Suppressor of cytokine signaling 3 (SOCS3) is an important intracellular protein that inhibits cytokine signaling in numerous cell types and has been implicated in several inflammatory diseases. However, the expression and function of SOCS3 in osteoblasts are not known. In this study, we demonstrated that SOCS3 expression was transiently induced by LPS in osteoblasts, and apparently contributed to the inhibition of IL-6 induction by LPS treatment. We found that tyrosine 204 of the SOCS3 box, the SH2 domain, and the N-terminal kinase inhibitory region (KIR) of SOCS3 were all involved in its IL-6 inhibition. Furthermore, we demonstrated that CCAAT/enhancer-binding protein (C/EBP) β was activated by LPS (increased DNA binding activity), and played a key role in IL-6 expression in osteoblasts. We further provided the evidence that SOCS3 functioned as a negative regulator for LPS response in osteoblasts by suppressing C/EBPβ DNA binding activity. In addition, tyrosine 204 of the SOCS3 box, the SH2 domain, and the N-terminal kinase inhibitory region (KIR) of SOCS3 were all required for its C/EBPβ inhibition. These findings suggest that SOCS3 by interfering with C/EBPβ activation may have an important regulatory role during bone-associated inflammatory responses.

SOCS3 belongs to the family of suppressors of cytokine signaling (SOCS)2 proteins, which is induced by a number of mediators, including LPS, TNF-α, as well as IL-6 and IL-10 (1–3). SOCS3 has been shown to function as a proinflammatory mediator by suppressing IL-6-gp130 signaling, interfering with its ability to inhibit LPS signaling (4, 5). For example, mice lacking SOCS3 in macrophages and neutrophils are resistant to LPS-induced shock (4). In contrast, accumulating data suggest that SOCS3 may suppress inflammatory responses (6). Thus, the function of SOCS3 during inflammation seems to be dependent on the particular disease model used and cell type studied. Moreover, the precise role of SOCS3 in LPS responses remains enigmatic.

The stimulation of Toll-like receptor (TLR) 4 by LPS plays a critical role in innate immune responses in mammals. Although most studies on LPS-induced inflammation and the ensuing tissue destruction have been focused on immune systems, recent studies demonstrate that osteoblasts also express functional TLR4, which may play an important role in the pathogenesis of LPS-mediated bone disorders (2, 7, 8). For example, LPS stimulates osteoblasts to secrete receptor activator of NF-κB ligand (RANKL), IL-6, IL-1, TNF-α, GM-CSF, and PGE2, each of which seems to be involved in LPS-mediated bone resorption (9). Among these proinflammatory mediators, IL-6 regulation in bone is extremely important for tissue homeostasis. Inappropriate expression of IL-6 has been suggested to have an impact on the increase in bone resorption observed in several bone inflammatory diseases (10, 11). Stimulation of IL-6 mRNA synthesis by LPS in human osteoblasts has been suggested to occur through CD14, p38 MAPK, and MEK (12). Several transcription factors such as NF-κB and CCAAT/enhancer-binding protein (C/EBP)β seem to be involved in IL-6 gene regulation in osteoblasts (13, 14).

C/EBPβ belongs to a family of basic region-leucine zipper (bZIP) transcription factors comprised of C/EBPα, β, δ, ε, γ, and ζ. These proteins dimerize through their leucine zippers and bind to DNA through their adjacent basic regions. C/EBPβ has been implicated in the regulation of proinflammatory cytokines as well as other gene products associated with the activation of macrophages and the acute phase inflammatory responses (15). For example, C/EBPβ has been shown to activate a reporter gene controlled by the IL-6 promoter in transient expression assays (16, 17). Furthermore, the stable expression of C/EBPβ in a murine B lymphoblast cell line is sufficient to confer LPS inducibility of IL-6 expression (18), and the activity of the C/EBPβ bZIP in the absence of N-terminal motifs...
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required the NF-κB site of the IL-6 promoter (19). Interestingly, we have previously demonstrated that augmentation of C/EBPβ activity on the IL-6 and IL-8 promoters by C/EBPγ required formation of a heterodimeric leucine zipper and corepression of NF-κB (20). All of these studies suggested a mechanism for IL-6 activation whose essential feature is the requirement for the bZIP region of C/EBPβ to synergize with NF-κB, although this remains to be further investigated. Interestingly, recent data demonstrate that C/EBPβ inhibits NF-κB-mediated transcription in TNF-α tolerant cells by blocking p65 phosphorylation (21). These data, taken together, suggest that C/EBPβ has complex effects during the inflammatory responses. Furthermore, the role of C/EBPβ in LPS-induced bone inflammation remains investigated.

Given the important but variable roles for SOCS3 and C/EBPβ in inflammation, this study was designed to examine their possible contribution to LPS-stimulated IL-6 expression in osteoblasts. We demonstrated, for the first time, that SOCS3 expression was transiently induced by LPS in osteoblasts, and apparently contributed to the inhibition of IL-6 induction by LPS treatment. We found that tyrosine 204 of the SOCS box, the SH2 domain, and the KIR of SOCS3 were all involved in its IL-6 inhibition. In addition, we showed that C/EBPβ was activated by LPS, and functioned as a key regulator of LPS-induced IL-6 expression in osteoblasts. Furthermore, we found that SOCS3 inhibited LPS-induced IL-6 expression in osteoblasts by suppressing C/EBPβ activity. We further showed that tyrosine 204 of the SOCS box, the SH2 domain, and the KIR of SOCS3 were all required for its inhibition of C/EBPβ DNA binding activity. The data suggest that SOCS3 by interfering with C/EBPβ activation such as DNA binding may have an important regulatory role during bone-associated inflammatory responses.

