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SOCS-1 Participates in Negative Regulation of LPS Responses

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Summary

SOCS-1 is a negative regulatory molecule of the JAK-STAT signal cascade. Here, we demonstrate that SOCS-1 is a critical downregulating factor for LPS signal pathways. SOCS-1 expression was promptly induced in macrophages upon LPS stimulation. SOCS-1-deficient mice were highly sensitive to LPS-induced shock and produced increased levels of inflammatory cytokines. Introduction of SOCS-1 inhibited LPS-induced NF-κB and STAT1 activation in macrophages. Furthermore, LPS tolerance, a refractory state to second LPS stimulation, was not observed in SOCS-1-deficient mice. These results suggest SOCS-1 as an essential, negative regulator in LPS responses that protects the host from harmful overresponses to LPS and may provide new insight into the endotoxin-induced fatal syndrome that occasionally occurs following infection.

Introduction

Lipopolysaccharide (LPS) is an integral cell wall component of Gram-negative bacteria and can provoke a life-threatening condition called endotoxic shock (for review see Ulevitch and Tobias, 1995). LPS has been shown to be recognized by a member of Toll-like receptors (TLRs), TLR4 (Poltorak et al., 1998; Qureshi et al., 1999; Hoshino et al., 1999). TLRs have now been established to be essential for the recognition of specific patterns of microbial components (for review see Aderem and Ulevitch 2000; Medzhitov, 2001; Akira et al., 2001). Activation of TLRs including TLR4 induces production of inflammatory cytokines through the sequential activation of intracellular signaling molecules such as myeloid differentiation factor (MyD) 88, IL-1R-associated kinase (IRAK), IRAK-4, and TNFR-associated factor (TRAF) 6 (Medzhitov et al., 1998; Muzio et al., 1998). This process results in the activation of two different pathways that involve the c-Jun NH_2-terminal kinase (Jnk) and p38 mitogen-activated protein kinase (MAPK) family and the Rel family transcription factor NF-κB (Muzio et al., 1998). Critical involvement of MyD88, IRAK-4, and TRAF6 in LPS-induced inflammatory responses was demonstrated in gene-targeted mice (Lomaga et al., 1999; Naito et al., 1999; Kawai et al., 1999; Suzuki et al., 2002).

LPS-activated cells sometimes induce microcirculatory dysfunction as well as inflammatory changes, which cause various tissue damage, circulatory failure, and occasionally death (Michalek et al., 1980; Freudenberg et al., 1986; for review see Hinshaw, 1985, 1990; Ulevitch and Tobias, 1995). To prevent the excessive and prolonged responses of host innate immune cells to LPS, host may acquire downregulating system that ensures safety responses to LPS and/or unresponsiveness to a second stimulation with LPS named LPS tolerance (Ziegler-Heitbrock, 1995). Pretreatment with a sublethal dose of LPS reduces febrile responses and even mortality in these animals after subsequent challenge with a lethal dose of LPS (Fraker et al., 1988; Biberstine et al., 1996; Kreutz et al., 1997; for review see Zeisberger and Roth, 1998). Several factors have been proposed to be involved in LPS tolerance, including the downregulation of LPS signaling receptor, TLR4/MD2, expression (Nomura et al., 2000) and decreased activation of NF-κB (Ziegler-Heitbrock et al., 1994; Goldring et al., 1998). However, the underlying mechanisms are largely unknown.

SOCS-1 (suppressor of cytokine signaling), also called SSI-1 (STAT-induced STAT inhibitor-1) and JAB-1 (JAK binding protein-1), was initially identified as an intracellular negative-feedback molecule that inhibits overactivation of the JAK-STAT-mediated signal cascade initiated by various stimuli, including IFN-γ, IL-4, IL-6, and LIF (Starr et al., 1997; Endo et al., 1997; Naka et al., 1997). SOCS-1 induced by these cytokines binds to JAKs to inhibit the following signal transduction (Narazaki et al., 1998; Nicholson et al., 1999; Yasukawa et al., 1999; Fujimoto et al., 2000; for review see Naka et al., 1999, 2001b; Yasukawa et al., 2000; Nicola and Greenhalgh, 2000; Starr, 2001). SOCS-1 is a key molecule for IFN-γ signaling in vivo. SOCS-1-deficient (SOCS-1 KO) mice show lethal pathological changes in various organs including fulminant hepatitis and die within 3 weeks after birth, whereas disruption of the IFN-γ gene reduces these pathological alterations as well as early death in SOCS-1 KO mice (Alexander et al., 1999; Marine et al., 1999). However, our recent study demonstrates that SOCS-1 is also essential for crosstalk inhibition in cytokine sig-

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naling between IFN-γ and IL-4 in vivo, because lethal tissue alterations are abrogated equally in SOCS-1 and STAT1 double-knockout (SOCS-1/STAT1 DKO) mice and SOCS-1 and STAT6 double-knockout (SOCS-1/STAT6 DKO) mice (Naka et al., 2001a). Furthermore, it has also been clarified that SOCS-1 has the capacity to associate with other types of intracellular molecules, such as Vav, Tec, Syk, ITAM motif of CD8, and Kit receptor in vitro (Ohya et al., 1997; Sepulveda et al., 1999; Matsuda et al., 2000). Additionally, SOCS-1 is required for safety responses to TNF-α and insulin that do not primarily utilize the JAK-STAT signal pathway (Morita et al., 2000; Kawazoe et al., 2001).

In this study, we investigated whether SOCS-1 plays an essential role in LPS signaling and in induction of LPS tolerance because SOCS-1 KO mice spontaneously show elevation of serum levels of proinflammatory cytokines in addition to the multiple inflammatory organ diseases, which are hallmarks of LPS-associated diseases in wild-type (WT) mice (Starr et al., 1998; Naka et al., 1998; Michalek et al., 1980; Freudenberg et al., 1986; for review see Hinshaw, 1985, 1990). SOCS-1 KO mice died after challenge with much lower amounts of LPS than those required for induction of lethal shock in WT mice. SOCS-1 KO mice that had been treated with their sublethal dose of LPS still showed lethality to the second challenge with their lethal dose of LPS, while WT similarly pretreated were free from lethal shock after challenge with their lethal dose of LPS. These findings suggest that SOCS-1 may act as an essential negative regulatory molecule in innate immune responses.

