

Short communication

**GENE EXPRESSION PATTERNS IN BONE FOLLOWING  
LIPOPOLYSACCHARIDE STIMULATION**

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**Abstract.** Bone displays suppressed osteogenesis in inflammatory diseases such as sepsis and rheumatoid arthritis. However, the underlying mechanisms have not yet been clearly explained. To identify the gene expression patterns in the bone, we performed Affymetrix Mouse Genome 430 2.0 Array with RNA isolated from mouse femurs 4 h after lipopolysaccharide (LPS) administration. The gene expressions were confirmed with real-time PCR. The serum concentration of the N-terminal propeptide of type I collagen (PINP), a bone-formation marker, was determined using ELISA. A total of 1003 transcripts were upregulated and 159 transcripts were downregulated (more than twofold upregulation or downregulation). Increased expression levels of the inflammation-related genes interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were confirmed from in the period 4 h to 72 h after LPS administration using real-time PCR. Gene ontogene analysis found four bone-related categories involved in four biological processes: system development, osteoclast differentiation, ossification and bone development. These processes involved 25 upregulated genes. In the KEGG database, we further analyzed the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway, which is strongly related to osteogenesis. The upregulated bone morphogenetic protein 2 (BMP2) and downregulated inhibitor of DNA binding 4 (Id4) expressions were further confirmed by real-time PCR after LPS stimulation. The osteoblast

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Abbreviations used: BMP2 – bone morphogenetic protein; BMSC – bone mesenchymal stem cells; Cbfa1 – core binding factor 1; Id4 – inhibitor of DNA binding 4; IL-1 $\beta$  – interleukin-1 $\beta$ ; IL-6 – interleukin-6; KEGG – *Kyoto Encyclopedia of Genes and Genomes*; LPS – lipopolysaccharide; PINP – N-terminal propeptide of type I procollagen; OC – osteocalcin ; TGF- $\beta$  – transforming growth factor  $\beta$ ; TNF- $\alpha$  – tumor necrosis factor  $\alpha$

function was determined through examination of the expression levels of core binding factor 1 (Cbfa1) and osteocalcin (OC) in bone tissues and serum PINP from 4 h to 72 h after LPS administration. The expressions of OC and Cbfa1 decreased 6 h after administration ( $p < 0.05$ ). Significantly suppressed PINP levels were observed in the later stage (from 8 h to 72 h,  $p < 0.05$ ) but not in the early stage (4 h or 6 h,  $p > 0.05$ ) of LPS stimulation. The results of this study suggest that LPS induces elevated expressions of skeletal system development- and osteoclast differentiation-related genes and inflammation genes at an early stage in the bone. The perturbed functions of these two groups of genes may lead to a faint change in osteogenesis at an early stage of LPS stimulation. Suppressed bone formation was found at later stages in response to LPS stimulation.

**Keywords:** Lipopolysaccharide, Microarray, Bone, Mouse, Inflammation, Bone morphogenetic protein 2, N-terminal propeptide of type I collagen, Osteogenesis, Core binding factor 1, Osteocalcin

## INTRODUCTION

Sepsis is the main cause of admission to critical care units. Most studies of sepsis focus on the vital organs or systems, such as the circulatory, immune and hematological systems. Although numerous therapeutic strategies for sepsis prevention and treatment have been tested, the mortality rate remains high. Recently, bone has been found to play a critical role in regulating many important physiological functions including hematopoiesis, the immune response and energy metabolism [1–4]. These results suggest bone may play a crucial role in the pathogenesis of sepsis.

Generally, bone resorption activity is enhanced in inflammatory diseases. Inflammation induced by lipopolysaccharides (LPS) mimics bacterial infection and stimulates bone resorption in animals, including humans [5–7], which may be directly mediated by TLR4 activation in osteoclasts or secretion of inflammatory factors [8, 9]. Plasma endotoxin absorption could reduce this elevated bone resorption in sepsis patients [10]. However, the change in bone formation function is controversial in inflammatory diseases. We found decreased bone-specific alkaline phosphatase (bALP) levels in the serum of infected neonates, including septic ones, compared with non-infected neonates [11]. In previous *in vitro* studies, LPS was found to depress bone nodule formation in primary fetal rat calvaria cells and osteoblast differentiation in MC3T3E1 cells [12, 13]. Activated Notch pathway coupling with suppressed canonical Wnt/beta-catenin signaling is involved in this process, as previously reported [13]. By contrast, increased levels of the serum bone formation biomarker N-terminal propeptide of type I procollagen (PINP) were detected in cases of human endotoxemia [7]. The mechanisms of this perturbed osteoblast function in response to LPS stimulation have not been clearly elucidated. Clarification of

the expression pattern of genes in this pathophysiological process is helpful to understand the mechanisms.