MATERIALS AND METHODS

Cells and Reagents—Osteoblast-like MC3T3-E1 cells were obtained from American Type Culture Collection (CRL-2593™), and cultured in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FBS), and maintained in a humidified incubator at 37 °C with 5% CO₂. Culture of bone marrow stromal/osteoblastic cells (BMSC) has been described previously (22). Briefly, one tibia and one femur from each mouse were immersed in 70% ethanol, and stored in primary medium (α-MEM containing L-glutamine, nucleosides, supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% fungizone) temporarily before BMSC were isolated and cultured as outlined below. The bones were rinsed four times (2 min each) in a calcium- and magnesium-free PBS (PBS-CMF) containing antibiotics (penicillin-streptomycin) and fungizone under sterile conditions. The epiphyses of each bone were removed with a razor blade. The marrow was flushed out from the diaphysis, and collected in primary culture medium. The marrow cell suspension was gently drawn through an 18-gauge needle to mechanically dissociate the mixture into a single cell suspension. The cells were plated at 10 x 10⁶ cells/10 cm tissue culture dish. On day 5, nonadherent cells were removed by aspiration, and adherent cells (osteoblast-like cells) were replenished with secondary medium (primary medium supplemented with 50 μg/ml l-ascorbic acid and 3 mM β-glycerophosphate) to induce mesenchymal cells to form osteoblasts. LPS (Escherichia coli, serotype 0111:B4) was purchased from Sigma.

Expression Vectors and Promoter Reporters—Construction and amplifying of recombinant adenosine containing mouse SOCS3 (pLP-Ad-SOCS3) was described previously (23). Adenoviral siRNA for SOCS3 was generated by Welgen under the control of the cytomegalovirus promoter. Recombinant adenosine viruses were purified by BD Adeno-X virus purification kit (BD Biosciences, Palo Alto, CA), and stored in aliquots at −80 °C. The viral stocks were titered using Adeno-X Rapid Titer Kit (Biosciences, Palo Alto, CA). The mouse IL-6 promoter-lucerase construct (−250 to +1), IL-6 promoter-lucerase construct harboring a mutated NF-κB binding site, C/EBPβ-dependent promoter-lucerase construct, the DEI-4 (DEI4 (-35alb)LUC) containing four copies of a C/EBP binding site tandemly arrayed upstream of the albumin minimal promoter, as well as the expression vectors for C/EBPβ and C/EBPγ were kindly provided by Richard C. Schwartz (Michigan State University). Mouse IL-6 promoter-lucerase construct harboring a mutated C/EBP binding site (−161 to −147) was kindly provided by Gail A. Bishop (University of Iowa). NF-κB-dependent promoter-lucerase construct was obtained from Promega, Madison, WI. SOCS3 mutants in various domains have been described previously (24).

Luciferase Assay—Transient transfections were performed with 4 x 10⁴ cells plated in 12-well plates by using 0.5 μg of DNA and 1.5 μl of Fugene® 6 Transfection Reagent (Roche, Indianapolis, IN) in 50 μl of Opti-MEM I medium (Invitrogen, Carlsbad, CA). Unless otherwise indicated, 24 h after transfection, the cells were either incubated with or without LPS for the indicated time. Cell lysates were subjected to luciferase activity analysis by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

ELISA-MC3T3—E1 cells were stimulated by LPS for the indicated time. The supernatants were centrifuged at 900 x g for 5 min. The cell-free supernatants were harvested for IL-6 measurement by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

RNA Isolation and Detection of mRNA by Semi-quantitative RT-PCR—Total RNAs were extracted from cells with Trizol (Invitrogen). After isolation, total cellular RNA was incubated with RQ1 RNase-free DNase (Promega) to remove contaminating DNA. Reverse transcription was performed with 2 μg of RNA using the Superscript II RNase H- Reverse Transcriptase (Invitrogen). PCR was performed with primers for SOCS3: 5’ primer, 5’-CCC GCG GGC ACC TTG CTT A-3’ and 3’ primer, 5’-AGG CAG CTT GGT CAC TTT CTCATAA-3’; C/EBPβ: 5’ primer, 5’-CAA GCT GAG CGA CGA GTA CA-3’ and 3’ primer, 5’-AGC TGC TCC ACC TTC TTG TG-3’; GAPDH: 5’ primer, 5’-GCC TCG TCT CAT AGA CAA GAT G-3’ and 3’ primer, 5’-CAG TAG ACT CCA CGA CAT AC-3’. After a “hot-start” for 5 min at 94 °C, 28–33 cycles were used for amplification with a melting temperature of 94 °C, an annealing temperature of 60 °C, and an extending temperature of 72 °C, each for 1 min, followed by a final extension at 72 °C for 8 min. PCR was performed using different cycle numbers for all primers, to
assure that DNA was detected within the linear part of the amplifying curves for both primers.

**Western Blot Analysis**—MC3T3-E1 cells were lysed in ice-cold radioimmunoprecipitation (RIPA) buffer. Samples containing 50 μg protein were electrophoresed in a 10% polyacrylamide gel and then transferred to a PVDF membrane. Membranes were incubated with rabbit anti-SOCS3 antibody (Santa Cruz Biotechnology), rabbit anti-C/EBPβ antibody (Santa Cruz Biotechnology), and rabbit anti-GAPDH antibody (Cell Signaling, Boston, MA), respectively. After three washes in TBST, the membranes were incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Piscataway, NJ). The membrane was developed by enhanced chemiluminescence technique (Thermo Fisher Scientific, Rockford, IL).