Results

Hyperresponsiveness to LPS in SOCS-1 KO Mice

SOCS-1 KO mice started to develop a complex disease around 1 to 2 weeks after birth (Starr et al., 1998; Naka et al., 1998). To investigate sensitivity of SOCS-1 KO mice to LPS, we intraperitoneally injected various doses of LPS into 3-day-old, pre-onset SOCS-1 KO mice that did not exhibit any obvious pathological changes as reported in our previous study (Naka et al., 1998). SOCS-1 KO mice but not WT littermate mice died within 6 hr after challenge with 5 μg of LPS (Figure 1Aa). The mortality of SOCS-1 KO mice is significantly higher than WT mice ($p < 0.01$). Administration of 0.5 μg of LPS did not cause mortality in SOCS-1 KO mice (described below, Figure 6). As described below, LD_{50} of LPS for SOCS-1 KO mice was 2–3 μg, which was less than 1% of LD_{50} (800 μg) for WT mice (Figure 6). To exclude the possibility that the low dose LPS simply accelerates natural fatality in pre-onset SOCS-1 KO mice, we examined LPS response of SOCS-1 heterozygous (He) mice that show normal growth after birth but have partial impairment in cytokine signaling (Naka et al., 1997, Fujimoto et al., 2002). Four-week-old SOCS-1 He mice also exhibited hypersensitivity to LPS as compared with WT littermate mice ($p < 0.01$), although it took a much longer time for SOCS-1 He mice to succumb after LPS challenge than for SOCS-1 KO mice (Figure 1Ab). Because TNF-α is a major relevant factor involved in the lethality induced by LPS (Beutler et al., 1985; Tracy et al., 1987; Freudenberg and Galanos, 1991), we measured serum levels of TNF-α in pre-onset SOCS-1 KO mice, SOCS-1 He mice, or WT mice at 2 hr after LPS challenge. Serum TNF-α levels in SOCS-1 KO and SOCS-1 He mice were more than six to seven times and 2.5 to three times, respectively, of those in WT mice (Figures 1Ba and 1Bb). Moreover, SOCS-1 KO mice but not SOCS-1 He or WT mice were highly susceptible to a TNF-α -induced lethality (data not shown), indicating that the hyperacute lethality of LPS-treated SOCS-1 KO mice (Figure 1A) is attributed to their hyperproduction of and hyperresponsiveness to endogenous TNF-α.

As a major cell source of TNF-α in LPS-challenged mice is macrophages (Carswell et al., 1975; Michie et al., 1988), we investigated the ability of SOCS-1-deficient macrophages to produce TNF-α or IL-12 in response to various doses of LPS in vitro. The splenic adherent cells from pre-onset SOCS-1 KO mice produced much greater amounts of TNF-α or IL-12 in response to 1 to 1000 ng/ml of LPS as compared with WT splenic adherent cells (Figures 2A and 2B). The increased production of IL-12 was also the case for treatment with 5–500 nM of CpG DNA or with 1–30 ng/ml of macrophage-activating lipopeptide 2 kDa (MALP-2) (Figure 2C and data not shown). These findings indicate that downregulatory mechanisms of TLR2 and TLR9 as well as TLR4 signal cascade may be disturbed in splenic adherent cells from SOCS-1 KO mice. The cell yield from SOCS-1 KO mice was almost equivalent to that from WT littermate mice. The purity of macrophages in the splenic adherent cells prepared from WT or the mutant mice was more than 85%, determined by their expression of F4/80 (Figure 2D). In addition to the case of pre-onset SOCS-1 KO mice, the splenic adherent cells from 4-week-old SOCS-1 He mice also produced much greater amounts of TNF-α and IL-12 in response to 1–1000 ng/ml of LPS as compared with these from WT littermates (Figure 2E and data not shown). To exclude the possibility that SOCS-1-deficient macrophages might be endogenously and spontaneously activated even under SPF conditions, we compared the levels of activation markers on splenic macrophages between SOCS-1 KO mice and WT mice. SOCS-1-deficient splenic macrophages express almost equivalent levels of MHC class II and activation molecules, CD80, and CD86 as compared to WT macrophages (Figure 2D), suggesting that no endogenous activation of macrophages occurs under SOCS-1-deleted conditions. As previously reported, SOCS-1 is also a potent downregulating molecule for TNF-α signaling (Morita et al., 2000). Taken together, these results suggested that SOCS-1 might inhibit the signaling of both LPS and TNF-α, eventually resulting in downregulated biological responses to LPS in WT mice.

Loss of LPS Tolerance in SOCS-1 KO Mice

The results that SOCS-1 downregulates LPS-induced biological responses (Figures 1 and 2) prompted us to investigate whether SOCS-1 is also involved in LPS tolerance, a possible protection system to prevent harmfully excessive and prolonged responses to LPS. Pre-onset SOCS-1 KO mice or WT littermate mice had been treated with a low dose of LPS (0.5 μg), which is less than the lethal dose for pre-onset SOCS-1 KO mice