In this study, to profile the gene expression patterns in bone at the early stage after LPS simulation, we used gene chip and bioinformatics software to cluster genes into different expression patterns and signaling pathways. Then we detected the expression levels of osteogenesis marker genes with real-time PCR and the concentrations of serum PINP with ELISA to evaluate osteoblast function.

## **MATERIALS AND METHODS**

### **Animals**

Male C57BL/6 mice aged 6 weeks were purchased from the Animal Center of Daping Hospital of the Third Military Medical University. Mice were divided into the LPS group and control group randomly. The LPS group mice were intraperitoneally injected with 15 mg/kg LPS *E. coli* 0111:B4 (Sigma Chemical Co.) while the control group mice were given an equal volume of saline. The Institutional Animal Care and Use Committee of Daping Hospital approved all of the experiments involving animals.

### **Microarray**

A total of 10 mice (5 per group, control or LPS), were used for the microarray experiment. First, total RNA extracted with TRIzol Reagent (Invitrogen Life Technologies) was purified using an RNeasy MinElute Cleanup kit (Qiagen). Double-stranded cDNA was synthesized from 5 µg total RNA and purified with an Affymetrix GeneChip Sample Cleanup Kit. Biotin-labeled cRNA prepared from double-stranded cDNA with Affymetrix IVT Labeling Kit was purified and fragmented to 35–200 nt. An Affymetrix Mouse Genome 430 2.0 Array was hybridized with fragmented biotin-labeled cRNA (0.05 µg/µl) for 16 h at 45°C in an Affymetrix Hybridization Oven 640. Then the washed array was scanned using an Affymetrix GeneChip Scanner 3000. The chip signals were analyzed with Affymetrix GeneChip Operating Software Version 1.4. The bioinformatics analysis was performed using Affymetrix microarray analysis software microarray suite 4 (MAS 4; <http://bioinfo.capitalbio.com/mas/login.do>).

### **RNA extraction**

Total RNA from femurs was isolated with TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Briefly, 5 mice per group were killed by cervical dislocation 4, 6, 12, 24, 48 and 72 h after injection of LPS or 4 h after saline injection. The femurs were rinsed quickly in diethylpyrocarbonate-treated PBS and ground with liquid nitrogen. The bone powder was then mixed with TRIzol Reagent and extracted with chloroform, isopropanol and ethanol in turn to get total RNA. The concentration of total RNA was measured with a spectrophotometer at a 260/280 absorbance ratio (Eppendorf).

### Quantitative real-time PCR

The total RNA was treated with DNase (Qiagen) at 37°C and resuspended in RNase-free ddH<sub>2</sub>O. cDNA (20 µl) was prepared from 1 µg total RNA using an Exscript Reverse kit (TAKARA) according to the manufacturer's protocol. Quantitative real-time PCR was performed with SYBR Primer Ex Taq kit (TAKARA) on an MX3000P PCR machine (Stratgene). The sequences of primers used for quantitative real-time PCR are listed in Table 1. The fold changes of the tested genes were normalized against the cycle threshold (Ct) values of cyclophilin A based on dRn.

Table 1. Real-time PCR primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
TNF- $\alpha$	TCCCCAAAGGGATGAGAAGTTC	TCATACCAGGGTTTGAGCTCAG
IL-1 $\beta$	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
IL-6	TTCCATCCAGTTGCCTTCTT	CAGAATTGCCATTGCACAAC
BMP2	TGAGGTGCACAGACTTGGA	TGTACGCGTGAATGACCTA
Id4	CAGTGCATATGAACGACTGC	GACTTTCTTGTTGGGCGGGAT
Cbfa1	TGAGGAAGAAGCCCATTAC	ACTTCTTCTCCCGGTGTG
OC	TCTGACAAAGCCTTCATGTCC	AAATAGTGATACCGTAGATGCG
Cyclophilin A	CGAGCTCTGAGCACTGGAGA	TGGCGTGTAAGTCACCACC

### Enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained from the orbital sinus of 5 mice per group 4, 6, 12, 24, 48 and 72 h after LPS administration or 4 h after saline injection under pentobarbital anesthesia. Serum was collected and stored at -70°C until use. Levels of PINP were quantified with a Mouse PINP EIA Kit (IDS) according to the manufacturer's protocol.

### Statistical analysis

All the statistical analyses were carried out with SPSS 11.0 software. One-sample T tests were used to compare the gene expression levels between groups. One-way ANOVA tests were used to compare serum PINP levels.  $p < 0.05$  was considered significant.

