Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK–STAT

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Suppressor of cytokine signaling-1 (SOCS-1) is one of the negative-feedback regulators of Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling. We previously showed that SOCS-1 participates in LPS signaling, but it is not entirely clear yet how SOCS-1 suppresses LPS signaling. In this study, we demonstrate that SOCS-1 selectively inhibits LPS-induced IL-6 production through regulation of JAK–STAT but not production of TNF- α , granulocyte colony-stimulating factor, IFN- β , and other cytokines. We found that LPS directly activated Jak2 and Stat5, whereas SOCS-1 inhibited LPS-induced Jak2 and Stat5 activation. Furthermore, AG490, a Jak-specific inhibitor, and dominant negative Stat5 only reduced LPS-induced IL-6 production. Additionally, Stat5 interacted with p50, resulting in recruitment of Stat5 to the IL-6 promoter together with p50 in response to LPS stimulation. These findings suggest that the JAK–STAT pathway participates in LPS-induced IL-6 production and that SOCS-1 suppresses LPS signaling by regulating JAK–STAT.

innate immunity | Jak2 | Stat5 | Toll-like receptor signal

Bacterial LPS is the principal active agent in the pathogenesis of endotoxin shock, which is triggered by the interaction of LPS with host cells, such as monocytes and macrophages, and leads to the production of cytokines and other inflammatory mediators, including IL-1, IL-6, TNF- α , IL-12, and IFNs (1). It is well known that Toll-like receptors (TLRs) are involved in innate immune responses to a variety of pathogens (2). LPS is recognized by TLR4, which is associated with the following downstream molecules: myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain-containing adaptor (TIRAP)/MyD88-adaptor-like (Mal), IL-1 receptor-associated kinase (IRAK), and TNF receptor-associated factor 6 (TRAF6) (3–5). The transmitted signal triggers MyD88- and TRIF-dependent pathways, resulting in the activation of transcription factors NF- κ B and IFN regulatory factor-3 (IRF-3), respectively, which then leads to the production of LPS-induced physiological events.

Suppressor of cytokine signaling-1 (SOCS-1) was initially identified as an intracellular negative-feedback molecule that inhibits Janus kinase (JAK)–signal transducer and activator of transcription (STAT) activation initiated by various stimuli, including IFN- γ , IL-6, IL-4, and IL-12 (6–8). SOCS-1-deficient (KO) mice are born healthy but develop various abnormalities as they age, including growth retardation, thymic atrophy, and fulminant hepatitis accompanied by serious fatty degeneration and lung damage caused by infiltration with mononuclear cells; all of these mice die within 3 weeks of birth (9, 10). These pathological alterations are reduced in IFN- γ -deficient SOCS-1 KO mice (11, 12). In an earlier study, we demonstrated that SOCS-1 is also essential for cross-talk inhibition in cytokine signaling between IFN- γ and IL-4 *in vivo* because lethal tissue alterations are eliminated equally in SOCS-1 and Stat1 double-KO mice and SOCS-1 and Stat6 double-KO mice (13). Moreover, we showed that SOCS-1 inhibits insulin and TNF- α -

induced apoptosis signals and affects the various cytokine signal pathways (14, 15), and in a more recent study (16, 17), we found that SOCS-1 participates in LPS signaling and is also essential for innate immunity. Nevertheless, the molecular mechanisms involved in LPS signaling have not yet been clearly identified (16, 17).

In this study, we demonstrate that SOCS-1 selectively inhibits LPS-induced IL-6 production but not the production of TNF- α , granulocyte colony-stimulating factor, IFN- β , or other cytokines. To explore the relevant mechanisms, we first investigated whether JAK–STAT was directly involved in LPS signaling because JAK–STAT proteins are the target of SOCS-1. We found that LPS directly activated Jak2 and Stat5, whereas SOCS-1 inhibited LPS-induced Jak2 and Stat5 activation. In addition, we show that Jak2 and Stat5 are required for LPS-induced IL-6 production. These findings suggest that SOCS-1 directly inhibits LPS signaling through regulation of the JAK–STAT pathway.

Materials and Methods

Mice and Cells. Three-week-old SOCS-1 mutant (SOCS-1 He) mice, as described in ref. 8, were used. WT and SOCS-1 He mice were injected i.p. with 1 mg of *Escherichia coli* LPS (Sigma) for 2 h.