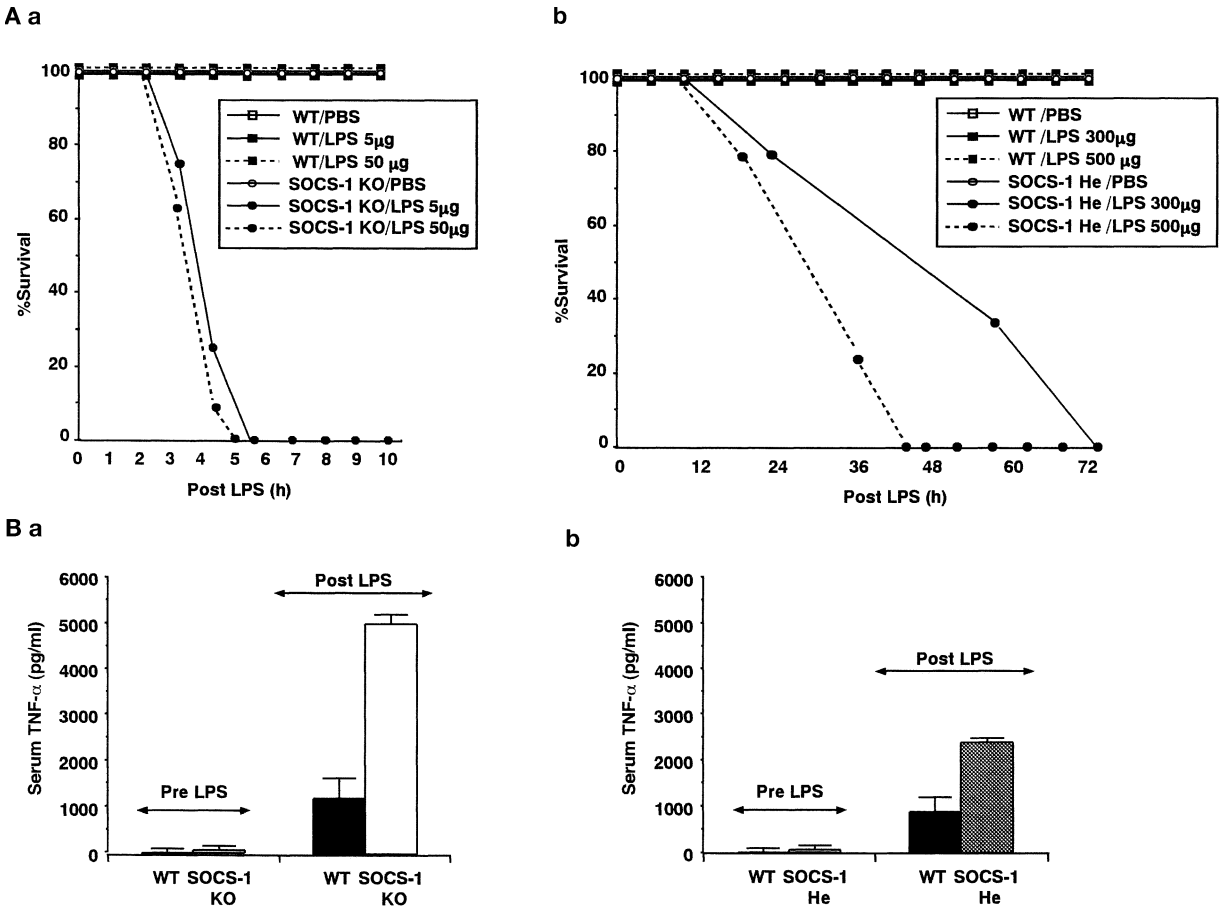


Figure 1. Hypersensitivity of SOCS-1 KO Mice to LPS In Vivo

(Aa) Three-day-old SOCS-1 KO and littermate WT mice were i.p. administered with 0, 5, or 50 μg LPS. Survival was monitored until 12 hr after LPS challenge. SOCS-1 KO mice showed lower survival than WT mice upon challenge with 5 μg ($p < 0.01$) or 50 μg LPS ($p < 0.01$).

(Ab) Four-week-old SOCS-1 He and littermate WT mice were i.p. administered with 0, 300, or 500 μg LPS. Survival was monitored until 72 hr after LPS challenge. SOCS-1 He mice showed lower survival than WT mice upon challenge with 5 μg ($p < 0.01$) or 50 μg LPS ($p < 0.01$).

(Ba) Serum TNF-α levels from SOCS-1 KO or littermate WT mice (3-day-old) were measured at 0 and 2 hr after intraperitoneal injection of LPS (50 μg).

(Bb) Serum TNF-α levels from SOCS-1 He or littermate WT mice (4-week-old) were measured at 0 and 2 hr after intraperitoneal injection of LPS (500 μg).

Data in (Aa) and (Ab) represent 15 mice in each experimental group and are the result of two independent experiments with similar results. Data in (Ba) and (Bb) show the mean ± SD for three mice from each group and are representative of three independent experiments with similar results.

(described below, Figure 6), and were subsequently challenged with the corresponding lethal doses of LPS, 50 μg for the mutant mice and 1 mg for WT mice, respectively. WT mice that had been treated with only vehicle died within 7 hr, while WT mice pretreated with the low dose of LPS evaded the death after challenge with their own lethal doses of LPS, indicating that the initial dose of LPS is adequate for induction of LPS tolerance in WT mice (Figure 3A). In contrast, SOCS-1 KO mice that had been administered the same initial dose of LPS failed to evoke LPS tolerance (Figure 3Aa). SOCS-1 He mice, like SOCS-1 KO mice, showed impairment in LPS tolerance (Figure 3Ab). In a separate experiment, we found no significant difference of survival rate and its kinetics upon LPS challenge between PBS- and LPS-primed SOCS-1 KO mice or SOCS-1 He mice (data not shown). These in vivo findings suggested that LPS tolerance is abrogated in SOCS-1KO or SOCS-1 He mice.

To analyze LPS tolerance in cell levels, splenic adherent cells from pre-onset SOCS-1 KO mice or WT littermate mice had been pretreated with 100 ng/ml of LPS for 24 hr and then stimulated with 100 ng/ml of LPS for an additional 12 hr following vigorous washing. LPS-pretreated WT splenic adherent cells showed a decreased TNF-α production after the second activation with LPS (Figure 3B). In contrast, LPS pretreatment did not reduce TNF-α production by SOCS-1-deficient cells in response to the second stimulation with LPS (Figure 3B). SOCS-1 mRNA was detected in WT splenic adherent cells from 4–24 hr after LPS stimulation (Figure 3C).

As SOCS-1 is induced by stimulation with other TLR ligands such as CpG-DNA (Dalpke et al., 2001) and MALP2 (data not shown), we analyzed a role of SOCS-1 in crosstolerance to LPS induced by TLR9 or TLR2 ligands. In contrast to WT cells, SOCS-1-deficient macrophages lost crosstolerance to LPS induced by CpG DNA