## RESULTS

### Gene expression patterns of LPS-treated femurs

To identify the profiles of gene expression in bone induced by LPS, total RNA of mouse femur was isolated 4 h after LPS administration. The expressions of a total of 45037 transcripts were examined with a microarray. The subset of transcripts with a greater than +/-2-fold change in expression and  $p < 0.05$  (Student's t-test) between groups were defined as being regulated. Of the 1162

transcripts considered to be differentially expressed, 1003 were upregulated and 159 were downregulated 4 h after LPS administration. Persistent increased expression patterns of three representative cytokine genes interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were detected using real-time PCR from 4 to 12 h after LPS stimulation (Fig. 1A–C).

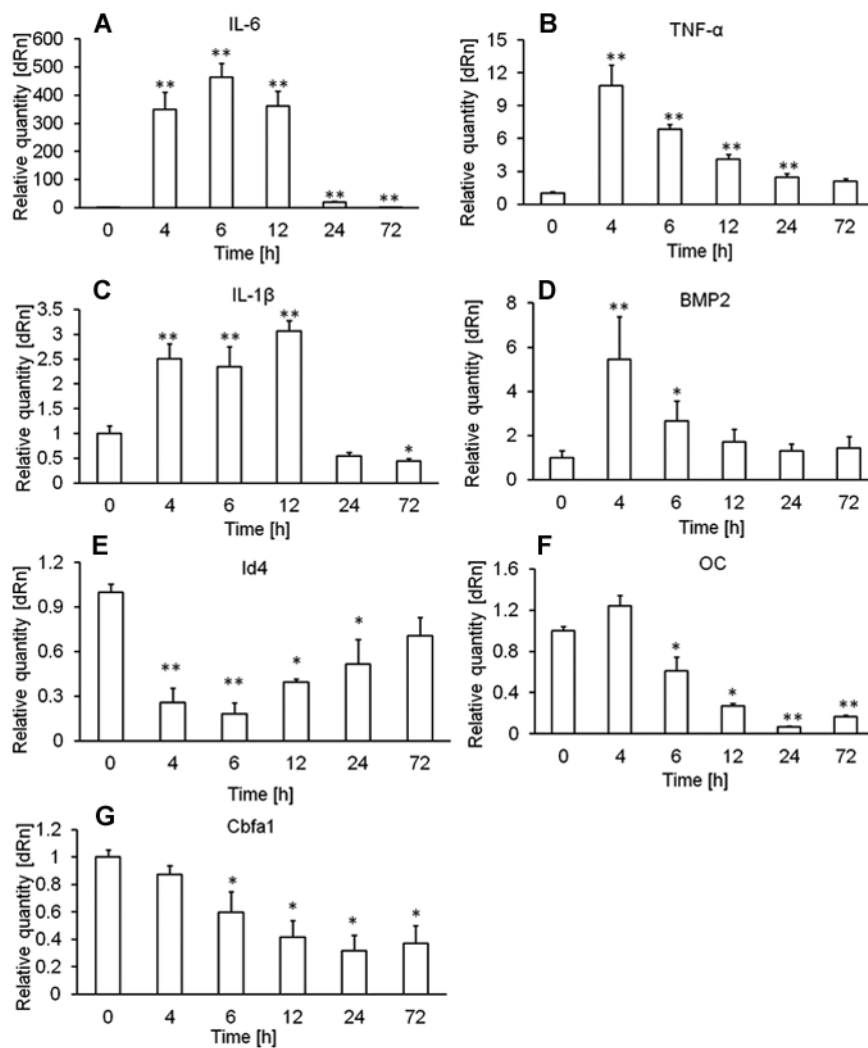


Fig. 1. Expression levels of the preinflammatory mediator genes BMP2 and Id4 and the osteogenic marker genes in mice femurs after LPS administration. A–C – The expression level of TNF- $\alpha$  peaked at 4 h, while those of IL-6 and IL-1 $\beta$  respectively peaked 6 h and 12 h after LPS stimulation. D–E – Similar to chip results, increased expression of BMP2 and suppressed expression of Id4 were detected from 4 to 6 h after LPS stimulation. F–G – Significantly decreased expression levels of cbfa1 and OC were observed from 6 to 72 h after LPS stimulation. \* $p < 0.05$ , \*\* $p < 0.01$  compared with the control group.

