF- κ B activation [27]. It is previously reported that LPS activated NF- κ B through TLR4/MyD88 signaling [50,51,55]. Thus, down-regulation of MyD88 by siRNA or specific inhibitors should significantly inhibit activation of NF- κ B induced by LPS or other NF- κ B agonists [56,57]. In this study, the robust inhibitory effects on NF- κ B activation by using TLR4 or MyD88 gene knock-out mice or specific antibodies confirmed the crucial roles of TLR4 and MyD88 in activation of NF- κ B signaling.

It is well established that BMP-2-induced osteogenic differentiation is mainly via the Smad-dependent canonical pathway [1,58]. In the canonical BMP/Smad signaling, phosphorylated Smad1/5/8 assemble into complexes with Smad4 and then translocate into the nucleus to activate Runx2-mediated regulation of osteoblast differentiation. In this study, we observed that activation of NF- κ B by LPS hampered BMP-2-induced activation of BMP/Smad signaling, characterized by suppression of pSBE-luciferase activity, inhibition of Smad1/5/8 phosphorylation and nuclear translocation, and eventually down-regulation of osteogenic transcriptional factor, Runx2. These findings are consistent with the results of recent studies [42,59,60], which reported that TNF- α elicited BMPs-induced osteogenic inhibition by suppressing phosphorylation of Smad1/5/8, Smad receptor

FIG. 7. Overexpression of p65 inhibits BMP-2-induced ALPase activity and activation of BMP/Smad signaling. **(A)** C2C12 cells were transiently transfected with p65, p65 siRNA or I κ B α siRNA for 24 h, and then stimulated with BMP-2 in the presence or absence of LPS for 7 days. ALPase staining assay of ALPase activity. **(B, D)** C2C12 cells were transiently transfected with p65, p65 siRNA, I κ B α siRNA and pSBE-Luc for 24 h; then, the cells were incubated with BMP-2 in the presence or absence of LPS for additional 48 h. Luciferase activity assay of cell lysis, normalized versus pRL-TK-Luciferase activity. **(C, E)** C2C12 cells were transiently transfected with p65, p65 siRNA or I κ B α siRNA for 24 h, and then stimulated with LPS for 30 min and followed with BMP-2 for 30 min. WB analysis of p65 and Smad1/5/8 phosphorylation, normalized with GAPDH. Results are representative of three independent experiments. Results are shown as mean $\pm$ SD. ** P < 0.01 compared with the untreated cells or the indicated groups. Color images available online at www.liebertpub.com/scd

transactivation, or attenuating Smad1 activity. Interestingly, it is also reported that TNF- α inhibited BMP/Smad signaling by interfering with the DNA binding of Smad proteins instead of inhibiting phosphorylation of Smad1/5/8 or nuclear translocation of the Smad1/Smad4 complex [32]. In the present study, the LPS-induced inflammatory environment is characterized by secretion of a cocktail of inflammatory cytokines in supernatants. Therefore, the agonists of NF- κ B signaling in LPS-treated medium are much more complex than in TNF- α -treated medium, and that may result in these discrepant results. Moreover, several other factors, such as the differentiation stage of the starting cells, the origin of the cell types and the methods of the experiments, could also be accounted for by the opposing results.

Accumulating evidences implied that NF- κ B signaling is probably involved in transmitting intracellular signaling of Smads [61]. Furthermore, BMP/Smad and NF- κ B signaling system seem to have antagonistic effects on cell growth, apoptosis, and differentiation. However, the exact effect of NF- κ B on BMP/Smad signaling remains ambiguous. Here, we focused on the interplay between NF- κ B and BMP/Smad signaling on regulation of BMP-2-induced osteoblast differentiation. We clearly demonstrated that NF- κ B and BMP/

Smad signaling play conflicting roles in regulation of BMP-2-induced osteoblast differentiation. On one hand, pretreatment with specific NF- κ B signaling inhibitors such as BAY-11-7082, TPCK and PDTC could reverse the inhibitory effect of inflammation via inhibit activation of NF- κ B in BMSCs. On other hand, overexpression of p65 and silencing of p65 or I κ B α gene showed opposite results in BMP-2-induced BMP/Smad signaling and osteoblastic differentiation in C2C12 cells. These results are similar to the results of previous reports that inhibition of NF- κ B in differentiated osteoblasts and Saos2 osteosarcoma cells could substantially increase osteoblastic differentiation and bone formation [33,34], and suggest that crosstalk between NF- κ B and BMP/Smad signaling negatively regulates BMP-2-induced osteoblast differentiation.

In conclusion, the LPS-mediated inflammatory environment inhibits BMP-2-induced osteogenic differentiation through the crosstalk between TLR4/MyD88/NF- κ B and BMP/Smad signaling. Our findings highlight two important points regarding the clinical application of BMP-2: (1) An intracellular balance of signal intensities between NF- κ B and BMP/Smad is crucial for BMP-2-induced osteoblast differentiation; (2) inhibition of the TLR4/MyD88/NF- κ B

FIG. 8. Cartoon describing the possible crosstalk between TLR4/MyD88/NF- κ B and BMP/Smad signaling. BMP-2 induces osteoblast differentiation of BMSCs through activation of Smad1/5/8 signaling, characterized by up-regulation of ALPase activity and the expression of osteoblastic genes such as ALP, COL1A1, OCN, OPN, Runx2 and osterix. LPS activates NF- κ B by increasing IkB α phosphorylation via a TLR4-MyD88-dependent pathway. Meanwhile, inflammatory cytokines such as TNF- α and IL-1 β , both of which are secreted in the LPS-triggered inflammatory environment, create a cytokines-NF- κ B loop to potentially stimulate NF- κ B activity. Ultimately, activated NF- κ B inhibits Smad1/5/8 phosphorylation, resulting in down-regulation of osteogenic markers and suppression of osteogenic differentiation. Color images available online at www.liebertpub.com/scd

pathway may present a new approach for raising the osteoinductive efficacy of BMP-2.

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Author Disclosure Statement

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