Peritoneal macrophages were prepared as described in ref. 18. The thioglycolate-elicited peritoneal macrophages and a mouse macrophage cell line (Raw cells) were cultured in RPMI medium 1640 with 10% FCS, 100 μ g/ml streptomycin, and 100 units/ml penicillin G. Raw cells were stably transfected with Stat5 1*6 and Stat5 DN cDNAs (kindly donated by Toshio Kitamura, University of Tokyo, Tokyo), as shown in ref. 8. Stable transfected Raw mutant lines (Raw/Neo, Raw/SOCS-1, Raw/Stat5 1*6, and Raw/Stat5 DN) were also maintained in the presence of 500 μ g/ml G418. COS-7 cells were cultured in DMEM with 10% FCS, 100 μ g/ml streptomycin, and 100 units/ml penicillin G.

RT-PCR. Total RNA was prepared by using RNeasy (Qiagen, Germantown, MD), and cDNA was prepared as described in ref. 16. Reaction conditions consisted of a 45-s denaturation step at 94°C, a 30-s annealing step at 58°C, and a 30-s elongation step at 72°C for 25–35 cycles. The specific primers were as follows: IL-6, sense 5'-GATGCTACCAAACTGGATATAATC-3' and antisense 5'-GGTCCTTAGCCACTCCTTCTGTG-3'; TNF- α , sense 5'-GTGACAAGCCTGTAGCCCA-3' and antisense 5'-

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Abbreviations: TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IRF-3, IFN regulatory factor-3; SOCS-1, suppressor of cytokine signaling-1; JAK, Janus kinase; STAT, signal transducer and activator of transcription; KO, knockout; SOCS-1 He, SOCS-1 mutant; I κ B ζ , NF- κ B inhibitor; ChIP, chromatin immunoprecipitation.

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Fig. 1. SOCS-1 selectively inhibits IL-6 production by LPS. (A) Raw/Neo and Raw/SOCS-1 cells were stimulated by LPS at the indicated time points. Expression of LPS-induced cytokines genes was examined by RT-PCR. (B) Raw/Neo and Raw/SOCS-1 cells were stimulated by LPS as indicated. IL-6 and TNF- α production was measured by using ELISA. (C) SOCS-1 He mice and WT mice were injected i.p. with 1 mg of LPS. Serum IL-6 levels were measured by ELISA at 2 h. Data show means $\pm$ SE of three independent experiments.

AAAGTAGACCTGCCCGGAC-3'; granulocyte colony-stimulating factor, sense 5'-CTCAACTTTCTGCCCA-GAGG-3' and antisense 5'-AGCTGGCTTAGGCACTGTGT-3'; NF- κ B inhibitor (I κ B ζ), sense 5'-TGTTGCCTTCTCACT-TCGTG-3' and antisense 5'-GAGTCTCAGTTTGGGGT-GGA-3'; G3PDH, sense 5'-TCCACCACCTGTTGCTGTA-3' and antisense 5'-ACCACAGTCCATGCCATCAC-3'.

Cytokine ELISA. The cells were stimulated with 2 mg/ml LPS for 60, 90, 120, 150, or 180 min. Mouse IL-6 and TNF- α (R & D Systems) from either the supernatants or the serum were measured by ELISA according to the manufacturer's instructions.

Activation of Jak2-Stat5. Raw/Neo, Raw/SOCS-1, or peritoneal macrophages were incubated with 2 mg/ml LPS for the time indicated, and cells were lysed with a lysis buffer (1% Nonidet P-40/20 mM Tris-HCl, pH 7.5/150 mM NaCl/10 mM Na₂VO₄/0.5 mM DTT/1:100 protease inhibitor mixture). By using anti-Jak2 and anti-Stat5 Abs (Santa Cruz Biotechnology), immunoprecipitation was performed as described in ref. 15. Whole-cell lysates and the immunocomplex were analyzed with Western blotting by using anti-4G10 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Stat5 (Tyr-694) (Cell Signaling Technology, Beverly, MA), or anti-Jak2 Abs.

Immunoprecipitation and Western Blotting. COS-7 cells were transfected with 5 μ g of pEF-BOS-Jak2 or pcDNA3.1-Stat5 and 5 μ g of pEF-BOS-TLR4-Flag (kindly donated by Kensuke Miyake, University of Tokyo, Tokyo) or pEF-BOS-MyD88-Flag with the DEAE-dextran method. Cells were lysed with lysis buffer and lysates were immunoprecipitated with anti-Flag M2 (Sigma). Immunoprecipitated samples were analyzed by means of West-

ern blotting by using anti-Jak2 or anti-Stat5 Abs. Similarly, pEF-BOS-p50-Flag and pcDNA3.1-Stat5 WT, Stat5 1*6, or Stat5 DN were introduced into COS-7 and Raw/Stat5 1*6 cells stimulated by LPS. Cell lysates were immunoprecipitated with the aid of anti-p50 Ab, and immunoprecipitated samples were analyzed by means of Western blotting by using anti-Stat5 (BD Transduction Laboratories).

