Myeloid depletion of SOCS3 enhances LPS-induced acute lung injury through CCAAT/enhancer binding protein δ pathway

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ABSTRACT **Although uncontrolled inflammatory response plays a central role in the pathogenesis of acute lung injury (ALI), the precise molecular mechanisms underlying the development of this disorder remain poorly understood. SOCS3 is an important negative regulator of IL-6-type cytokine signaling. SOCS3 is induced in lung during LPS-induced lung injury, suggesting that generation of SOCS3 may represent a regulatory product during ALI. In the current study, we created mice lacking SOCS3 expression in macrophages and neutrophils (*LysM-cre* $SOCS3^{fl/fl}$). We evaluated the lung inflammatory response to LPS in both *LysM-cre* $SOCS3^{fl/fl}$ mice and the wild-type (WT) mice ($SOCS3^{fl/fl}$). *LysM-cre* $SOCS3^{fl/fl}$ mice displayed significant increase of the lung permeability index (lung vascular leak of albumin), neutrophils, lung neutrophil accumulation (myeloperoxidase activity), and proinflammatory cytokines/chemokines in bronchial alveolar lavage fluids compared to WT mice. These phenotypes were consistent with morphological evaluation of lung, which showed enhanced inflammatory cell influx and intra-alveolar hemorrhage. We further identify the transcription factor, CCAAT/enhancer-binding protein (C/EBP) δ as a critical downstream target of SOCS3 in LPS-induced ALI. These results indicate that SOCS3 has a protective role in LPS-induced ALI by suppressing C/EBPδ activity in the lung. Elucidating the function of SOCS3 would represent prospective targets for a new generation of drugs needed to treat ALI.—Yan, C., Ward, P. A., Wang, X., Gao, H. Myeloid depletion of SOCS3 enhances LPS-induced acute lung injury through CCAAT/enhancer binding protein δ pathway. *FASEB J.* 27, 2967–2976 (2013). www.fasebj.org**

Key Words: C/EBP · LPS

Suppressor of cytokine signaling 3 (SOCS3) inhibits STAT/JAK signaling by either mediating cytokine receptor/JAK degradation or inhibiting JAK catalytic activity. SOCS3 expression can be induced by a variety of stimuli, including cytokines, Toll-like receptor (TLR) ligands, bacteria, and immune complexes (1–3). SOCS3 can be activated by LPS in both neutrophils and macrophages (4–6). Of note, SOCS3 is one of the most abundantly induced proteins in LPS-treated macrophages (4). The role of SOCS3 in inflammation has been studied in both innate and adaptive immune cells. Both pro- and anti-inflammatory functions of SOCS3 have been reported. For example, IL-6 promotes both *in vivo* acute and chronic inflammation in the absence of SOCS3 (7). In addition, SOCS3 plays a key role in regulating IFN-γ-like responses induced by IL-6 (8, 9). Of note, SOCS3 overexpression mitigates inflammatory arthritis induced by antigen/IL-1β or collagen, as well as acute inflammation induced by staphylococcal enterotoxin B and LPS (10–13). In contrast with the above findings, myeloid SOCS3-deficient mice are shown to be resistant to LPS-induced shock (14, 15). This result indicates that SOCS3 may function as a proinflammatory mediator (14, 15). Supporting this hypothesis, Liu *et al.* (16) show that SOCS3 inhibits the TGFβ1/Smad3 signaling pathway, which leads to enhancing TLR4-mediated inflammatory response in macrophages. Recently, it has been shown

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility shift assay; H&E, hematoxylin and eosin; KC, keratinocyte-derived chemokine; KO, knockout; MIP-2, macrophage inflammatory protein 2; MPO, myeloperoxidase; PBS, phosphate-buffered saline; SOCS3, suppressors of cytokine signaling 3; TLR, Toll-like receptor; WT, wild type

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that both T-cell-specific SOCS3-deficient mice and transgenic mice overexpressing the SOCS3 gene in T cells are susceptible to infection by *Leishmania major* (17, 18). Based on these studies, targeting SOCS3 signaling pathway may offer an attractive strategy for preventing or resolving inflammation associated with various diseases.

The role of SOCS3 in the lung has also been investigated. Enhanced SOCS3 expression in T cells has been linked to the onset and maintenance of human allergic diseases (19). Moreover, a recent study shows that treatment of T cells with SOCS3-specific siRNA significantly suppresses airway responses in a murine model of ovalbumin (OVA)-induced allergic asthma (20). Data for SOCS3 in acute lung injury (ALI) are limited. Our previous work demonstrates that adenovirus-mediated overexpression of SOCS3 enhances IgG immune complex-induced lung injury (2). A recent study suggests a link between SOCS3 and acid-induced ALI in mice (21). However, due to the embryonic lethality of SOCS-knockout (KO) mice (22), the function of SOCS3 in ALI remains to be established. In this report, we used myeloid-specific SOCS3-KO mice to demonstrate for the first time that SOCS3 deficiency significantly enhances acute lung inflammation and injury induced by intratracheal LPS administration. Moreover, our data implicate the transcription factor CCAAT/enhancer-binding protein (C/EBP) δ as a critical downstream target of SOCS3 in LPS-induced ALI.

MATERIALS AND METHODS

Animals, cells, and reagents

Specific pathogen-free male C57BL/6 mice, $SOCS3^{fl/fl}$ mice, and *LysM-cre* mice expressing Cre recombinase from the endogenous lysozyme M locus were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and maintained in a specific pathogen-free facility. Conditional SOCS3-KO mice that lack SOCS3 in myeloid (*LysM-cre* $SOCS3^{fl/fl}$) were generated as described previously (14), and all mice were used at the age of 8–12 wk old. Generation of *C/EBP*$\beta^{-/-}$ and *C/EBP*$\delta^{-/-}$ mice by homologous recombination has been described previously (23, 24). Mouse alveolar macrophage-derived cell line, MH-S, was purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.01 M HEPES, and maintained in a humidified incubator at 37°C with 5% CO_2. ELISA kits for mouse IL-6, TNF-α, macrophage inflammatory protein 2 (MIP-2), and keratinocyte-derived chemokine (KC) were obtained from R&D Systems (Minneapolis, MN, USA).