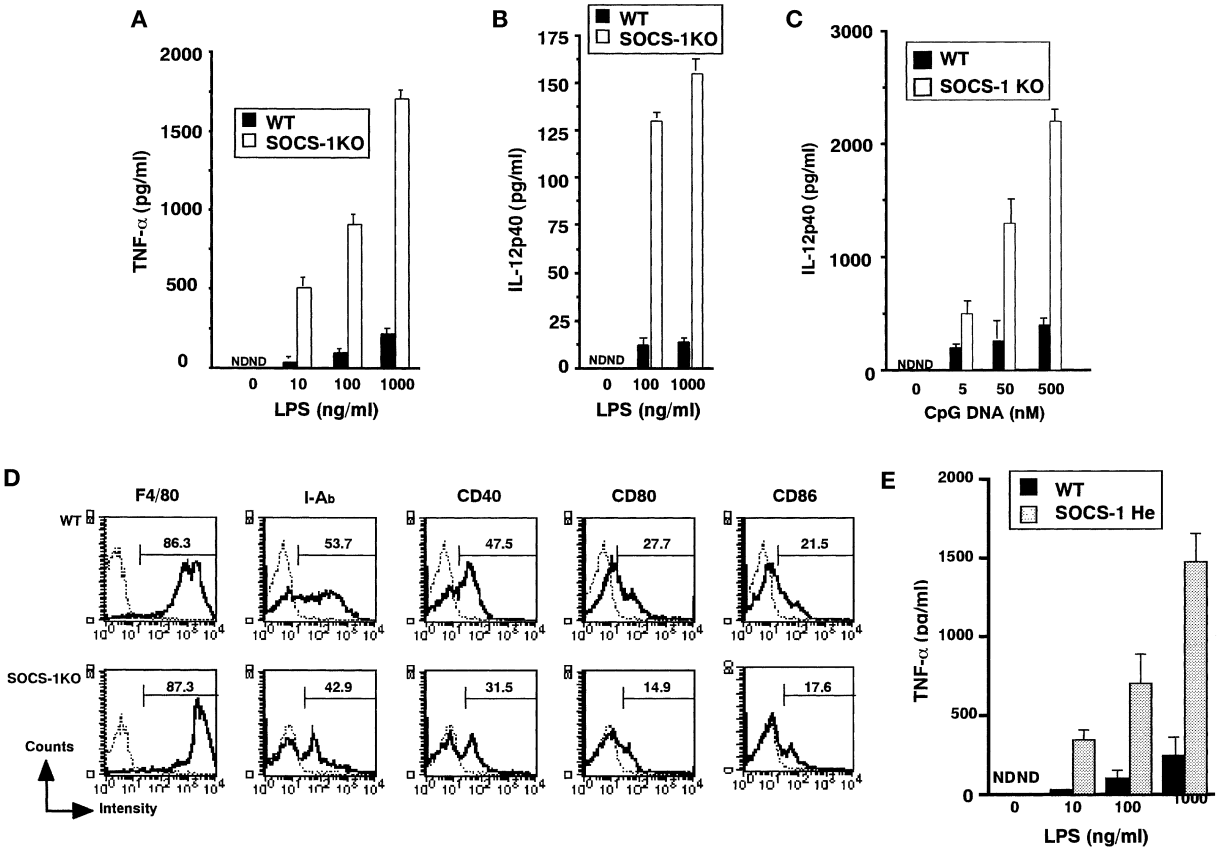


Figure 2. Hyperresponse to LPS of SOCS-1-Deficient Macrophages

(A, B, C, and E) Splenic adherent cells isolated from mice with various genotypes were primed with different amount of LPS (A, B, and E) or CpG DNA (C) for 12 hr, after which culture supernatants were harvested for measurement of TNF-α (A and E) or IL-12p40 (B and C). (D) The same splenic adherent cells from the mice with various genotypes were incubated with FITC-conjugated anti-I-A, anti-CD40, anti-CD80, anti-CD86, or a combination of PE-conjugated streptavidin, biotinylated anti-rat IgG2b, and anti-F4/80. The cell yield of SOCS-1 KO mice was $(2.3 \pm 0.8) \times 10^5$/mouse, while that of WT was $(2.0 \pm 1.3) \times 10^5$/mouse. Data in (A)–(E) are representative of three independent experiments with similar results. ND, not detected.

or MALP-2 (Figure 3B). Therefore, SOCS-1 seems to play an important role in induction of crosstolerance among TLR ligands.

Recently, it has been shown that LPS tolerance is, in part, due to downregulation of expression of TLR4/MD-2 complex, a functional receptor for LPS, which is induced by the initial stimulation with LPS (Nomura et al., 2000). We compared the expression of the TLR4/MD-2 complex on splenic adherent cells from SOCS-1 KO mice before and after treatment with LPS. Any differences between the mutant and control cells were not detected in the reduction of expression of theTLR4/MD-2 complex after stimulation with LPS (Figure 3D). Interestingly, mean intensity of the TLR4/MD-2 complex expressed on TLR4/MD-2 complex-positive macrophages from the mutant mice is obviously lower as compared with WT mice (Figure 3D). These in vivo and extra vivo findings indicate that SOCS-1 is involved in LPS tolerance without affecting TLR4/MD2 downregulation.

LPS-Induced SOCS-1 Induction in Macrophages

To explore the roles of SOCS-1 in LPS signaling, we further examined whether SOCS-1 directly downregulates LPS responses in macrophages. As LPS or IFN-γ induces nitric oxide (NO) production, we examined whether overexpression of SOCS-1 impaired NO production by macrophages. LPS or IFN-γ stimulates Raw/Neo, a mouse macrophage cell line (Raw264.7 cells, Raw cells) transfected with an empty vector to produce NO (Figure 4A), and the combination of these two stimuli resulted in the strongest induction of NO production (Figure 4A). Expectedly, Raw cells overexpressing SOCS-1 (Raw/SOCS-1) produced little NO in response to IFN-γ. Moreover, Raw/SOCS-1 did not produce NO in response to LPS or LPS plus IFN-γ, suggesting that SOCS-1 inhibits LPS signaling. Next, we examined whether LPS promptly induces SOCS-1 in mouse macrophages, as LPS reportedly induces SOCS-1 directly or indirectly via IFN-α/β (Dalpke et al., 2001; Crespo et al., 2000). SOCS-1 mRNA was detected in parental Raw cells at 60 min after LPS stimulation under the condition that new protein synthesis was inhibited by treatment with cycloheximide (Figure 4B), suggesting direct induction of SOCS-1 by LPS in macrophages.

Inhibitory Action of SOCS-1 in LPS-Induced STAT1 Activation

It is well known that NO is exclusively generated by iNOS (Xie and Nathan, 1994), which requires both NF-κB and STAT1 for its activation (Pine, 1997; Gao et al.,

A

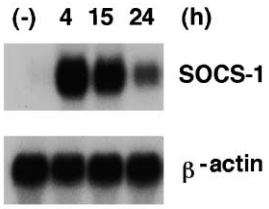
a Mortality of SOCS-1 KO mice (3 day-old mice)

WT (LPS 0.5 μg / LPS 1mg)	0 / 20
WT (PBS / LPS 1mg)	20 / 20
SOCS-1 KO (LPS 0.5 μg / LPS 50 μg)	20 / 20
SOCS-1 KO (PBS / LPS 50 μg)	20 / 20

b Mortality of SOCS-1He mice (4 week-old mice)

WT (LPS 0.5 μg / LPS 1mg)	2 / 20
WT (PBS / LPS 1mg)	17 / 20
SOCS-1 He (LPS 0.5 μg / LPS 500 μg)	20 / 20
SOCS-1 He (PBS / LPS 500 μg)	20 / 20