**siRNA Transfection**—MC3T3-E1 cells were transfected by using Lipofectamine™ 2000 (Invitrogen) with 40 nM control siRNA or C/EBPβ siRNA (Santa Cruz Biotechnology). 24 h after transfection, the cells were incubated with 100 ng LPS/ml for 4 h. RNAs were harvested for RT-PCR to analyze C/EBPβ expression. Supernatants were collected for ELISA.

**Adenovirus Transfection**—Cells were grown to 90% confluence, and infected with various adenovirus (Adeno-X-DsRed2, pLP-Ad-SOCS3, Ad-Control-sh, and Ad-SOCS3-sh) at 200 multiplicity of infection (MOI) for 4 h before adding fresh medium. 48 h later, proteins or RNAs were harvested for the analysis of SOCS3 expression. In some experiments, the cells were treated with 100 ng LPS/ml for the indicated time. The supernatants were collected for ELISA analysis.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts of MC3T3-E1 cells were prepared as follows. Cells were lysed in 15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Roche, Indianapolis, IN) for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 × g for 20 s at 4 °C. Proteins were extracted from nuclei by incubation at 4 °C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Roche, Indianapolis, IN). Protein concentrations were determined by BioRad protein assay kit (Thermo Fisher Scientific). The EMSA probes were double-stranded oligonucleotides containing a murine IL-6/C/EBP binding site (5'-CTAAAACGACGTACAGGTCACTTAAATAGGT-3') annealed with 5'-TGGAAACCTTATTAAGATTG-CACAATGTGACGTCACATTGTGCAATCTTAATAAG-3', kindly provided by Richard Schwartz, Michigan State University), or a NF-κB consensus oligonucleotide (AGTTGGGAGGACTTTCCAGGC, Promega, Madison, WI). C/EBP probes were labeled with α[³²P]ATP (3,000 Ci/mmol at 10 mCi/ml, GE Healthcare, Piscataway, NJ). NF-κB probes were labeled with γ[³²P]ATP (3,000 Ci/mmol at 10 mCi/ml, GE Healthcare). DNA binding reactions were performed at room temperature in a 25-μl reaction mixture containing 6 μl of nuclear extract (1 ng/ml in buffer C) and 5 μl of 5X binding buffer (20% (w/v) Ficoll, 50 mM HEPES pH 7.9, 5 mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained KCl at a final concentration of 50 mM, Nonidet P-40 at a final concentration of 0.1%, 1 μg of poly (dl-dC), 200 pg of probe, bromphenol blue at a final concentration of 0.06%, Samples were electrophoresed through 5.5% polyacrylamide gels and dried under vacuum. For supershifts, nuclear extracts were preincubated with antibodies (1 to 2 μg) for 0.5 h at 4 °C prior to the binding reaction. The following antibodies were purchased from Santa Cruz Biotechnology: anti-p50, anti-p52, anti-p65, anti-RelB, anti-c-Rel, anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ, anti-C/EBPε, and anti-C/EBPγ antibodies.

![FIGURE 1. Silencing LPS-induced expression of SOCS3 enhances IL-6 secretion in MC3T3-E1 cells. A, MC3T3-E1 cells were incubated with 100 ng LPS/ml for indicated times. Total cellular RNA was isolated for RT-PCR with primers for SOCS3 and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. B, total proteins were extracted to conduct Western blot using rabbit anti-SOCS3 antibody, and rabbit anti-GAPDH antibody, respectively. The level of GAPDH was shown at the bottom as a loading control. C, MC3T3-E1 cells were infected with Ad-Control-sh or Ad-SOCS3-sh at an MOI of 200. 48 h after infection, the cells were incubated with 100 ng LPS/ml for 1 h. RNAs were isolated, and RT-PCR was performed by using primers for SOCS3 and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. D, MC3T3-E1 cells were infected with Ad-Control-sh or Ad-SOCS3-sh at a MOI of 200. 48 h later, cells were stimulated by 100 ng LPS/ml for indicated times, and supernatants were harvested and subjected to ELISA. Data were means of six independent experiments ± S.E. **, p < 0.01; *** p < 0.001 compared with Ad-Control-sh-infected group.](image-url)
FIGURE 2. Overexpression of SOCS3 inhibits LPS-mediated IL-6 production in MC3T3-E1 cells. A, MC3T3-E1 cells were infected with pLP-Ad-SOCS3 at indicated MOI. 48 h after infection, the total protein extracts were subjected to Western blot using antibodies against SOCS3 and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. B, MC3T3-E1 cells were infected with Adeno-X-DsRed2 or pLP-Ad-SOCS3 at indicated MOI. 48 h later, the cells were stimulated either with or without 100 ng LPS/ml for 6 h. The supernatants were used to perform ELISA to determine IL-6 protein level. Data were expressed as means ± S.E., of six independent experiments. C, MC3T3-E1 cells were infected with Adeno-X-DsRed2 or pLP-Ad-SOCS3 at a MOI of 200. 48 h later, the cells were incubated with 100 ng/ml LPS for 4 h. Then proteins were harvested and subjected to Western blot using antibodies against SOCS3 and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. D, primary osteoblasts were infected with Adeno-X-DsRed2 and pLP-Ad-SOCS3 at 200 MOI, respectively. The total proteins were harvested 48 h after infection, and Western blot was conducted using antibodies against SOCS3 and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. E, primary osteoblasts were infected with Adeno-X-DsRed2 and pLP-Ad-SOCS3 at 200 MOI, respectively. 48 h later, the cells were stimulated either with or without 100 ng LPS/ml for 6 h. IL-6 secretion was measured by ELISA. Data were means of six independent experiments ± S.E. *** indicates a statistically significant difference (p < 0.001).
Pulse-chase Analysis—MC3T3-E1 cells were infected with Adeno-X-DsRed2 or pLP-Ad-SOCS3 at a MOI of 200. 48 h later, medium was removed, and the cells were washed twice with PBS. Met/Cys-free DMEM (MP Biomedicals, Solon, OH) supplemented with 5% dialyzed fetal calf serum (Hyclone, South Logan, UT) was added, and the cells were incubated for 2 h at 37 °C with 5% CO₂. Subsequently, the cells were incubated for 2 h in medium containing 160 μCi/ml of trans[^35]S-label (MP Biomedicals, Solon, OH) in the presence or absence of 100 ng LPS/ml. Radiolabeled proteins were then chased for 5 h in fresh complete medium. The cell lysates were immunoprecipitated by using anti-C/EBPβ antibody and separated on 10% SDS-polyacrylamide gel. The dried gel was exposed for autoradiography.