LPS-induced ALI

All procedures involving mice were approved by the Animal Care and Use Committee of Harvard Medical School. Mice were anesthetized intraperitoneally with ketamine HCl (100 mg/kg), followed by intratracheal instillation of 50 μl of LPS dissolved in phosphate-buffered saline (PBS; 1 mg/ml) during inspiration. Negative control mice received 50 μl PBS intratracheally. Unless otherwise indicated, mice were exsanguinated 18 h after LPS deposition, and the pulmonary circulation was flushed with 1 ml of PBS *via* the pulmonary artery. The lungs were immediately frozen in liquid nitrogen.

Myeloperoxidase (MPO) activity

Lungs were perfused *via* the right ventricle with 3 ml of PBS. For MPO activity, the lungs were homogenized in 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The samples were sonicated for 1 min, and centrifuged at 10,000 rpm for 10 min. Next, 10 μl of the recovered supernatants was added to a 96-well plate, followed by addition of 100 mM potassium phosphate buffer containing 1.5 M H_2O_2 and 167 μg/ml *o*-dianisidine dihydrochloride. The enzyme activity was determined by measuring the change in optical density (OD) at 450 nm over a period of 4.5 min using a 96-well plate reader.

Histological assay

At 18 h after LPS deposition, 1 ml of 10% buffered (pH 7.2) formalin was instilled into the lung *via* the trachea. The lungs were then surgically removed and further fixed in 10% buffered formalin solution for morphological assay by tissue sectioning and staining with hematoxylin and eosin (H&E).

Bronchial alveolar lavage (BAL) fluid collection, differential white blood cell counts, albumin, and chemokine/cytokine ELISAs

At 18 h after initiation of the ALI, the thorax was opened, and 0.8 ml of ice-cold, sterile PBS was instilled into the lung *via* a tracheal incision. The recovered BAL fluid was centrifuged at 450 *g* for 6 min, and the cell-free supernatants were used for chemokine and cytokine measurements by ELISAs. Cell pellets were resuspended in 1 ml of Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA), and differential cell analyses were performed by Diff-Quik-stained cytospin preparations (Dade, Duedingen, Switzerland) counting a total of 300 cells/slide in randomly selected high-power fields (×1000). Mouse albumin levels in BAL fluid were measured using a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). The permeability index was expressed as the ratio of the albumin in the LPS-injured lungs *vs.* that in the control-treated lungs of same type of mice.

Alveolar macrophage isolation and culture

Mouse lungs were lavaged 3 times with ice-cold PBS. BAL fluids were then centrifuged at 1000 rpm for 10 min, and cell pellets were suspended in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum. Next, 1.5×10^4 cells were seeded in each well of 96-well plate. After 2 h, the medium was removed, and the cells were challenged with 100 ng/ml LPS for indicated time periods. Supernatants were harvested to conduct ELISA.

Assessment of C/EBP activation by electrophoretic mobility shift assay (EMSA)

Lung nuclear extracts were prepared as described previously (21). Briefly, frozen lungs were homogenized in 0.6% (v/v) Nonidet P-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin. The homogenate was incubated on ice for 5 min and then

centrifuged for 5 min at 5000 *g* at 4°C. Proteins were extracted from the pelleted nuclei by incubation at 4°C with solution B (420 mM NaCl; 20 mM HEPES, pH 7.9; 1.2 mM $MgCl_2$; 0.2 mM EDTA; 25% (v/v) glycerol; 0.5 mM dithiothreitol; 0.5 mM phenylmethylsulphonyl fluoride; 2.5 μg/ml leupeptin; 5 μg/ml antipain; and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 13,000 *g* for 30 min at 4°C, and the supernatant extract was collected and stored at −80°C. The EMSA probes were double-stranded oligonucleotides containing a C/EBP consensus oligonucleotide (5′-TGCAGATTGCGCAATCTGCA-3′; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). C/EBP probes were labeled with [^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Biosciences Corp, Sunnyvale, CA, USA). DNA binding reactions were performed at room temperature in a 25-μl reaction mixture containing 6 μl of nuclear extract (1 mg/ml in buffer C or solution B) and 5 μl of 5× binding buffer (20% (w/v) Ficoll; 50 mM HEPES, pH 7.9; 5 mM EDTA; and 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(dI-dC), 200 pg of probe (unless otherwise noted), bromphenol blue at a final concentration of 0.06% (w/v), and water to volume of 25 μl. Samples were electrophoresed through 5.5% polyacrylamide gels, dried under vacuum, and exposed to X-ray film. For supershifts, nuclear extracts were preincubated with antibodies (1–2 μg) for 0.5 h at 4°C prior to the binding reaction. The following antibodies were purchased from Santa Cruz Biotechnology: C/EBPβ, C/EBPδ, and normal rabbit immunoglobulin G.

Luciferase assay

MH-S cells were transfected with indicated reporter plasmids by using Fugene6 transfection reagent (Roche, Indianapolis, IN, USA). At 48 h after transfection, cells were treated with or without 100 ng/ml LPS. After 4 h, cells were lysed in Passive Lysis 5X buffer (Promega, Madison, WI, USA), and luciferase activity was measured. Mouse SOCS3 expression plasmids were described previously (25). The reporter plasmid containing 2 copies of a C/EBP binding site, 2 × C/EBP-Luc, the mouse IL-6 promoter-reporter, and TNF-α promoter-reporter, as well as the C/EBPδ expression plasmid, have been described in our previous publications (26, 27).

RNA isolation and detection of SOCS3 mRNA by real-time PCR

Total RNAs were extracted from lungs with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. Total RNA (2 μg) was submitted to reverse transcription by using the Superscript II RNase H- Reverse Transcriptase (Invitrogen). PCR was performed with primers for SOCS3: 5′ primer, 5′-AGCTCCAAAAGCGAGTACCA-3′, and 3′ primer, 5′-TGACGCTCAACGTGAAGAAG-3′. Following reverse transcription, the cDNA was amplified and quantified using a Sequence Detection System (SDS 7300; Invitrogen) and a PCR universal protocol as follows: AmpliTaq Gold (Invitrogen) activation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The fluorescence of the double-stranded products accumulated was monitored in real time. The relative mRNA levels were normalized to levels of GAPDH mRNA in the same sample.