### Gene ontology (GO) analysis

To further explore microarray data, GO analysis was performed using Molecule Annotation System (MAS) V4.0 software. After applying a p-value cut-off of 0.05, 320 GO categories in biological processes domains were considered as significantly over-represented. Four bone-related categories (skeletal system development, osteoclast differentiation, ossification and bone development) involved 25 upregulated genes, such as bone morphogenetic protein 2 (BMP2), vitamin D receptor (VDR) and bone sialoprotein (IBSP; Table 2).

Table 2. The classification of differentially expressed genes in bone after LPS stimulation (assessed using GO analysis).

GO name	P-value	Count in category	Gene symples
Skeletal system development	0.002	22	BMP2, VDR, IBSP, TBX3, PTGS2, ARID5B, TIPARP, EDN1, COL2A1, ZBTB16, MMP13, COL9A1, TNFSF11, HIF1A, CTGF, PDGFRA, ACAN, UBD, ROR2, RIPPLY2, IGFBP3, MYC
Osteoclast differentiation	0.003	4	CALCR, TNF, TNFSF11, CD300LF
Ossification	0.017	10	BMP2, IBSP, TNFSF11, PTGS2, CTGF, UBD, COL2A1, RIPPLY2, IGFBP3, MMP13
Bone development	0.032	10	IBSP, BMP2, TNFSF11, PTGS2, CTGF, UBD, COL2A1, RIPPLY2, IGFBP3, MMP13

Table 3. Fold change in the expression of genes involved in the TGF- $\beta$  signaling pathway at the early stage of LPS stimulation in bone.

Gene	Description	Fold change
ACVR1	Activin A receptor, type 1	2.18
BMP2	Bone morphogenetic protein 2	2.79
FST	Follistatin	4.49
Id2	Inhibitor of DNA binding 2	2.13
Id3	Inhibitor of DNA binding 3	2.04
Id4	Inhibitor of DNA binding 4	-2.12
IFNG	Interferon gamma	2.64
INHBA	Inhibin beta-A	2.49
INHBB	Inhibin beta-B	3.33
LTBP1	Latent transforming growth factor beta binding protein 1	2.13
Myc	Myelocytomatosis oncogene	2.29
THBS1	Thrombospondin 1	3.13
TNF	Tumor necrosis factor	2.37

### ***Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis***

The bioinformation analysis using MAS software also highlighted pathways that were significantly affected by LPS. According to the KEGG database, a total of 236 differentially expressed genes were recruited in 46 pathways when applying a p-value cut-off of 0.01.

Transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway, a well-known osteogenesis regulator, was further analyzed (Table 3). Of the thirteen differentially expressed genes involved in the TGF- $\beta$  pathway, Id4 was the only downregulated gene. A similar expression change of BMP2 and Id4 was also detected using real-time PCR (Fig. 1D, E). The peak expression level of BMP2 was observed at 4 h ( $p < 0.001$ ). There was no significant difference in the BMP2 expression levels between the 12, 24, 72 h and saline groups. Suppressed Id4 expression levels were observed from 4 to 24 h ( $p < 0.05$ ).

### **Change of osteoblast function in response to LPS stimulation**

According to the microarray results, the known osteogenesis marker genes, such as core binding factor alpha 1 (Cbfa1) and osteocalcin (OC) had no significant changes. The expressions of these genes were examined using real-time PCR over a time course of 4–72 h (Fig. 1F–H). Compared with the saline group, the expression levels of Cbfa1 showed no significant changes, while that of OC was slightly increased at 4 h. LPS significantly suppressed the mRNA expression of Cbfa1 and OC 6 h after administration ( $p < 0.05$ ).

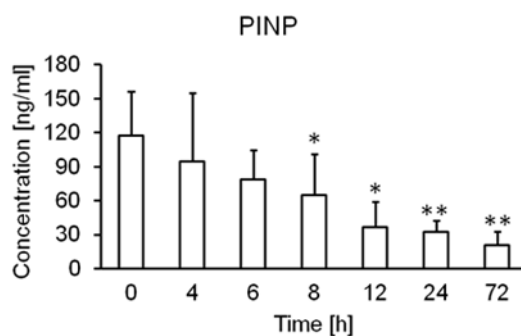


Fig. 2. The serum concentration of PINP after LPS administration. A significantly decreased serum level of PINP was observed from 8 to 72 h after LPS stimulation. \* $p < 0.05$ , \*\* $p < 0.01$  compared with saline group.

To further assess the effect of LPS on bone formation *in vivo*, the serum concentration of PINP was measured with ELISA. LPS time-dependently reduced the serum PINP concentration (Fig. 2). The serum PINP level significantly decreased from  $117.0 \pm 39.54$  to  $65.14 \pm 35.87$  ng/ml 8 h after LPS stimulation ( $p = 0.022$ ) but the levels at 4 h ( $94.8 \pm 59.95$  ng/ml,  $p = 0.593$ ) and 6 h ( $78.4 \pm 26.30$  ng/ml  $p = 0.361$ ) showed no statistical difference compared with the saline group. Significant decreases in PINP were also observed at 12 h ( $p = 0.037$ ), 24 h ( $p = 0.034$ ) and 72 h ( $p = 0.007$ ).