Statistical Analysis—All values are expressed as the mean ± S.E. Significance was assigned where p < 0.05. Data sets were analyzed using Student’s t test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

RESULTS

Expression of SOCS3 in MC3T3-E1 Cells Mediated Inhibition of LPS-stimulated IL-6 Secretion—LPS is an efficient inducer of SOCS3 expression in both macrophages and neutrophils (2, 4). To determine if the expression of SOCS3 at mRNA level could be induced by LPS in osteoblasts, we treated the MC3T3-E1 cells with LPS for different time periods, and then conducted RT-PCR experiments. As shown in Fig. 1A, SOCS3 mRNA expression was dramatically induced by LPS at 0.5 and 1 h time points, and its expression was gradually decreased to undetectable level 12 h after LPS stimulation. Western blot was then performed to identify the SOCS3 protein expression after LPS challenge. The data showed that the SOCS3 was induced 0.5 h after LPS treatment, and the protein level was gradually reduced 2 h later (Fig. 1B). The regulation of TLR4 signaling by SOCS3 is unclear, and the available data are controversial. Therefore, we sought to determine whether SOCS3 expression in osteoblasts has any effect on LPS-induced IL-6 secretion. To that end, we first showed that LPS-induced SOCS3 expression was significantly down-regulated by adenosivirus-mediated shRNA for SOCS3 (Ad-SOCS3-sh) when compared with that treated with adenosivirus expressing the control shRNA (Ad-Control-sh) in MC3T3-E1 cells (Fig. 1C). MC3T3-E1 cells infected with Ad-control-sh or Ad-SOCS3-sh were then stimulated with LPS for various periods. As shown in Fig. 1D, MC3T3-E1 cells expressed undetectable amounts of IL-6 without LPS stimulation. However, IL-6 secretion was gradually enhanced in a time-dependent fashion when the cells were incubated with LPS (Fig. 1D). Importantly, Ad-SOCS3-sh infected MC3T3-E1 cells consistently exhibited enhanced IL-6 secretion when compared with cells infected with Ad-Control-sh (Fig. 1D). The augmentation rate from 2.5 h to 6 h in Ad-SOCS3-sh-infected cells was more than 2-fold of that in control group (Fig. 1D).

To further confirm the negative influence of SOCS3 on IL-6 secretion, we infected MC3T3-E1 cells with adenosivirus that could induce SOCS3 expression (pLP-Ad-SOCS3). As shown in Fig. 2A, cells infected with pLP-Ad-SOCS3 exhibited high level of SOCS3 protein expression in a MOI-dependent manner. We then showed that expression of SOCS3 dramatically repressed LPS-induced IL-6 secretion (~75%) at a MOI of 50 (Fig. 2B). IL-6 secretion was further inhibited with the increase of MOI. When cells were infected with pLP-Ad-SOCS3 at a MOI of 400, IL-6 level was reduced to almost undetectable level. We next compared adenosivirus-mediated and LPS-induced SOCS3 expression in MC3T3-E1 cells. As shown in Fig. 2C, pLP-Ad-
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SOCS3-mediated SOCS3 expression was comparable to LPS-induced endogenous SOCS3 expression in the cells, and LPS treatment further enhanced SOCS3 expression. Finally, the inhibitory effect of SOCS3 on LPS-induced IL-6 secretion was observed in primary osteoblasts (Fig. 2, D and E). Taken together, these results support the negative role of SOCS3 on LPS-induced IL-6 secretion in osteoblasts.

KIR, Tyrosine 204 of the SOCS Box, and SH2 domain Are Indispensable for SOCS3-mediated Inhibition of IL-6 Expression—We further examined the ability of SOCS3 inhibition on IL-6 expression in transient transfections with an IL-6 promoter-luciferase construct. As with the endogenous IL-6 promoter, LPS treatment induced luciferase expression by over 4-fold (Fig. 3A). However, SOCS3 expression resulted in an over 60% decrease in luciferase expression induced by LPS treatment (Fig. 3A). SOCS3 contains three conserved domains: SH2 domain, KIR, and the SOCS3 box which have been shown to play roles in distinct LPS-stimulated signal transduction events in macrophages (25). We thus examined the dispensability of various SOCS3 domains for its inhibitory role on LPS-induced IL-6 expression by luciferase assay. As shown in Fig. 3B, wild-type SOCS3 greatly impaired luciferase expression in response to LPS treatment. In contrast, SOCS3 carrying a mutation in the KIR (L22D), the SOCS3 box (Y204F), or the SH2 domain (R71E) failed to repress luciferase expression, while other mutations in SOCS3 box (Y211F and Cis DC41) had no influence on SOCS3-mediated impairment of luciferase expression in response to LPS (Fig. 3B). These results indicate that KIR, tyrosine 204 of the SOCS Box, and SH2 domain are all required for its inhibition of IL-6 expression in MC3T3-E1 cells.