Statistical analysis

All values are expressed as means ± SE. Data sets were analyzed using Student's *t* test or 1-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

RESULTS

Effect of myeloid SOCS3 deficiency on LPS-induced ALI

Alveolar macrophages are critical for initiation of the LPS-induced innate immune response in the lung (28). We generated *LysM-cre* SOCS3$^{fl/fl}$ mice, which lack SOCS3 expression in macrophages and neutrophils (14). To examine the role of SOCS3 in LPS-induced inflammation *in vivo*, SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice were intratracheally injected with LPS to mimic human gram-negative ALI. We investigated whether myeloid-specific depletion of SOCS3 affected pulmonary vascular permeability by measuring albumin levels in BAL fluids. LPS deposition led to an obvious increase in lung vascular permeability (**Fig. 1*A***). Notably, permeability index was significantly augmented in *LysM-cre* SOCS3$^{fl/fl}$ mice challenged with LPS when compared to their WT littermates (SOCS3$^{fl/fl}$; Fig. 1*A*). We further examined lung MPO content, which reflects the number of neutrophils recruited into the lung (Fig. 1*B*). MPO activity in lung homogenates was greatly elevated in LPS-injured mice compared with noninflamed mice (Fig. 1*B*). As with pulmonary vascular permeability, lung MPO activity was markedly increased in *LysM-cre* SOCS3$^{fl/fl}$ mice when compared with SOCS3$^{fl/fl}$ mice during LPS-induced acute lung inflammation (Fig. 1*B*). We next determined the total leukocyte and neutrophil counts in BAL fluids, which contributed to lung damage. As shown in **Fig. 2*A***, in the

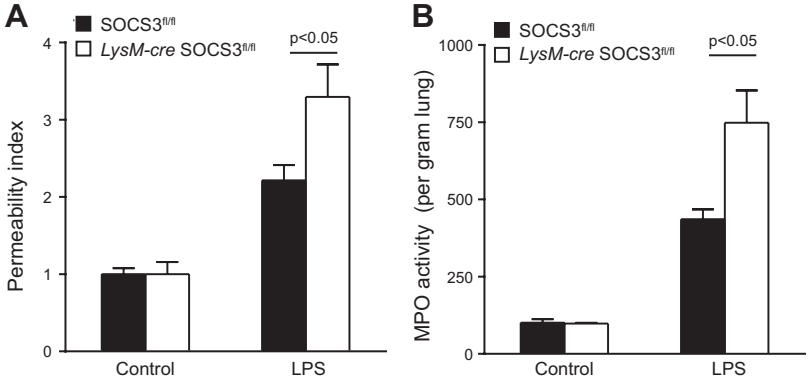

Figure 1. Effects of myeloid deletion of SOCS3 on LPS-induced ALI. SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice received airway injection of LPS. After 18 h, BAL fluids and whole lungs were harvested. *A*) Mouse albumin content in BAL fluids was determined using ELISA as an index of lung microvascular permeability. *B*) Changes in lung MPO activity were measured as an index of lung neutrophil accumulation. Data are expressed as means ± SE; $n = 3$, control-treated groups; $n = 5$–6, injured groups.

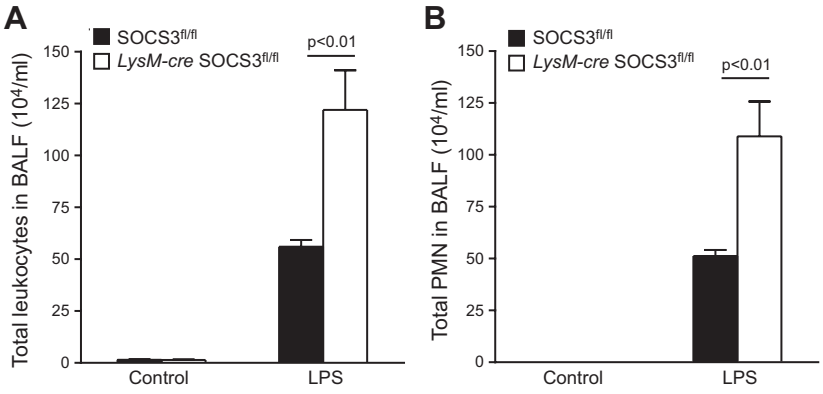

Figure 2. Effects of myeloid deletion of SOCS3 on leukocyte counts in BAL fluid from LPS-injured lungs. SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice received airway injection of LPS. After 18 h, BAL fluids and whole lungs were harvested. Total leukocytes (*A*) and neutrophils (*B*) in BAL fluids were quantitated. Data are expressed as means ± SE; n = 3, control-treated groups; n = 5–6, injured groups.

presence of LPS, the number of total leukocytes recovered from BAL fluids was markedly elevated as compared with control mice received PBS alone. In inflamed lungs, the major cells present in BAL fluids were neutrophils (Fig. 2). Notably, *LysM-cre* SOCS3$^{fl/fl}$ mice exhibited enhanced recruitment of total leukocytes and neutrophils into alveolar spaces as compared with SOCS3$^{fl/fl}$ mice during LPS-induced ALI (Fig. 2). Therefore, SOCS3 expression in myeloid-derived cells mitigated lung injury by suppression of leukocytes, especially neutrophils recruited into airway spaces in the LPS-induced acute lung inflammation model.

To further evaluate the effect of SOCS3 on LPS-induced ALI, we performed histological assays. As shown in **Fig. 3*A, B***, mice treated with PBS exhibited normal pulmonary architecture without evidence of inflammatory features. Mice receiving airway administration of LPS showed lung hemorrhage, edema, and neutrophil accumulation (Fig. 3*C*). Notably, all of these features were significantly enhanced in LPS-challenged mice lacking SOCS3 (Fig. 3*D*). Taken together, these data indicate that

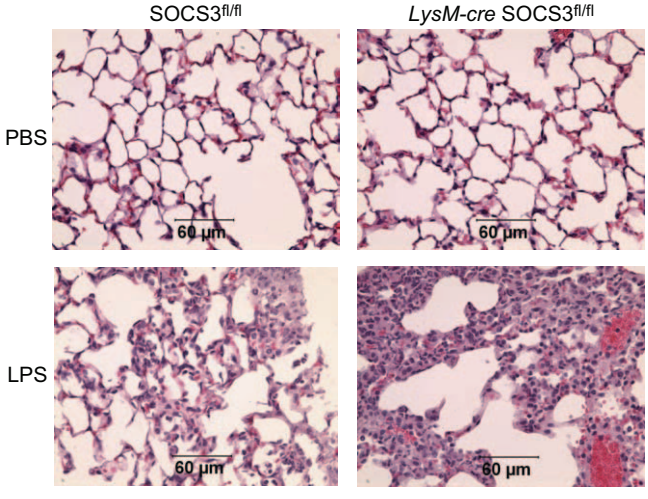

Figure 3. Effects of myeloid deletion of SOCS3 on lung histology during LPS-induced lung injury. SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice received airway injection of LPS. Sections from lungs harvested 18 h after PBS or LPS deposition in SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice were stained with H&E (×40 view). Lung sections shown include SOCS3$^{fl/fl}$ + PBS (*A*), LysM-cre SOCS3$^{fl/fl}$ + PBS (*B*), SOCS3$^{fl/fl}$ + LPS (*C*), and LysM-cre SOCS3$^{fl/fl}$ + LPS (*D*). Scale bars = 60 μm.