C



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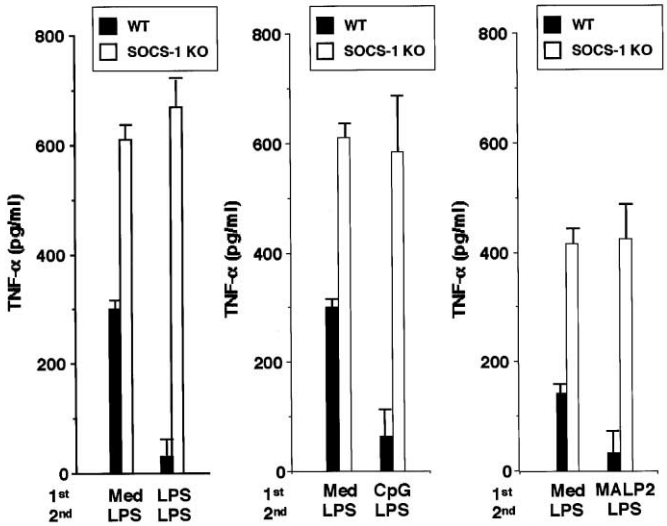

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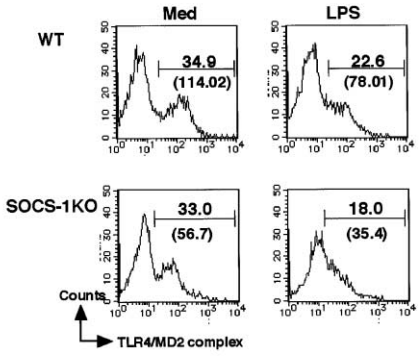

Figure 3. Essential Role of SOCS-1 in Induction of LPS Tolerance

(Aa) Three-day-old SOCS-1 KO and WT littermate mice were intraperitoneally injected with 0.5 μg LPS, which is less than the lethal dose for SOCS-1 KO mice (see Figure 6). After 24 hr, these mice were treated with the corresponding lethal doses of LPS (50 μg for SOCS-1 KO mice and 1 mg for WT mice). The lethality was observed until 12 hr after LPS rechallenge.

(Ab) Four-week-old SOCS-1 He and WT littermate mice were intraperitoneally injected with 0.5 μg LPS, which is less than the lethal dose for SOCS-1 He mice. After 24 hr, these mice were treated with the corresponding lethal doses of LPS (500 μg for SOCS-1 He mice and 1 mg for WT mice). The lethality was observed until 72 hr after LPS rechallenge.

(B) Splenic adherent cells isolated from WT or SOCS-1 KO mice had been incubated with 100 ng/ml LPS (LPS), 50 nM CpG DNA, 3 ng/ml of MALP-2, or only medium (Med) for 24 hr. These cells were subsequently incubated with a second dose of LPS (100 ng/ml) for a further 12 hr, after which culture supernatants were harvested for measurement of TNF-α.

(C) Splenic adherent cells were incubated with 100 ng/ml of LPS for the indicated hours, and their SOCS-1 mRNA expression was determined by Northern blotting.

(D) Splenic adherent cells from WT or SOCS-1 KO mice had been incubated with 100 ng/ml LPS (LPS) or only medium (Med) for 24 hr. Subsequently, their expression of TLR4/MD-2 was determined by FACS. The proportion of TLR4/MD-2$^+$ cells was shown as a percentage. Geometric mean intensity of TLR4/MD-2 in TLR4/MD-2$^+$ cells was shown in the parentheses. Data in (Aa) and (Ab) represent ten mice in each experimental group and are the result of two independent experiments with similar results. Data in (B) and (D) are representative of three independent experiments with similar results.

1997). As LPS can solely induce NO production in Raw cells and introduction of SOCS-1 completely inhibits their NO production (Figure 4A), we analyzed whether SOCS-1 inhibits LPS-induced STAT-1 phosphorylation. LPS induced both serine and tyrosine phosphorylation of STAT1 in Raw cells (Figure 5A), suggesting that stimulation with LPS alone may be able to activate STAT1. The electrophoretic mobility shift assay for STAT1 revealed that LPS stimulation induced STAT1 activation (data not shown). In contrast, Raw/SOCS-1 exhibited abrogation of serine and tyrosine phosphorylation of STAT1 after stimulation with LPS compared with Raw/Neo (Figure 5A). Thus, SOCS-1 is capable of repressing LPS-induced STAT1 activation by inhibiting both serine and tyrosine phosphorylation of STAT1. It is believed that LPS directly induces serine phosphorylation of STAT1 (Kovarik et al., 1998), albeit its tyrosine phosphorylation is presumably evoked by LPS-induced IFN-β via MyD88-independent signal cascade (Toshchakov et al., 2002). In fact, the treatment with translational inhibitor, cycloheximide, did not reduce the serine phosphorylation of STAT1 in LPS-activated Raw cells (data not

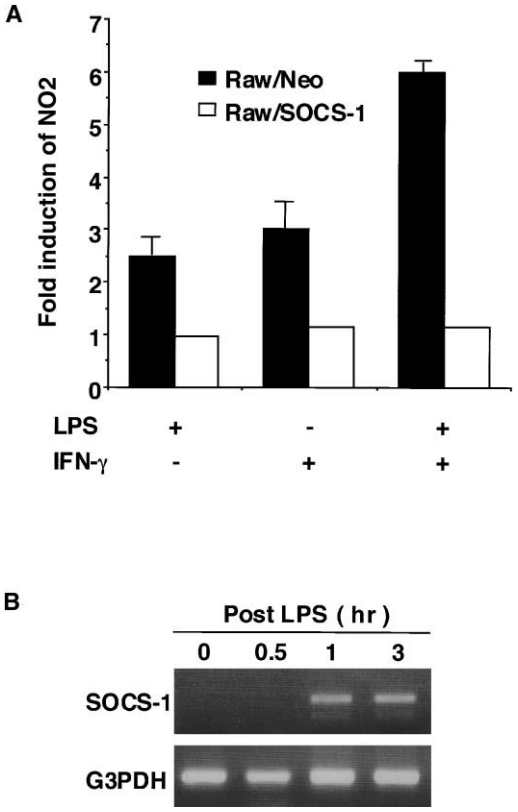

Figure 4. LPS-Induced SOCS-1 Induction in Macrophages

(A) Raw/Neo cells or Raw/SOCS-1 cells were incubated with 2 μg/ml LPS and/or 10 μg/ml IFN-γ for 16 hrs. NO_2+NO_3 concentration in each supernatant was measured, and the relative fold of nitrite in the supernatant of stimulated cells was calculated to that of unstimulated cells.