C/EBPs and NF-κB Are Indispensable for IL-6 Expression in MC3T3-E1 Cells—The above data suggested that IL-6 expression in osteoblasts was up-regulated by LPS at the transcription level. It has been shown that C/EBPβ and NF-κB can synergistically activate the IL-6 promoter requiring the cognate binding sites of both factors (26). To determine whether this synergistic effect exists in osteoblasts, we first examined the activation of C/EBPs and NF-κB by LPS treatment. As shown in Fig. 4A, strong NF-κB DNA binding activity (mainly p65, data not shown) was induced as early as 0.5 h after LPS stimulation, and lasted for 1 h. NF-κB DNA-binding activity was then gradually decreased. LPS treatment also led to the induction of C/EBP DNA-binding activity in the MC3T3-E1 cells (Fig. 4A). The C/EBPβ gene can produce several N-terminally truncated isoforms including Liver-enriched activator protein (LAP) and liver-enriched inhibitory protein (LIP). LAP is a transcriptional activator in many systems, whereas LIP is regarded as a functional LAP antagonist. Using supershift assay, we found that C/EBP complex contained C/EBPβ, C/EBPδ, and C/EBPγ, both in LPS treated and untreated cells (Fig. 6C). Furthermore, we found that both C/EBPβ and C/EBPδ DNA binding activity (LAP/LIP, C/EBPδ/δ, LAP/LIP, and C/EBPδ/LIP) to the IL-6 C/EBP binding site increased dramatically at 2 h and steadily over time with LPS treatment (Figs. 4B and 6C). Interestingly, LPS reduced the DNA binding activity of C/EBPγ (Fig. 6C), supporting the general belief that it is a trans-dominant inhibitor of C/EBP activators (27). Taken together, these results indicated that DNA binding activities of both NF-κB and C/EBPs were induced by LPS in the osteoblasts, and they might be involved in LPS-induced IL-6 expression.

We next examined whether forced expression of NF-κB p65 and/or C/EBPβ could affect the IL-6 promoter-luciferase activity. As shown in Fig. 5, A and B, transient transfection with p65 expression vector caused an over 5-fold increase of luciferase activity when compared with control vector, whereas ectopic C/EBPβ or C/EBPδ expression resulted in ~40 and ~30% increase of luciferase activity, respectively. Concurrent forced expression of p65 and C/EBPβ led to a ~60% increase of promoter activity compared with p65 alone. However, the IL-6 promoter-luciferase activity was marginally enhanced by the co-expression of p65 and C/EBPδ. We further showed that p65 had no effect on the expression of C/EBPβ itself (data not shown). To further address the ability of NF-κB and C/EBPβ to synergistically mediate the IL-6 expression in osteoblasts, we transfected MC3T3-E1 cells with an IL-6 promoter-luciferase construct or an IL-6 promoter-luciferase construct harboring a

FIGURE 4. LPS induces C/EBPs and NF-κB binding to IL-6 promoter region. MC3T3-E1 cells were treated with 100 ng LPS/ml for the time as indicated. The nuclear proteins were extracted to perform EMSA to detect NF-κB activity (A) and C/EBP activation (B), respectively.
mutant in either the NF-κB binding site or the C/EBP binding site. As shown in Fig. 5C, a mutation in either the NF-κB binding site or in the C/EBP binding site led to a decrease of IL-6 promoter-luciferase activity by ~70% following LPS stimulation compared with un-mutated IL-6 promoter-luciferase. Thus, interaction of both NF-κB and C/EBPβ with the pro-

FIGURE 5. C/EBPβ and NF-κB p65 synergistically stimulate IL-6 expression. A and B, MC3T3-E1 cells were transiently transfected with total of 0.5 μg indicated genes. 24 h later, luciferase activity was measured by using cell lysates. Luminometer values were normalized for expression from a co-transfected thymidine kinase-luciferase gene. C, MC3T3-E1 cells were transiently transfected with 0.5 μg of DNA consisting of thymidine kinase-luciferase gene, and either a wild type IL-6 promoter-luciferase construct or an IL-6 promoter-luciferase constructs harboring a mutation in either NF-κB binding site or C/EBP binding site. 24 h after transfection, the cells were incubated with 100 ng LPS/ml for 4 h. Cell lysates were used to perform luciferase activity assay. The data were expressed as means of three experiments ± S.E. *, **, and *** indicate a statistically significant difference, p < 0.05, p < 0.01, and p < 0.001, respectively.
moter region was required for the LPS-induced IL-6 expression in MC3T3-E1 cells.