depletion of SOCS3 expression in myeloid-derived cells enhances LPS-induced acute lung inflammation by increasing the permeability index and neutrophil accumulation.

Effect of myeloid SOCS3 deficiency on cytokine/chemokine expressions in LPS-injured lung

We determined the influence of SOCS3 on proinflammatory cytokine/chemokine production in BAL fluids harvested 18 h after intratracheal administration of LPS. As shown in **Fig. 4**, in the absence of LPS, there were only very low levels of inflammatory mediators in BAL fluids collected from either SOCS3$^{fl/fl}$ or *LysM-cre* SOCS3$^{fl/fl}$ mice, and LPS challenge significantly induced TNF-α, IL-6, KC, and MIP-2 production. Notably, in LPS-injured lungs, myeloid-deletion of SOCS3 resulted in a significant increased expression of TNF-α (Fig. 4*A*), IL-6 (Fig. 4*B*), KC (Fig. 4*C*), and MIP-2 (Fig. 4*D*) by 97, 77, 147, and 128%, respectively, when compared with WT mice, indicating that SOCS3 expression in myeloid cells might play a central role in cytokine and chemokine generation during LPS-induced ALI. Our previous study indicates that SOCS3 overexpression has a positive regulatory role in IgG immune complex-induced lung inflammatory response (2). We next determined the influence of SOCS3 deficiency on proinflammatory cytokine/chemokine production in BAL fluids harvested 4 h after IgG immune complex deposition in the lung. As shown in Supplemental Fig. S1, IgG immune complex deposition significantly induced TNF-α, IL-6, KC, and MIP-2 production in the BAL fluids collected from SOCS3$^{fl/fl}$ mice. In contrast to the LPS model (Fig. 4), SOCS3 deficiency (*LysM-cre* SOCS3$^{fl/fl}$) resulted in a significantly decreased production of TNF-α, IL-6, KC, and MIP-2 in IgG immune complex-injured lungs, when compared with SOCS3$^{fl/fl}$ mice (Supplemental Fig. S1). Furthermore, SOCS3 deficiency resulted in a significant decrease in production of TNF-α from IgG immune complex-stimulated alveolar macrophages, when compared with cells from SOCS3fl/fl mice (Supplemental Fig. S2). These data together suggest that SOCS3 plays an opposite role in the LPS- and IgG immune complex-induced lung inflammatory responses.

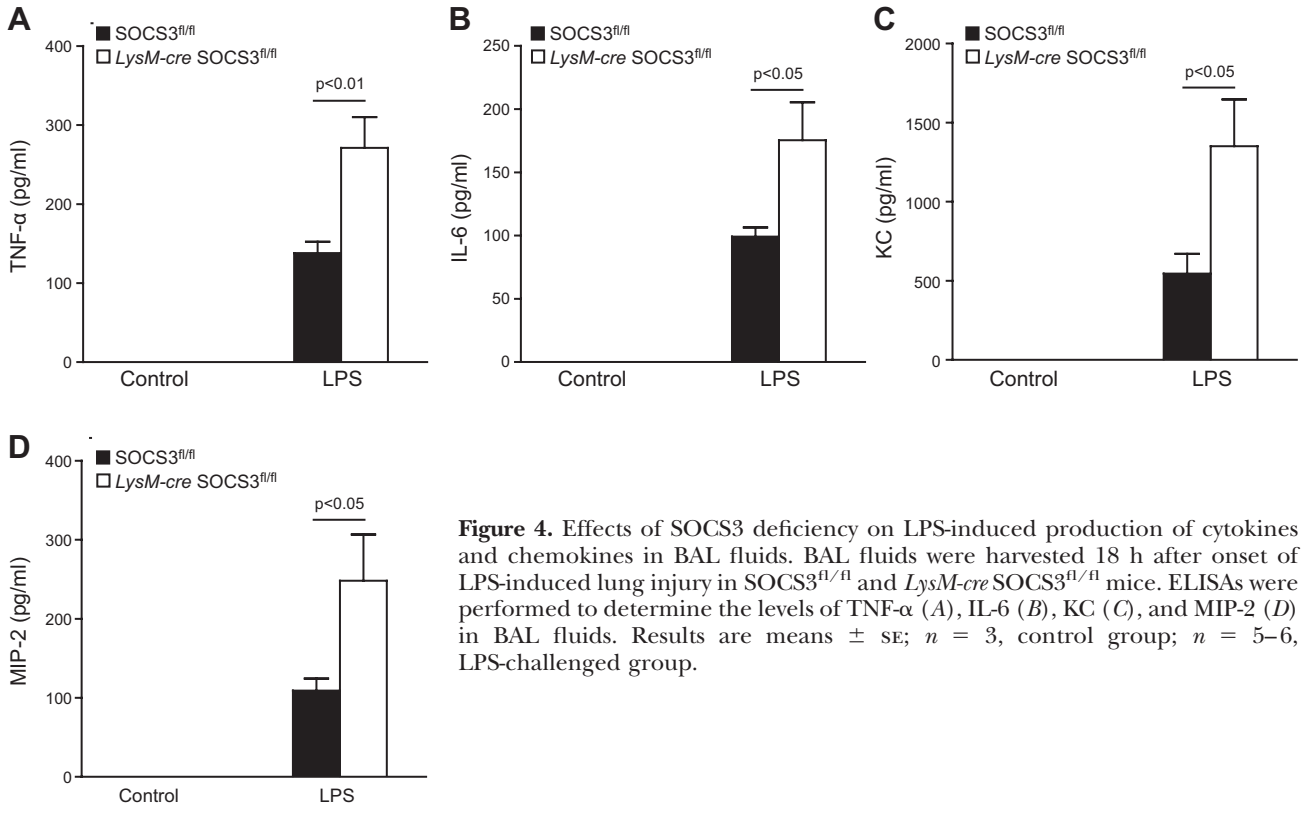

Figure 4. Effects of SOCS3 deficiency on LPS-induced production of cytokines and chemokines in BAL fluids. BAL fluids were harvested 18 h after onset of LPS-induced lung injury in $SOCS3^{fl/fl}$ and *LysM-cre* $SOCS3^{fl/fl}$ mice. ELISAs were performed to determine the levels of TNF-α (*A*), IL-6 (*B*), KC (*C*), and MIP-2 (*D*) in BAL fluids. Results are means ± SE; $n = 3$, control group; $n = 5–6$, LPS-challenged group.