(B) Raw cells were incubated with 2 μg/ml of LPS for the indicated hours, and their SOCS-1 mRNA expression was determined by RT-PCR. The data are representative of three independent experiments with similar results.

shown). Therefore, SOCS-1 has the potential to directly inhibit LPS-induced signaling.

We next analyzed whether MyD88 is critical for LPS-induced STAT1 activation because recent studies revealed the importance of MyD88-independent LPS signaling pathway (Akira et al., 2001; Toshchakov et al., 2002). MyD88-deficient Kupffer cells showed both serine and tyrosine phosphorylation of STAT1 upon LPS stimulation as was demonstrated in WT cells (Figure 5B), indicating LPS-induced MyD88-independent STAT1 activation. Collectively, our present results indicate that SOCS-1 downregulates LPS-induced phosphorylation of STAT1 at both sites through MyD88-independent pathways, eventually ensuring safety response to LPS as well as LPS tolerance.

Partial Involvement of STAT1 in Susceptibility of SOCS-1 KO Mice to LPS

Next, we wished to know whether SOCS-1-depletion-induced oversignaling of STAT1 determines the susceptibility of SOCS-1 KO mice to LPS in vivo. To test this possibility, we examined the susceptibility to LPS of SOCS-1 KO mice lacking STAT1 (Meraz et al., 1996). SOCS-1/STAT1 DKO mice and SOCS-1 KO littermate mice exhibited substantial and remarkable susceptibility to LPS, respectively, whereas WT mice were resistant to higher doses of LPS than those used for these mutant mice. LD_{50} of LPS for SOCS-1 KO mice was around 2–3 μg, while LD_{50} for SOCS-1/STAT1 DKO was 300 μg (Figure 6). LD_{50} for WT was 800 μg (Figure 6), while LD_{50} for STAT1 KO mice was more than 3 mg (data not shown). These findings indicate that STAT1 is involved in the susceptibility of SOCS-1 KO mice or WT mice to LPS.

Inhibitory Effect of SOCS-1 on LPS-Induced NF-κB Activation

Although deletion of STAT1 substantially altered the LPS responsiveness of SOCS-1 KO mice, these DKO mice were still more susceptible to LPS than STAT1 single KO mice or WT littermate mice (Figure 6), suggesting that STAT1 is a major but not exclusive target molecule of SOCS-1 upon LPS challenge. LPS directly induces NF-κB activation through both MyD88-dependent and -independent signal pathways (Adachi et al., 1998). We next examined whether SOCS-1 suppressed LPS-induced NF-κB activation by using the method of reporter gene assay. Introduction of SOCS-1 attenuated activation of NF-κB reporter gene activity by LPS stimulation in a dose-dependent manner (Figure 7A). To clarify potential target molecules bound by SOCS-1 in LPS signal pathways, we performed coimmunoprecipitation analyses. Cotransfection of COS7 cells with SOCS-1 and IRAK revealed the association of SOCS-1 with IRAK via the SH2 region of SOCS-1 (Figure 7B), although SOCS-1 could not associate with MyD88 (data not shown). These findings suggest that SOCS-1 may have the capacity to directly inhibit LPS signal pathways presumably by binding to IRAK.

Discussion

Our present study demonstrates that SOCS-1 is an essential downregulating factor for protecting host from fatal responses to LPS. SOCS-1 KO and He mice show hyperproduction of TNF-α after LPS challenge compared with WT mice (Figure 1B). This is also the case for macrophage levels (Figures 2A, 2B, and 2E), although both types of macrophages express comparable levels of activation markers (Figure 2D and data not shown), excluding the possibility that hyperresponse of SOCS-1-deficient cells to LPS might be due to endogenous activation by intrinsic proinflammatory cytokines such as IFN-γ. Moreover, SOCS-1 KO and He mice lack the LPS tolerance that is observed in WT mice (Figures 3A and 3B). Therefore, SOCS-1 deficiency seems to determine hypersusceptibility to LPS. In fact, introduction of the SOCS-1 gene induces inability to respond to LPS in Raw cells (Figure 4A). In particular, SOCS-1-transfected Raw cells do not show STAT1 activation after LPS stimulation (Figure 5A). As STAT1 is activated by the MyD88-independent LPS signal cascade (Figure 5B), it is conceivable that inhibitory effect of SOCS-1 on LPS signaling is partly due to inactivation of STAT1 through downregulation of the MyD88-independent signal pathway. In fact,

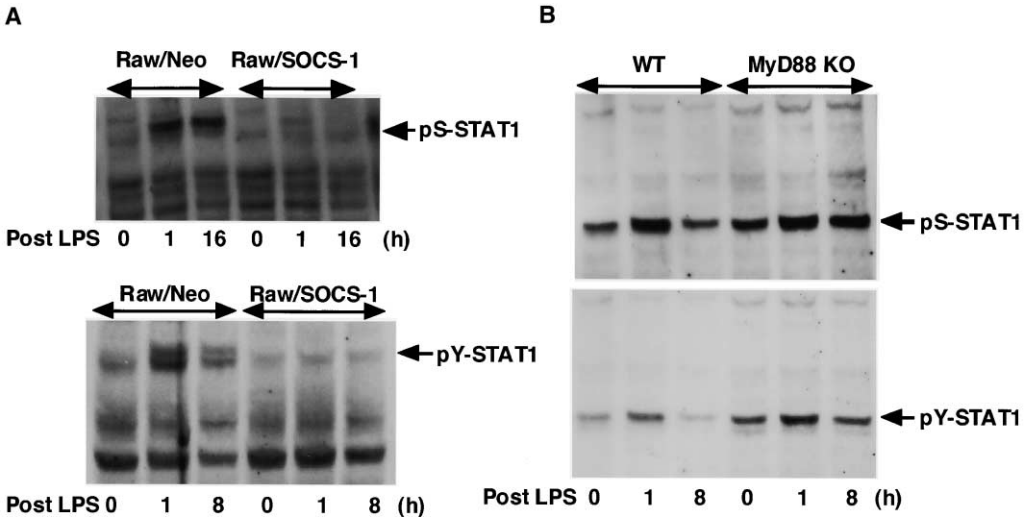

Figure 5. SOCS-1 Overexpression Inhibits the LPS Signaling Pathway

(A) Both serine 727 and tyrosine 701 phosphorylation of STAT1 was prevented in SOCS-1-transfected Raw cells. Raw cells transfected with (Raw/SOCS-1) or without (Raw/Neo) SOCS-1 construction were incubated with 2 μg/ml LPS for the indicated hours. Whole-cell lysates were used for immunoblotting analysis with anti-phospho Ser727 STAT1 Ab (upper panel) or anti-phospho Tyr701 STAT1 Ab (lower panel).

