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 (GCSF), 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-κB 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 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 μg/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 μg/ml G418. COS-7 cells were cultured in DMEM with 10% FCS, 100 μg/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′-GATGCTACAACTGCATATACT-3′ and antisense 5′-GGTCTTAMCCACCTCCCTGTT-3′; TNF-α, sense 5′-GTGACAAGCCTGTAGCCCA-3′ and antisense 5′-GTGACAGGCTGTAGCCCA-3′.

Conflict of interest statement: No conflicts declared.

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, SOCS-1 mutant; IL-6, IL-6; NF-κB, NF-κB inhibitor; CHIP, chromatin immunoprecipitation.

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AAAGTAGACCTGCCCGGAC-3'; granulocyte colony-stimulating factor, sense 5'-CTCAACTTTCTGCCCA-GAGG-3'; NF-κB inhibitor (1κBz), sense 5'-TGTGGCTTTCTCATTGTA-3' and antisense 5'-TGTTGCCTTCTCACTTCGTG-3'; G3PDH, sense 5'-TCCACCACCCTGTTGCTGTA-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, and 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 μg of pEF-BOS-Jak2 or pcDNA3.1-Stat5 and 5 μg 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 Western 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'-CTCCAGAGCAGAATGAGCTACAGACAT-3' for the κB 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. L4). To determine whether the changes in mRNA
Jak2 Is Directly Activated by LPS and Involved in IL-6 Production. It is possible that JAK participates in LPS signaling because JAK proteins are the target of SOCS-1. To determine the validity of this hypothesis, we first examined whether JAK is activated by LPS. Raw/Neo and Raw/SOCS-1 were stimulated by LPS, and JAK activation in the form of tyrosine phosphorylation of the proteins was measured with the aid of immunoprecipitation and Western blotting. The results demonstrated that Jak2 was directly activated between 1 and 5 min after LPS stimulation, whereas SOCS-1 inhibited Jak2 activation (Fig. 2A). To determine whether Jak2 associates with the TLR4-MyD88 complex, COS-7 cells were transiently introduced with Jak2, TLR4, and MyD88 and subjected to coimmunoprecipitation analysis. As shown in Fig. 2B, there was an association between Jak2 and the TLR4-MyD88 complex, but Jak2 could not interact separately with TLR4 or MyD88 (Fig. 2B).

We next investigated whether Jak2 affects the various cytokines induced by LPS to help us explore the role of Jak2 in LPS signaling. Because AG490 is known as the tyrosine kinase inhibitor specific for Jak2 and Jak3 activity (20), Raw cells were stimulated by LPS with or without AG490, and RT-PCR was used for cytokine induction analysis. Interestingly, it was found that AG490 inhibited only the induction of IL-6 in response to LPS stimulation and did not inhibit the induction other cytokines, including TNF-α (Fig. 2C). Moreover, LPS-induced IL-6 production was significantly impaired by AG490 at the protein level as well as at the mRNA level (Fig. 2D). These findings indicate that Jak2 may participate directly in LPS signaling and has an important function in the LPS-induced IL-6 production pathway.

Stat5 Is Involved in IL-6 Production by LPS. Because STAT proteins are activated by JAK in various types of cytokine signaling, we investigated the involvement of STAT in the LPS signaling pathway. Tyrosine phosphorylation of STAT in Raw/Neo and Raw/SOCS-1 cells was examined by Western blotting after treatment with LPS. It was found that Stat5 was activated 5 and 15 min after LPS treatment in Raw/Neo; yet such activation was considerably reduced in Raw/SOCS-1 (Fig. 3A). In addition, Stat5 was activated by LPS in peritoneal macrophages as well as in Raw cells (Fig. 3B). We next examined the interaction between Stat5 and TLR4. For this purpose, Stat5 and TLR4 were introduced into COS-7 cells and subjected to coimmunoprecipitation analysis, which demonstrated the interaction of Stat5 with TLR4. In addition, Stat5 was recruited to TLR4 in response to LPS in Raw cells (Fig. 3C). These findings suggest that Stat5 is...
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 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 ± SE of three independent experiments.

**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 ± SE of three independent experiments.

**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
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 IkB protein IkBζ, which is induced by the MyD88-dependent NF-κB pathway, is necessary for LPS-induced IL-6 production (18). In this study, SOCS-1 did not inhibit IkBζ induction by LPS, which is consistent with the previous finding that SOCS-1 does not affect the NF-κB 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 IkBζ. The association of IkBζ 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 (IkBζNS) of the IκB 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 IκB 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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