SOCS3 negatively regulates LPS-induced production of inflammatory mediators in alveolar macrophages

SOCS3 expression is induced by LPS in many cell types, including peritoneal macrophages, neutrophils, osteoblasts, and microglia (6, 14, 29, 30). To determine whether SOCS3 expression can be induced in alveolar macrophages, we challenged MH-S cells with LPS for different time periods, and conducted real-time PCR experiments. As shown in **Fig. 5*A***, SOCS3 mRNA expression was dramatically induced by LPS at 2 and 4 h time points, and thereafter, its expression was gradually decreased. Next, we sought to investigate the role of SOCS3 in LPS-induced inflammation in alveolar macrophages. As shown in Fig. 5*B*, *C*, LPS treatment stimulated TNF-α and IL-6 promoter-driven luciferase expression by >2- and 13-fold, respectively. Furthermore, overexpression of SOCS3 decreased LPS-induced TNF-α and IL-6 reporter expression by 45 and 30%,

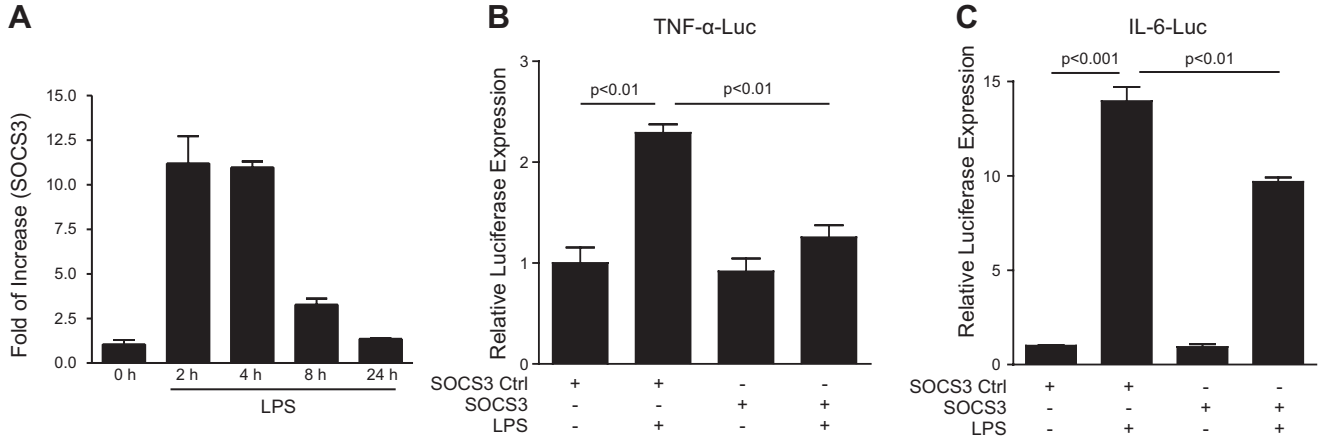

Figure 5. Effects of SOCS3 expression on LPS-induced TNF-α and IL-6 expression in alveolar macrophage cells. *A*) MH-S cells were treated with 100 ng/ml LPS for indicated time periods. Total cellular RNAs were extracted and subjected to real-time PCR for the expression of SOCS3 and GAPDH. Data are expressed as means ± SE (n=3). *B*, *C*) Transient transfections were performed in MH-S cells by using 0.5 μg of indicated DNA. After 48 h, the cells were treated with or without 100 ng/ml LPS for 4 h. TNF-α (*B*) and IL-6 (*C*) luciferase activity was measured. Luminometer values were normalized for expression from a cotransfected thymidine kinase reporter gene. These values were then normalized to a relative value of 1 for the control plasmid transfected cells that was not stimulated by LPS. Data are expressed as means ± SE (n=3).

respectively. Next, we isolated primary alveolar macrophages from SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice and treated these cells with LPS for various times. As shown in **Fig. 6**, SOCS3-deficient alveolar macrophages released significantly higher levels of TNF-α, IL-6, KC, and MIP-2 at all time points after LPS treatment compared to SOCS3-proficient cells. Taken together, these data indicated that SOCS3 negatively regulates TLR4-mediated inflammation in alveolar macrophages.

SOCS3 regulates C/EBPβ and δ activity in LPS-injured lung and alveolar macrophages stimulated with LPS

The C/EBPs (C/EBPα, -β, -δ, -ε, -γ, and -ζ) comprise a family of basic region-leucine zipper (bZIP) transcription factors that dimerize through a leucine zipper and bind to DNA through an adjacent basic region (31–33). Among them, C/EBPβ and C/EBPδ are regulators of proinflammatory cytokines, as well as other gene products of the acute-phase response (32, 34–40). C/EBPβ has 2 major isoforms: liver-enriched activating protein (LAP) and its short isoform, liver-enriched inhibiting protein (LIP), which is translated from an alternative start site in the same messenger RNA (41). Our recent studies demonstrated that the binding activities of both C/EBPβ and C/EBPδ were significantly induced in LPS-injured lungs (42). Notably, C/EBPδ, but not C/EBPβ, is a key regulator of LPS-induced lung injury (42). We sought to determine whether SOCS3 regulates LPS-induced C/EBPβ and C/EBPδ activation in the lung. As shown in **Fig. 7*A, B***, DNA-binding activity of lung C/EBPβ and C/EBPδ was significantly higher in *LysM-cre* SOCS3$^{fl/fl}$ mice than in SOCS3$^{fl/fl}$ mice. We also assessed the role of SOCS3 in regulating C/EBP activity in alveolar macrophages by performing reporter assays using 2×C/EBP-Luc, a promoter-reporter whose expression is driven by 2 copies of a C/EBP binding site. MH-S cells (an alveolar macrophage cell line) were transiently transfected with 2×C/EBP-Luc in the presence or absence of a SOCS3-expressing plasmid. As shown in Fig. 7*C*, LPS treatment caused a >2.5-fold increase in luciferase activity. Notably, ectopic expression of SOCS3 led to a significant decrease in LPS-induced luciferase activity (Fig. 7*C*). In contrast, although we observed >8-fold LPS induction of NF-κB-luciferase expression, this activity was not inhibited by SOCS3 (data not shown). To further evaluate the influence of SOCS3 on C/EBPδ-driven 2×C/EBP-Luc expression, we transiently transfected MH-S cells with 2×C/EBP-Luc in the presence or absence of C/EBPδ expression plasmid. As shown in Fig. 7*D*, C/EBPδ expression resulted in a 1.6-fold increase of 2×C/EBP-Luc luciferase activity, while SOCS3 decreased luciferase expression to basal levels.