(B) Kupffer cells from WT or MyD88 KO mice were incubated with 2 mg LPS for the indicated hours, and serine phosphorylation (upper) or tyrosine phosphorylation (lower) in each cell lysate was determined by immunoblotting as shown in (A).

depletion of the STAT1 gene increases LD_{50} of LPS in SOCS-1 KO mice, indicating that SOCS-1 inhibits overshooting of LPS-induced STAT1 activation to evade lethality. However, LD_{50} of SOCS-1/STAT1 DKO mice is lower than that for STAT1 KO mice and even for WT mice (Figure 6). These findings clearly indicate that SOCS-1 does not solely target LPS-induced STAT1 activation. Indeed, SOCS-1 has the capacity to inhibit LPS-induced NF-κB activation (Figure 7A). Moreover, coimmunoprecipitation analyses demonstrated association of SOCS-1 with IRAK in COS 7 cells overexpressing these molecules (Figure 7B). This finding suggests that SOCS-1 might suppress MyD88-dependent signal pathways at least by binding to IRAK. Recently, it was reported that MyD88-independently induced IFN-β plays an essential role in tyrosine phosphorylation of STAT1 upon LPS stimulation (Toshchakov et al., 2002). It is conceivable that SOCS-1 negatively regulates this phosphorylation on the basis of its well-established nature against IFN-β-induced STAT1 activation. However, the mechanism by which SOCS-1 suppresses serine phosphorylation of STAT1 directly and MyD88 independently induced by LPS stimulation still remains unknown (Figure 5A). Therefore, SOCS-1 seems to suppress LPS-induced both MyD88-independent STAT1 activation and MyD88-dependent NF-κB activation. Collectively, the overshooting of STAT1 signaling, which is induced directly by LPS and/or indirectly by LPS-induced proinflammatory cytokines including IFN-γ, and the overactivation of LPS-induced NF-κB signaling render SOCS-1 KO mice to be hypersensitive to LPS.

Intriguingly, splenic adherent cells from SOCS-1 KO mice illustrated hypersensitivity to CpG DNA and MALP-2 (Figure 2C and data not shown) and also disturbed crosstolerance to LPS induced by CpG DNA or MALP-2 (Figure 3B). Indeed, SOCS-1 is induced by stimulation with CpG DNA (Dalpke et al., 2001) or MALP-2 (data not shown). Therefore, SOCS-1 may be involved

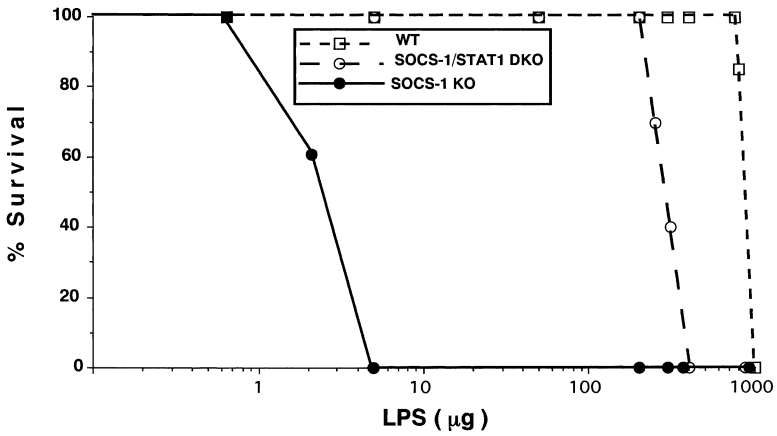

Figure 6. Partial Involvement of STAT1 in Susceptibility of SOCS-1 KO Mice to LPS In Vivo

SOCS-1 KO, SOCS-1/STAT1 DKO, or WT littermate mice were intraperitoneally injected with various amounts of LPS. Lethality was observed at 12 hr after LPS challenge. Five mice in each experimental group were used for this study. The data are representative of two independent experiments with similar results.

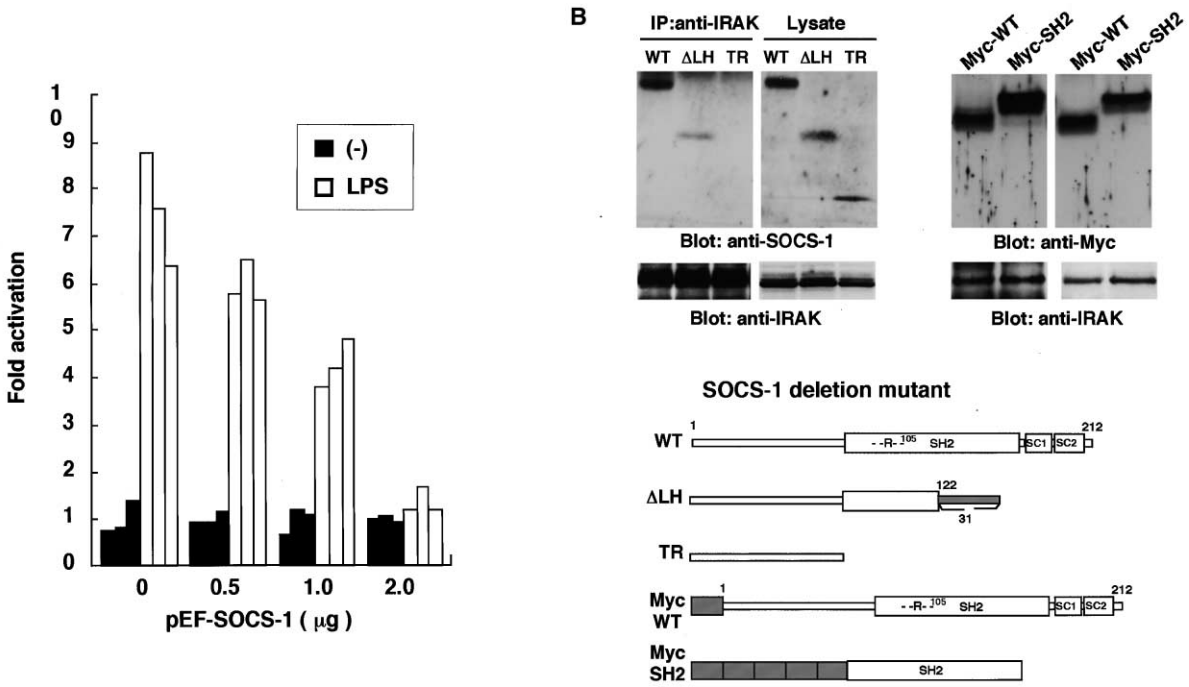

Figure 7. SOCS-1 Suppressed LPS Induction of NF-κB Reporter Gene Activity by Binding to IRAK

(A) Raw cells were transfected with pEF-BOS-SOCS-1 and NF-κB reporter plasmid, p55kB-Luc. Cells were treated with or without various doses of LPS for 3 hr and then luciferase activity was detected. Data are expressed as relative fold activation to that of nonstimulated (−) set. The data are representative of two independent experiments with similar results.