The ChIP Assay. The chromatin immunoprecipitation (ChIP) assay was performed essentially following the protocol of Upstate Biotechnology. In brief, Raw cells were stimulated with 1 mg/ml LPS for 1 or 2 h and then fixed with formaldehyde for 10 min. The cells were lysed, sheared by sonication, and incubated overnight with specific Abs, followed by incubation with protein A-agarose saturated with salmon sperm DNA (Upstate Biotechnology). Precipitated DNAs were analyzed by quantitative PCR (35 cycles) by using the primers 5'-CGATGCTAAACGACGT-CACATTGTGCA-3' and 5'-CTCCAGAGCAGAATGAGC-TACAGACAT-3' for the κ B site in the IL-6 promoter.

Results

SOCS-1 Selectively Inhibits LPS-Induced IL-6 Production but Not That of Other Cytokines Including TNF- α . We established a mouse macrophage-like cell line (Raw cells) that constitutively expressed SOCS-1 (Raw/SOCS-1) to examine the effects of SOCS-1 on LPS signaling. With Raw/Neo as a control, Raw/SOCS-1 was treated with LPS, and the production of various cytokines induced by LPS was examined by means of RT-PCR. There were no differences between Raw/SOCS-1 and Raw/Neo in LPS-induced cytokine production, including that of TNF- α . Nevertheless, only IL-6 production by LPS was impaired in Raw/SOCS-1 (Fig. 1A). To determine whether the changes in mRNA

Fig. 3. Stat5 participates directly in LPS-induced IL-6 production. (A) Raw/Neo and Raw/SOCS-1 cells were incubated with LPS at the indicated time points. Whole-cell lysates were used for immunoblotting (IB) analysis with anti-phospho-tyrosine Stat5 Ab. (B) Peritoneal macrophages were isolated from BALB/c mice and stimulated by LPS at the indicated time points. Tyrosine phosphorylation of Stat5 was examined by means of immunoprecipitation (IP) and Western blotting. (C) The interaction of Stat5 with TLR4 was examined by immunoprecipitation and Western blotting in COS-7 cells, which were induced with Stat5, TLR4, and Raw cells. Raw/Neo, Raw/Stat5 1*6, and Raw/Stat5 DN were stimulated by LPS at the indicated time points, followed by an examination of IL-6 and TNF-α induction by means of RT-PCR (D) and ELISA (E and F). Data show means $\pm$ SE of three independent experiments.

a downstream molecule of the LPS signal and that SOCS-1 inhibits Stat5 activation by LPS.

Hence, we presumed that Stat5 might participate in IL-6 production induced by LPS. To ascertain whether Stat5 has a similar effect as that of Jak2 on LPS-induced IL-6 production, we established Raw cells overexpressing the constitutively active form of Stat5 (Raw/Stat5 1*6) or the dominant negative form of Stat5 (Raw/Stat5 DN). Raw/Neo, Raw/Stat5 1*6, and Raw/Stat5 DN were stimulated by LPS, and cytokine induction was determined by RT-PCR analysis. As shown in Fig. 3D, Stat5 DN reduced mRNA induction of IL-6 by LPS but not mRNA induction of TNF-α (Fig. 3D). In addition, it was found that LPS-induced IL-6 production was reduced at the protein level in Raw/Stat5 DN compared with production in Raw/Neo and Raw/Stat5 1*6 (Fig. 3E). Nevertheless, LPS-induced TNF-α production was partly enhanced in Raw/Stat5 DN as opposed to production in Raw/Neo and Raw/Stat5 1*6 (Fig. 3F). Taken together, these results suggest that Jak2 and Stat5 are directly activated by LPS and are required for LPS-induced IL-6 production.

Stat5 Is Associated with NF-κB p50 and Is Recruited to the IL-6 Promoter in Response to LPS. A recent study has demonstrated that NF-κB p50 associates with IκBζ induced by LPS and subsequently induces IL-6 production (18). To determine whether

Stat5 interacts with p50, we performed coimmunoprecipitation analysis. This analysis showed that cotransfection of COS-7 cells with Stat5 WT, Stat5 1*6, or Stat5 DN and p50 led to the association of Stat5 WT and Stat5 1*6 with p50 but did not lead to an association of Stat5 DN with p50 (Fig. 4A). We next examined whether Stat5 was associated with endogenous p50 in Raw/Stat5 1*6 in response to LPS and found that the interaction between Stat5 and endogenous p50 occurred 2 h after LPS stimulation (Fig. 4B). These findings indicate that Stat5 is associated with NF-κB p50 in LPS signaling.