To further establish a functional link between C/EBPδ and SOCS3 in LPS-stimulated macrophages, we transfected MH-S cells with TNF-α or IL-6 promoter-driven luciferase constructs in the presence or absence of a SOCS3- and/or C/EBPδ-expressing plasmids. As expected, expression of SOCS3 led to a significant decrease in LPS-induced luciferase activity (**Fig. 8**). Notably, overexpression of C/EBPδ significantly reversed SOCS3-mediated inhibition of the luciferase expression (Fig. 8). Taken together, these data suggest that C/EBPδ is a critical target of SOCS3 in the TLR4 pathway.

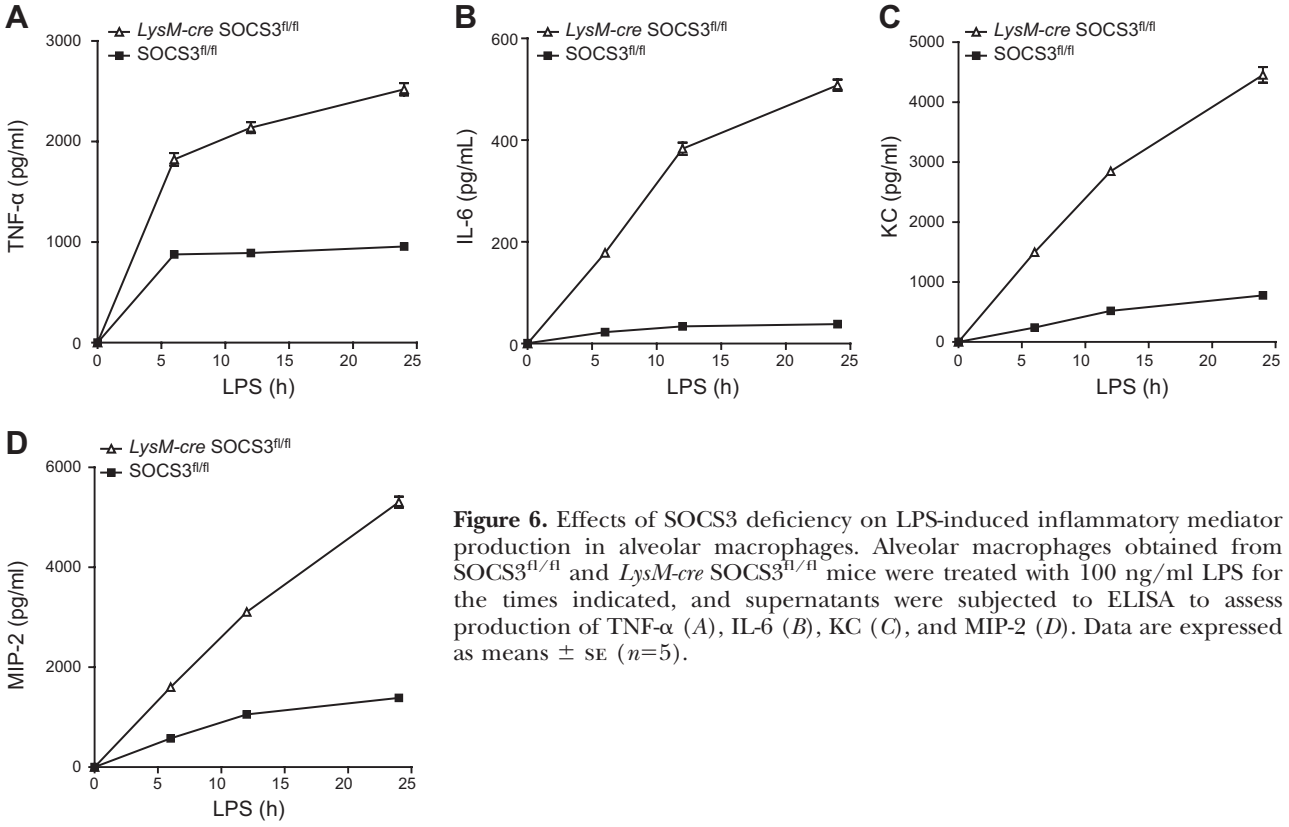

Figure 6. Effects of SOCS3 deficiency on LPS-induced inflammatory mediator production in alveolar macrophages. Alveolar macrophages obtained from SOCS3$^{fl/fl}$ and *LysM-cre* SOCS3$^{fl/fl}$ mice were treated with 100 ng/ml LPS for the times indicated, and supernatants were subjected to ELISA to assess production of TNF-α (*A*), IL-6 (*B*), KC (*C*), and MIP-2 (*D*). Data are expressed as means ± SE (n=5).