**SOCS3 Inhibits C/EBPs but Not NF-κB Binding to IL-6 Promoter Region**—To identify the potential mechanisms whereby SOCS3 suppressed IL-6 expression at the transcription level, we infected MC3T3-E1 cells with pLP-Ad-SOCS3, and performed EMSA assay. As shown in Fig. 6A, ectopic expression of SOCS3 could not affect LPS-induced DNA binding of NF-κB even when the cells were infected with pLP-Ad-SOCS3 at a MOI of 400. In contrast, DNA binding ability of C/EBPs was dramatically inhibited by SOCS3 (Fig. 6B). To determine which C/EBP family members were inhibited, supershift assay was performed. As shown in Fig. 6C, there was low but detectable level of C/EBPβ in nuclear proteins of untreated MC3T3-E1 cells, which was reduced to undetectable level by SOCS3. No basal level of C/EBPδ binding to DNA was identified. However, both C/EBPβ and C/EBPδ binding activities were dramatically enhanced by LPS challenge, which were greatly inhibited by SOCS3 (Fig. 6C). In addition, SOCS3 also significantly inhibited the DNA binding activities induced by exogenously expressed C/EBPβ (Fig. 6D). To determine if endogenous SOCS3 has the same effect on NF-κB and C/EBP binding activities as the exogenously expressed SOCS3, we infected MC3T3-E1 cells with Ad-Control-sh and Ad-SOCS3-sh, respectively. As shown in Fig. 7, DNA binding activity of C/EBPs was greatly enhanced by knocking-down LPS-induced SOCS3 expression while the NF-κB DNA binding was not affected.

To further determine the ability of SOCS3 to suppress the IL-6 expression through C/EBPs, we transfected MC3T3-E1 cells with a C/EBP-dependent (DEI4-luc) or a NF-κB-dependent promoter-luciferase construct (κB-luc) together with SOCS3 expression plasmids. As shown in Fig. 8A, LPS treatment resulted in a 2-fold increase of DEI4
C/EBP. We transfected MC3T3-E1 cells with DEI4 promoter-domains are required for the inhibitory role of SOCS3 on point mutation in the KIR (L22D), the SOCS3 box (Y204F), or cells (Fig. 3).

SOCS3-mediated inhibition of IL-6 expression in MC3T3-E1 and both the KIR and SH2 domain are indispensable for Binding Indispensable for SOCS3-mediated Suppression of C/EBP DNA

Luciferase expression, while SOCS3 led to a decrease of the luciferase expression to basal level. In contrast, although there is a more than 4-fold LPS induction of NF-κB-luciferase expression, this activity was enhanced in stead of inhibited by SOCS3 (Fig. 8B). Together, these results suggest the reduction of C/EBPs, but not NF-κB, DNA binding activity as a potential mechanism whereby SOCS3 suppresses IL-6 production at the transcriptional level.

KIR, Tyrosine 204 of the SOCS Box, and SH2 Domain Are Indispensable for SOCS3-mediated Suppression of C/EBP DNA Binding—We have shown that tyrosine 204 of the SOCS box and both the KIR and SH2 domain are indispensable for SOCS3-mediated inhibition of IL-6 expression in MC3T3-E1 cells (Fig. 3B). Thus, we sought to identify whether these domains are required for the inhibitory role of SOCS3 on C/EBP. We transfected MC3T3-E1 cells with DEI4 promoter-reporter and expression vectors for various SOCS3 mutants. As shown in Fig. 8C, SOCS3 could completely inhibit LPS-induced C/EBP DNA binding activity. In contrast, SOCS3 carrying a point mutation in the KIR (L22D), the SOCS3 box (Y204F), or the SH2 domain (R71E) failed to suppress LPS-induced luciferase expression, while other mutations in SOCS box (Y211F and Cis DC41) had no influence on SOCS3 actions (Fig. 8C).

C/EBPβ Inhibition by siRNA Decreased LPS-induced IL-6 Secretion in MC3T3-E1 Cells—Our results have demonstrated that SOCS3-mediated decrease of C/EBPβ DNA binding activity led to reduced IL-6 expression, which was based on an IL-6 promoter-luciferase, an exogenous indicator. To link the C/EBPβ activity in osteoblasts to IL-6 expression from its endogenous promoter, MC3T3-E1 cells were transfected with C/EBPβ-specific siRNA. As shown in Fig. 9A, C/EBPβ expression was almost completely abrogated by C/EBPβ-specific siRNA compared with control siRNA. Furthermore, C/EBPβ silencing significantly decreased IL-6 secretion after LPS stimulation (Fig. 9B).

C/EBPβ Expression Was Negatively Regulated by SOCS3 at Multiple Levels—To elucidate the potential mechanism whereby the amount of C/EBPβ binding to IL-6 promoter region was reduced by SOCS3, we infected MC3T3-E1 cells with pLP-Ad-SOCS3, and examined C/EBPβ protein levels. As shown in Fig. 10A, the amount of C/EBPβ proteins was greatly induced by LPS treatment (Fig. 10A, lanes 1 and 3). When SOCS3 was expressed, LPS-stimulated elevation of C/EBPβ proteins was significantly decreased (Fig. 10A, lanes 3 and 4), suggesting the amount of C/EBPβ proteins was negatively influenced by SOCS3. We then investigated whether the reduction of C/EBPβ proteins was due to the decreased level of C/EBPβ mRNA. We found that C/EBPβ mRNA expression was greatly induced by LPS treatment (Fig. 10B). However, SOCS3 negatively regulated the level of C/EBPβ mRNA (Fig. 10B). In addition, we determined whether SOCS3 had any effect on C/EBPβ protein degradation by pulse-chase experiment. First, we showed that C/EBPβ protein was greatly increased 2 h after LPS challenge, and the protein level was gradually reduced 4 h later (Fig. 10C). Based on this data, MC3T3-E1 cells were

FIGURE 7. Knocking-down SOCS3 expression enhances LPS-induced C/EBP but not NF-κB binding to IL-6 promoter region in MC3T3-E1 cells. MC3T3-E1 cells were infected with Ad-Control-si or Ad-SOCS3-si at an MOI of 200. 48 h after infection, the cells were incubated with 100 ng LPS/ml for 4 h. The nuclear proteins were harvested for EMSA to examine the influence of SOCS3 on NF-κB binding activity (A) and C/EBP binding activity (B), respectively.