We next performed the ChIP assay to confirm further the mechanism by which Stat5 specifically regulates IL-6 production in LPS signaling. Raw cells were stimulated with LPS for 2 h, and the ChIP assay was performed by using Abs, which can detect endogenous Stat5 or p50; it was found that Stat5 and p50 were recruited to the IL-6 promoter in response to LPS (Fig. 4C). Thus, these findings suggest that, like IκBζ, Stat5 associates with p50 and mediates LPS-induced IL-6 production.

Discussion

It has been shown that SOCS-1 affects not only various cytokine signals but also innate immune responses such as the LPS signal (16, 17). Although a recent study demonstrated that LPS-induced SOCS-1 transcription is regulated by early

Fig. 4. Stat5 associates with p50 and mediates LPS-induced IL-6 production. (A) COS-7 cells were cotransfected with Stat5 WT, Stat5 1*6, or Stat5 DN and p50. Whole-cell lysates were immunoprecipitated with anti-p50 Ab after which Stat5 was detected with Western blotting. (B) Raw/Stat5 1*6 cells were stimulated by LPS followed by examination of the association of Stat5 with endogenous p50 by means of immunoprecipitation (IP) and Western blotting. (C) Raw cells were stimulated with LPS for 2 h, and the ChIP assay was performed by using anti-Stat5a, anti-Stat5, and anti-p50 Abs. Purified DNA fragments were amplified by using primers specific for the IL-6 promoter, as described in *Materials and Methods*. IB, immunoblotting.

growth response-1 (21), it remains unclear how SOCS-1 participates directly in LPS signaling. The results of our study reveal that SOCS-1 suppresses the LPS signal by selective inhibition of LPS-induced IL-6 production, but not the production of other cytokines including TNF- α . Recently, it was reported that the nuclear I κ B protein I κ B ζ , which is induced by the MyD88-dependent NF- κ B pathway, is necessary for LPS-induced IL-6 production (18). In this study, SOCS-1 did not inhibit I κ B ζ induction by LPS, which is consistent with the previous finding that SOCS-1 does not affect the NF- κ B pathway in LPS signaling (19).

Next, we explored the target of SOCS-1 in LPS signaling. It has been reported that the JAK family member Tyk2 has an indirect but important role in LPS-induced endotoxin shock by the IFN- β signal (22). However, our data suggested that SOCS-1 directly participates in LPS signaling by selective inhibition of LPS-induced IL-6 production. A phosphorylation of Jak2 and Stat5 were induced by LPS stimuli between 1 and 15 min, whereas SOCS-1 inhibited this phosphorylation. In addition, Jak2 and Stat5 interacted with TLR4. These results indicate that Jak2 and Stat5 are directly activated by LPS, but not by indirectly LPS-inducible cytokines including IFN- β . Moreover, AG490, a JAK protein specific inhibitor, and Stat5 DN inhibited only IL-6 production by LPS, indicating that Jak2 and Stat5 share the regulatory mechanisms necessary to produce IL-6 in the LPS signal similarly to I κ B ζ . The association of I κ B ζ with p50 in response to LPS stimulation was shown to result in IL-6 production (18). In this article, we were able to show that Stat5

associates with p50 and is specifically recruited to the IL-6 promoter after LPS stimulation.

Recent studies have demonstrated that IRF-5 and IRF-7 of the IRF family and selective inhibitor of LPS-induced IL-6 production (I κ BNS) of the I κ B family play important roles in TLR signaling (23–25), suggesting that there are several regulatory mechanisms involved in TLR signaling. Similar to Stat5 activation by LPS, we found that Stat5 is also activated by CpG-DNA, which is known to be the TLR9 ligand (data not shown). Therefore, our study demonstrates that the JAK-STAT pathway, like the IRF family and I κ B family members, also participates in TLR signaling.

In conclusion, we have demonstrated that Jak2 and Stat5 are involved in LPS-induced IL-6 production and that SOCS-1 selectively inhibits LPS-induced IL-6 production by regulating Jak2 and Stat5 activation. Thus, these findings clearly show that SOCS-1 is directly and crucially involved in innate immunity. Given that Stat5 is a transcriptional factor, TLR-mediated Stat5 activation may have other functions in TLR signaling besides the induction of IL-6 production. Further *in vivo* analysis using Stat5 mutant mice is expected to provide new insights into TLR signaling by the JAK-STAT pathway.

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