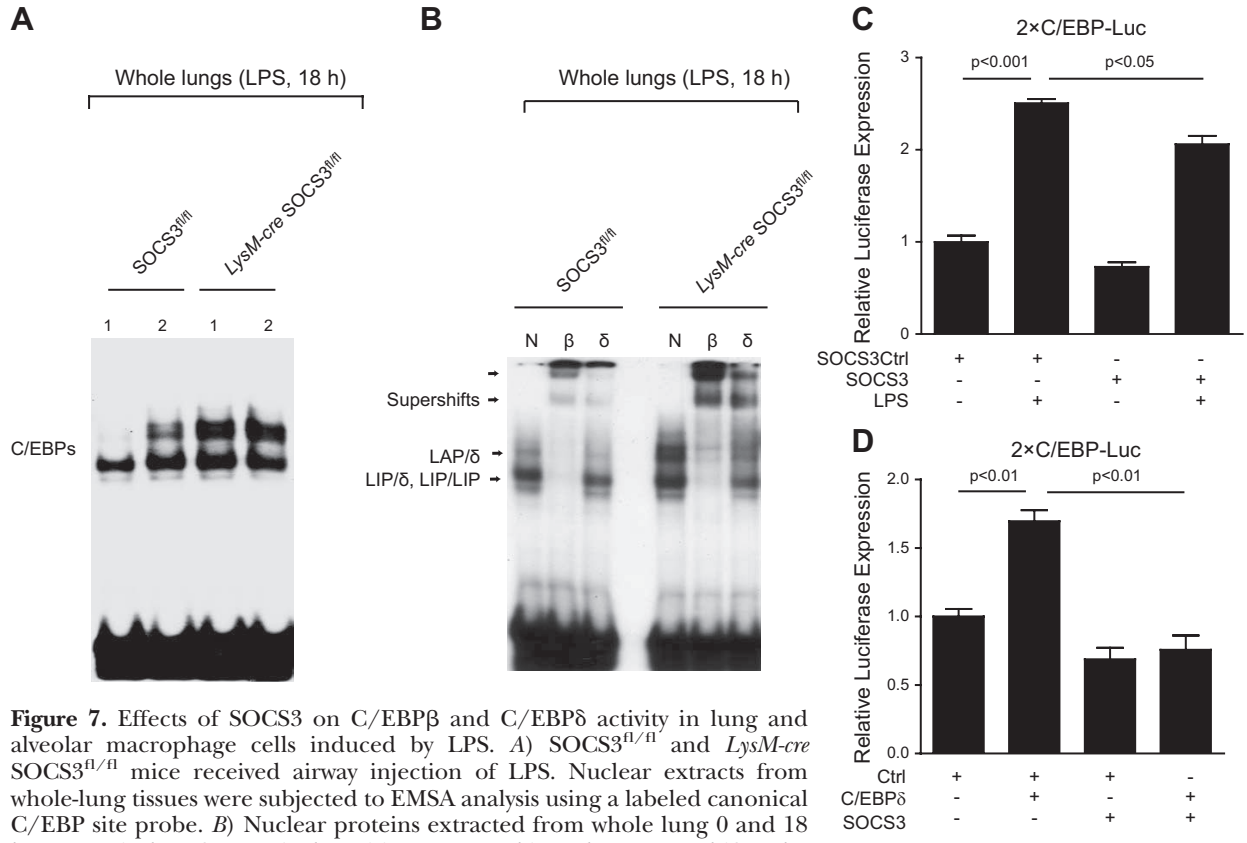

Figure 7. Effects of SOCS3 on C/EBPβ and C/EBPδ activity in lung and alveolar macrophage cells induced by LPS. *A*) $SOCS3^{fl/fl}$ and *LysM-cre* $SOCS3^{fl/fl}$ mice received airway injection of LPS. Nuclear extracts from whole-lung tissues were subjected to EMSA analysis using a labeled canonical C/EBP site probe. *B*) Nuclear proteins extracted from whole lung 0 and 18 h, respectively, after LPS deposition were subjected to supershift. The following antibodies were used: normal rabbit IgG (N), anti-C/EBPβ antibody (β), and anti-C/EBPδ antibody (δ). Supershifted species and C/EBP dimers are indicated. *C*, *D*) Transient transfections were performed in MH-S cells by using 0.5 μg of indicated DNA. After 48 h, the cells were treated with or without 100 ng/ml LPS for 4 h. The luciferase activity was measured. Luminometer values were normalized for expression from a cotransfected thymidine kinase reporter gene. These values were then normalized to a relative value of 1 for the control plasmid transfected cells that was not stimulated by LPS. Data are expressed as means ± SE (n=3).

DISCUSSION

Despite the recent development of protective lung ventilation strategies, ALI and its severe form, acute respiratory distress syndrome (ARDS), remain leading factors of morbidity and mortality in critically ill patients (43–46). One of the major cellular responses in lung initiated by various direct and indirect stimuli is the activation of genes encoding various inflammation-associated mediators. While a variety of factors, such as acute-phase proteins, lipid metabolites, coagulation factors, and antimicrobial peptides, are involved in the acute lung inflammatory response, cytokines constitute the largest and most pleiotropic group of such mediators (47). The critical role that cytokines play in the initiation, maintenance, and resolution of lung inflammation has been well recognized. For example, along

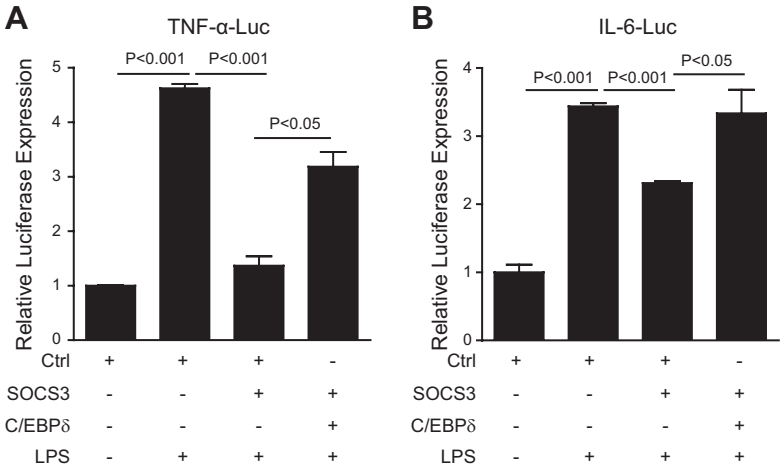

Figure 8. Function link of SOCS3 and C/EBPδ during LPS-induced inflammation in alveolar macrophage cells. Transient transfections were performed in MH-S cells by using 0.5 μg of indicated DNA. After 48 h, the cells were treated with or without 100 ng/ml LPS for 4 h. Luciferase activity (*A*) TNF-α expression or (*B*) IL-6 expression was measured. Luminometer values were normalized for expression from a cotransfected thymidine kinase reporter gene. These values were then normalized to a relative value of 1 for the control plasmid transfected cells that was not stimulated by LPS. The data were expressed as means ± SE (n=3).