FIGURE 6. SOCS3 inhibits both LPS-induced and exogenously expressed C/EBPs but not NF-κB binding to IL-6 promoter region in MC3T3-E1 cells. MC3T3-E1 cells were infected with Adeno-X-DsRed2 and pLP-Ad-SOCS3 at indicated MOI, respectively. 48 h later, the cells were treated or left untreated with 100 ng LPS/ml for 4 h. The nuclear proteins were harvested for EMSA to examine the influence of decreased SOCS3 on the NF-κB binding region in MC3T3-E1 cells. FIGURE 8. SOCS3 inhibited NF-κB activity but not NF-κB DNA binding activity as a potential mechanism whereby SOCS3 suppressed IL-6 production at the transcriptional level.
pulsed with Trans35S label for 2 h. As shown in Fig. 10D, LPS could significantly induce synthesis of C/EBPβ proteins in Adeno-X-DsRed2-infected cells. However, C/EBPβ protein synthesis was only slightly induced by LPS in the cells infected with pLP-Ad-SOCS3. This was mainly due to the decreased C/EBPβ mRNA mediated by SOCS3 (Fig. 10B). Importantly, SOCS3 expression could accelerate the degradation of C/EBPβ proteins (Fig. 10D). Taken together, our data suggested that C/EBPβ expression was negatively regulated by SOCS3 at both mRNA level and protein level, which led to a reduced amount of C/EBPβ binding to IL-6 promoter region.

**DISCUSSION**

SOCS-3 expression is regulated by a number of cytokines and bacterial products including LPS in a variety of cell types and tissues (28–30). However, the expression of SOCS3 in response to proinflammatory stimuli and its function in osteoblasts are unknown. Only one study shows that growth hormone can induce SOCS3 expression in osteoblasts-like cells (31). Here, we report that SOCS3 is transiently induced in response to LPS treatment of osteoblasts and contributes to the early stage inhibition of LPS-induced IL-6 expression. Additionally, we show that ectopic expression of SOCS3 suppresses IL-6 expression from both the endogenous IL-6 gene, as well as an IL-6 promoter-luciferase construct. Thus far, SOCS3 has been proven as inhibitor of signal transduction for IL-6, LIF, IL-11, GH, insulin, and leptin (32). In macrophages, SOCS3 is induced following TLR4 stimulation by LPS and regulates aspects of the TLR signaling. However, role of SOCS3 in the regulation of macrophage responses to LPS remains controversial. For example, SOCS3 has been shown to be an important mediator of IL-10 inhibition of LPS-induced macrophage activation (25). SOCS3 also functions in cells of the innate immune system by negatively regulating IL-1R signaling pathways (33). On the other hand, a most recent study shows that SOCS3 positively regulates TLR4 signaling by feedback inhibition of TGF-β/Smad3 signaling (34). These results suggest that SOCS3 can function as a positive regulator of TLR4 signaling in macrophages. Our current data suggest that SOCS3 plays an inhibitory role in the induction of IL-6 by LPS treatment in osteoblasts, which is consistent with SOCS3 having a role in the attenuation of LPS-induced inflammatory responses.

LPS induction of IL-6 operates through induction and cooperation of various transcription factors including NF-κB and C/EBPs. However, in most cases, this is cell specific. For example, Tanaka et al. (35) found that LPS stimulation of peritoneal
macrophages from mice deficient for C/EBPβ expression led to a normal induction of IL-6, while, in B cells, a C/EBP activity plays a critical role for LPS induction of IL-6 (18, 36). In osteoblasts, the signal transduction systems involved in stimulating IL-6 synthesis by LPS remain unclear. Using human osteoblastic cells, the transcriptional activation of AP-1 but not NF-κB has been shown to mediate IL-6 synthesis in response to LPS (12). On the other hand, a recent study suggests that 15-deoxy-(12, 14)-prostaglandin J(2) suppresses LPS-induced IL-6 expression in MC3T3-E1 cells via the Akt and NF-κB pathways (37). Data from the current study indicate that C/EBPβ is indispensable for LPS-induced IL-6 expression in osteoblasts. This is consistent with our previous work in B cells that C/EBPβ through its leucine zipper region is essential in the induction of IL-6 genes by LPS (20). Thus, it is tempting to speculate that C/EBPβ activation seems to be essential for LPS-stimulated bone resorption.

In macrophages, NF-κB has been proven to play a key role in the transcriptional up-regulation of the LPS-induced IL-6 gene. In current study, surprisingly, activation of NF-κB in

FIGURE 9. C/EBPβ inhibition by siRNA decreases LPS-induced IL-6 secretion in MC3T3-E1 cells. A, MC3T3-E1 cells were transiently transfected with 40 nM control siRNA or C/EBPβ-specific siRNA. 24 h after transfection, the cells were incubated with 100 ng LPS/ml for 4 h. RNAs were isolated, and RT-PCR was performed by using primers for C/EBPβ and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. B, MC3T3-E1 cells were transiently transfected with 40 nM control siRNA or C/EBPβ siRNA. 24 h later, the cells were incubated with or without 100 ng LPS/ml for 4 h. Supernatants were harvested for ELISA. The data were expressed as means of six experiments ± S.E. *** indicates a statistically significant difference (p < 0.001).