## DISSUSSION

Bone displays active bone remodeling in many inflammatory diseases. Generally, LPS and cytokines induced by LPS could directly enhance osteoclast function and increase bone resorption. However, the effects of LPS on osteoblasts are controversial. Previous studies reported that LPS could enhance bone formation and depress the expressions of osteogenesis marker genes, such as ALP and OC [7, 12]. The mechanisms underlying the perturbed osteoblast function are not clearly clarified. In this study, we used microarrays to explore the expression patterns of bone in LPS-treated mice.

We found that a relatively high proportion of genes exhibit a significant detectable difference in transcription levels (1003 upregulated and 159 downregulated, with a greater than  $\pm 2$  fold change) at an early stage of LPS stimulation. Persistently elevated expression levels of the three represented inflammatory genes, IL-6, IL-1 $\beta$  and TNF- $\alpha$ , were found at both early and later stages of LPS stimulation. As previously reported, a variety of cytokines are known to negatively regulate osteoblast function. TNF- $\alpha$  and IL-1 inhibit the differentiation of osteoblasts and bone mesenchymal stem cells (BMSCs) [14–16]. These cytokines also inhibit collagen synthesis and increase the apoptosis of osteoblasts [17–20]. However, the mechanisms of IL-6, IL-1 $\beta$  and TNF- $\alpha$  suppress bone formation are not clear and need to be studied.

GO analysis revealed that most of the significantly altered genes were involved in inflammation, the immune response and apoptosis. We also found 25 upregulated genes involved in four bone-related categories (skeletal system development, osteoclast differentiation, ossification and bone development). In line with this, KEGG analysis also highlighted the TGF- $\beta$  pathway, a well-known bone formation-related pathway. Thirteen differentially induced genes are involved in the TGF- $\beta$  pathway. The expression pattern of BMP2 was also confirmed with real-time PCR. BMP2 is known to play an important role in the embryonic development of neural crest, heart and limb bud [21–23]. BMP2 also contributes to postnatal bone formation. It could enhance osteoblast proliferation and differentiation [24].

Id2 and Id3, which were previously defined as the target genes of BMP2, were upregulated, while Id4 was downregulated. According to previous studies, BMP2 had different effects on the expression of Id4 mRNA depending on the cell types. BMP2 induced upregulation of Id4 in RD-C6 cells, while inhibition of its expression was observed in MC3T3E1 cells [25, 26]. Id4 is known as an essential factor for neural differentiation and lymphocyte development [27, 28]. However, the role of Id4 in osteoblastic differentiation has not been clarified. It will be important to investigate the regulatory mechanism of Id4 during LPS-induced osteoblastic differentiation.

Considering the perturbed roles of inflammation genes and BMP2 in osteogenesis, we detected the expressions of osteogenic genes and serum bone formation marker. Similarly to some previous studies, our findings show that



*E. coli* LPS time-dependently induced suppressed bone formation in mice. Significantly decreased expression levels of the osteogenic marker genes OC and Cbfa1 were observed at a later stage after LPS stimulation. Consistent with a change in bone osteogenic marker gene expressions, LPS also time-dependently suppressed the bone formation biomarker levels of PINP. This was in line with our previous finding that the serum levels of another bone formation marker, bALP, were lower in infected neonates than in non-infected ones [11]. Another study found increased PINP coupling with decreased C-terminal telopeptide of type I collagen (CTX), a marker for bone resorption, 6 h after LPS administration in humans [7]. This may be due to the different experimental conditions, such as observing time-point and LPS dosage.

Huang et al. found that LPS could inhibit BMP-2-induced osteogenesis, which is represented by suppressed ALP activity and downregulated osteogenic genes in bone marrow mesenchymal cells [29]. Thus, the suppressed osteogenesis function observed at the later stage of LPS administration may be due to persistent increased expression levels of inflammatory genes.

In conclusion, we found that LPS induces elevated expressions of inflammation genes and skeletal system development-related genes at an early stage in bone tissue. The opposite functions of these genes on the bone may lead to a slight change in osteogenesis at the early stage of LPS stimulation.

**Acknowledgement.** This study was supported by the Fundamental and Frontier Research Project of Chongqing, China (CSTC2013jcyjC00009) and Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT) (IRT1216).

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