with other soluble factors, as well as adhesion molecules, the cytokines contribute to the recognition of pathogens, the recruitment of neutrophils and mononuclear cells, and the removal of the invading microorganism (47). Accordingly, the levels of pro- and anti-inflammatory cytokines and their balance determine the magnitude of lung inflammation and outcome. An imbalance in the cytokine response may result in an uncontrolled systemic inflammatory response such as sepsis or allow progression of the infection, both of which often leads to organ dysfunction. Understanding how the inflammatory process in the lung is activated and how it is contained are key to developing strategies designed to block or reduce lung injury (48). Thus, the biological events and detailed molecular mechanisms that regulate the progression and resolution of inflammation in the lung remain of interest. *In vivo* animal model of LPS-induced ALI are widely used as experimental approaches to study the mechanisms of acute lung inflammation, which displays key features of microvascular injury as seen in patients with ARDS (49). Using this model, a recent study demonstrates that inhibiting Mer receptor tyrosine kinase suppresses STAT1, SOCS1/3, and NF-κB activation and enhances inflammatory responses in LPS-induced ALI (50). However, the exact role of SOCS3 in ALI and the underlying molecular mechanisms remain unknown. In the current study, we show that myeloid-specific SOCS3 deficiency significantly inhibits LPS-induced ALI as defined by enhanced albumin leakage into lung, MPO content, and histology change in the lung, suggesting that SOCS3 is a critical mediator of LPS-induced ALI and inflammatory response in alveolar macrophages.

The mechanisms whereby SOCS3 regulates cytokine signaling have been extensively studied. SOCS3 inhibits cytokine receptors/Janus kinases (JAKs)/STATs activities by either directly inhibiting the catalytic activity of JAK1/2/TYK2 or mediating ubiquitination of JAKs and cytokine receptors targeting them for proteasomal degradation (51). In addition, a recent study shows that SOCS3 interacts with both JAK and the gp130 receptor simultaneously, suggesting that SOCS3 at physiological level exerts its inhibitory function through cytokine receptors with SOCS3 binding sites (52). In contrast, the mechanism of how SOCS3 suppresses LPS-induced gene expression is still unclear. Both direct and indirect mechanisms have been proposed. SOCS3 has been shown to be the dominant mediator of the anti-inflammatory effects of IL-10 in macrophages (30, 53), suggesting a direct role of SOCS3 in mediating the anti-inflammatory response. The effect of SOCS on TLR signaling has also been proposed to occur indirectly through STAT3 activation (54). However, the target genes of SOCS3 necessary for this suppression are not known.

While our previous study shows that adenoviral-mediated overexpression of SOCS3 enhances IgG immune complex-induced ALI (2), the current study shows that SOCS3 negatively regulates TLR4 signaling in the lung. This duality of function suggests a new paradigm for SOCS3 in inflammatory response and lung injury. Of note, the duality of function is an emerging paradigm for not only SOCS3 but also many other immune mediators. For example, TLR4 agonists have opposing actions on lung function depending on the dose and timing of their administration (55). This study shows that low-level inhaled LPS signaling through TLR4 is necessary to induce Th2 responses to inhaled antigens in a mouse model of allergic sensitization. In contrast to low levels, inhalation of high levels of LPS with antigen results in Th1 responses. In another study, Cao *et al.* (56) show that the inositol 3-phosphatase PTEN negatively regulates FcγR signaling, but supports TLR4 signaling in murine peritoneal macrophages. Using the same SOCS3 myeloid-KO mice, we found that SOCS3 deficiency led to a significant decrease of IgG immune complex-induced cytokine and chemokine production in the BAL fluids (Supplemental Fig. S1). These data together suggest that SOCS3 is a negative regulator of TLR4 signaling but a positive regulator of FcγR signaling in the lung. In addition, the role of SOCS3 during lung inflammation remains largely unknown and is likely to be complex.

In the present study, we show that both C/EBPβ and C/EBPδ activation in the LPS-injured lung were significantly higher in myeloid-specific SOCS3-KO mice than in WT mice (Fig. 7), implicating C/EBPβ and/or C/EBPδ as possible targets of SOCS3 in the lung. Indeed, our recent study using C/EBPδ-deficient mice demonstrates that C/EBPδ is a critical mediator of acute inflammatory responses in LPS-injured lung and alveolar macrophages stimulated by LPS (42). Interestingly, we found that C/EBPβ deficiency had no effect on LPS-induced lung injury (42). These findings together indicate for the first time that C/EBPδ transcription factor is a key target of SOCS3 in LPS-induced lung inflammatory responses and injury. Several studies have shown that C/EBPβ and C/EBPδ play important roles in regulating inflammatory function (32, 57). The fact (57) that knocking down either C/EBPβ or C/EBPδ has no effect on LPS-induced IL-6 and TNF-α production in peritoneal macrophages, while the absence of both C/EBPβ and C/EBPδ results in a significant decrease in IL-6 and TNF-α expression, indicates a functional redundancy for these two transcription factors. In contrast, our recent finding (42) that C/EBPδ is a key regulator of LPS-induced inflammation in alveolar macrophages suggests that the function of C/EBPδ is cell-specific. Although both C/EBPβ and C/EBPδ can bind the same promoter regions of several inflammatory mediators, including IL-6 and TNF-α, their distinct transactivation domains may contribute the unique functions in different cells and/or tissues. Of note, while in the current study we provide the first evidence that SOCS3 inhibited C/EBP DNA binding activity induced by LPS, the same treatment did not affect NF-κB activation (Fig. 7 and data not shown). NF-κB has been proven to play a key role in the transcriptional up-regulation of many of the LPS-in-

duced inflammatory mediators. Hence, our study suggests that NF-κB itself is not sufficient to mediate inflammatory mediator expression, which may require its interaction with other transcription factors such as C/EBPs for LPS induction of these genes.

Activated alveolar macrophages play an indispensable role in LPS-induced lung inflammatory responses (28). In the current study we show that SOCS3 deficiency in alveolar macrophages results in a dramatic enhancement of LPS-induced cytokine and chemokine production (Fig. 6). Based on our results, we propose a novel SOCS3-C/EBPδ pathway in the LPS-injured lung, which encourages further evaluation of their therapeutic potential for the treatment of ALI. FJ

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