FIGURE 10. C/EBPβ expression is negatively regulated by SOCS3 at multiple levels. MC3T3-E1 cells were infected with Adeno-X-DsRed2 or pLP-Ad-SOCS3. 48 h after infection, the cells were treated or left untreated with 100 ng LPS/ml for 4 h. A, the total proteins were extracted to conduct Western blot using rabbit anti-C/EBPβ antibody, and rabbit anti-GAPDH antibody, respectively. The level of GAPDH was shown at the bottom as a loading control. B, total cellular RNA was isolated for RT-PCR with primers for C/EBPβ and GAPDH, respectively. The level of GAPDH was shown at the bottom as a loading control. C, MC3T3-E1 cells were incubated with 100 ng LPS/ml for indicated times. Total proteins were extracted to conduct Western blot using anti-C/EBPβ antibody and analyzed by SDS-PAGE. Data are representative for two independent experiments.
MC3T3-E1 cells shows a rapid and transitory pattern after LPS stimulation, which does not correlate with the induction of IL-6 expression. However, using an IL-6 promoter-luciferase construct, we demonstrate that both intact C/EBP and NF-κB binding sites are required for the LPS-induced IL-6 expression (Fig. 9). These data suggest that although NF-κB itself is not sufficient to mediate IL-6 expression, it may orchestrate C/EBPβ for LPS induction of the IL-6 promoter in osteoblasts.

Perhaps one of the most interesting results reported here is that SOCS3 suppressed LPS-induced DNA binding activity of C/EBPβ, which contributed to its inhibition on IL-6 expression. Whether SOCS3 exerts its regulatory function on TLR signaling by a direct or indirect mechanism is largely unknown. In murine monocytes/macrophages, Park et al. (38) found that SOCS3 did not induce any alteration in NF-κB activity induced by LPS or TNFα. However, it enhanced RelA-dependent κB promoter activity when co-transfected with RelA (38). In contrast, Baetz et al. (39) showed that SOCS3 inhibited an indirect signaling pathway following TLR stimulation, whereas neither MAP kinase nor NF-κB signaling were affected. The role for SOCS3 in C/EBPβ regulation is not reported. Here, we provide the first evidence that SOCS3 inhibited C/EBPβ DNA binding activity induced by LPS, while the same treatment did not affect NF-κB activation. However, employing a co-immunoprecipitation approach, we could not detect a direct interaction between SOCS3 and C/EBPβ (data not shown). Thus, the interaction between SOCS3 and C/EBPβ, if any, may be indirect in LPS-stimulated osteoblasts. Importantly, these data further support the hypothesis that C/EBPβ is indispensable for LPS-induced IL-6 expression in osteoblasts. In addition, our observation for the regulatory effect of SOCS3 on NF-κB activity is consistent with the previous reports (38, 39).

Our finding that LPS increases C/EBPβ expression levels in osteoblasts is a novel observation (Fig. 10). However, the molecular mechanism of LPS-induced C/EBPβ gene expression is unclear. A recent study showed that C/EBPβ expression was limited in MyD88- or IL-1R-associated kinase 4 (IRAK-4)-deficient macrophages treated with LPS (40). In addition, all three MAP kinases [ERK, JNK, and p38] were shown to be involved in the LPS-induced C/EBPβ expression in microglia cells (41). However, the exact mechanism whereby LPS signals control C/EBPβ expression remains an open question. Furthermore, it is possible that LPS signaling might be different between macrophages/microglia and osteoblasts. Thus, it will be interesting to dissect signaling pathways downstream of LPS in osteoblasts that lead to the expression of C/EBPβ.

Interestingly, our results showed that LPS-induced C/EBPβ expression was negatively regulated by SOCS3 at both mRNA level and protein level. Our data that the KIR, SOCS box, and SH2 domain are all required for SOCS3-mediated suppression of C/EBP DNA binding suggest SOCS3 may regulate the C/EBPβ expression by multiple mechanisms. For example, it is known that SOCS3 can mediate protein degradation through linking their substrates to the ubiquitination machinery via the SOCS box (42). Although it has been shown that both C/EBPγ and C/EBPζ (CHOP) are constitutively multiubiquitinated and subsequently degraded by the proteasome (43), so far, no data indicate that ubiquitination is involved in the degradation of C/EBPβ and -δ. Interestingly, a recent report demonstrates that proteasome-mediated C/EBPβ degradation is ubiquitin-independent (44). Our current finding that tyrosine 204 in the SOCS box is necessary for SOCS3 inhibition of C/EBPβ activity (Fig. 8C) suggests that tyrosine 204 may be involved in regulating SOCS3 interaction with Elongin C, a component of ubiquitin ligases, in the osteoblasts, which may contribute to target protein degradation. In addition, we showed that SOCS3 could accelerate the degradation of C/EBPβ proteins, and inhibition of the proteasome activity by the specific inhibitor, MG-132, attenuated the C/EBPβ degradation mediated by SOCS3 (data not shown). These results together suggest that SOCS3 may mediate C/EBPβ expression in osteoblasts through a proteasome-dependent mechanism. However, the molecular details for the association of SOCS box and Elongin B/C complex and its function to tune the activity of SOCS3 in osteoblasts, and whether and how ubiquitination contributes to C/EBPβ degradation remains to be investigated.

Taken together, we report here that SOCS3 in osteoblasts dramatically decreased the abundance of C/EBP DNA binding species from both the endogenous and ectopically expressed C/EBPβ, as well as the expression and secretion of IL-6 induced by LPS treatment. Although SOCS3 is involved in a variety of crucial processes including immune functions, growth, hematopoiesis, and metabolism, our finding is the first demonstration that SOCS3 expression has a functional role in osteoblasts. Our studies indicate that SOCS3 may function as an important regulator in bone-associated inflammatory diseases.

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