(B) COS7 cells were cotransfected with SOCS-1 or various deletion mutants of SOCS-1 and IRAK. After 2 days, cells were lysed and immunoprecipitated with anti-IRAK Ab, and then SOCS-1 was detected by Western blotting.

in interregulation of multiple TLR signalings as well, presumably by binding to IRAK.

More recently, it was reported that IRAK-M, a kinase-lacking member of the IRAK family, plays an essential role in the induction of LPS tolerance by inhibiting TLR signaling (Kobayashi et al., 2002). In fact, IRAK-M-deficient mice evade LPS tolerance. We need furfther studies to clarify whether SOCS-1 is involved in IRAK-M-mediated downregulation of TLR signaling.

Biological action of LPS, such as induction of TNF-α and IL-12 production, is potently augmented by costimulation with IFN-γ, the signaling of which is strongly inhibited by SOCS-1 (Sakamoto et al., 1998; Song and Shuai, 1998). However, IFN-γ was thoroughly undetectable in the sera of SOCS-1 KO and WT mice until 12 hr after administration with LPS (data not shown). Furthermore, the doses of LPS that caused death in SOCS-1/IFN-γ DKO mice were lower than those causing death in WT mice as well as IFN-γ single KO mice but higher than those causing death in SOCS-1 single KO mice (personal communication from A. Yoshimura). These results strongly indicate that SOCS-1 downregulates not only IFN-γ-dependent signaling events including IFN-γ-dependent STAT1 activation but also LPS-induced, IFN-γ-independent signaling.

Various treatments potentially elevate the sensitivity of mice to LPS. Treatment with bacteria, such as BCG and heat-killed *Propionibacterium acnes*, adrenectomy, or inoculation of tumors induces hyperresponses to LPS in mice with unknown mechanisms (Galanos and Freudenberg, 1993; Tsutsui et al., 1997). The present study suggests a possible contribution of SOCS-1 to these in vivo sensitization procedures to LPS.

SOCS-1 was initially identified as a potent downregulating element in adaptive immunity. It is critical for protection against oversignaling of various cytokines using the JAK-STAT signal pathways, including IL-4 and IFN-γ, which play major roles in adaptive immunity. Our study has made it clear that SOCS-1 also performs important functions in the regulation of innate immune responses. SOCS-1 should provide new insights leading to protection from and/or therapeutic regimens against lethal host responses brought on by serious infections, which are still encountered throughout the world. In addition, SOCS-1 is likely to become a rational target for therapeutic regimens against various immune diseases that occur on the basis of overshooting of the immune system, such as autoimmune diseases and chronic inflammatory diseases.

Experimental Procedures

Mice

STAT1 KO mice (Meraz et al., 1996), SOCS-1 KO mice (Naka et al., 1997), and STAT1/SOCS1 DKO mice (Naka et al., 2001a) were established as previously described, and 3-day-old mice were used. Four-week-old SOCS-1 He mice (Naka et al., 1997) were also used. All these mice were on the C57BL/6 background (backcrossed for more than seven generations). MyD88-deficient mice were described previously (Adachi et al., 1998). All mice were maintained under specific pathogen-free conditions. We performed all the experiments by using littermate mice.

Lethality Tests

For lethality tests, WT, SOCS-1 KO mice, SOCS-1 He mice, or SOCS-1/STAT1 DKO mice (eight to ten animals in each experimental group) i.p. received various amounts of salmonella LPS (0.5–1000 μg) in 100 μl of PBS, and the resulting lethality was observed until 12 hr after challenge.

Induction of LPS Tolerance In Vivo

Mice were i.p. injected with or without 0.5 μg of LPS in 100 μl of PBS, followed by an i.p. injection of various amounts of LPS at 24 hr after the initial challenge, and the resulting lethality was observed.

Induction of LPS Tolerance In Vitro

Splenic adherent cells were prepared as previously described (Tsutsui et al., 1999). In brief, single-cell suspensions from the spleens of various genotype mice (3 days after birth) were cultured for 1 hr under LPS-free conditions in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μM 2-ME, and 2 mM L-glutamine. Adherent cells collected were incubated with various doses of LPS or CpG DNA for 12 hr, and cytokine levels in each resulting supernatant were determined. In some experiments, the adherent cells collected were primed with or without 100 ng/ml LPS, 50 nM CpG DNA, and 3 ng/ml of MALP-2 for 24 hr. Cells (1×10^5) were subsequently washed three times with PBS and additionally incubated with another dose of LPS (100 ng/ml) for a further 12 hr, after which culture supernatants were harvested for cytokine analysis.

Cytokine Assay

Cytokine levels in culture supernatants or in sera were determined using commercial ELISA kit for TNF-α or IL-12p40 (Biosource, CA) according to the manufacturer's instructions. Each value represents the mean of triplicate values.

Flow Cytometric Analysis (FACS)

Cells were stained with the following monoclonal Abs (mAbs): FITC-conjugated anti-I-A, anti-CD40, anti-CD80, and anti-CD86 (PharMingen, San Diego, CA); anti-TLR4/MD2 complexes (MBL, Nagoya, Japan) and combination of anti-F4/80 (BMA, Augst, Switzerland); and biotinylated anti-rat IgG2b (PharMingen) and PE-conjugated streptavidin (Becton Dickinson, San Jose, CA). Stained cells were analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software (Becton Dickinson). Live lymphocytes were gated according to their forward and side scatter profiles.

RT-PCR

Raw cells were cultured for 16 hr before LPS treatment. After treatment with salmonella LPS (2 μg/ml) in the presence of 20 ng/ml of cycloheximide for the indicated time period, total RNA was prepared by RNAzol B (TEL-TEST, INC.), and then mRNA was purified by oligo (dT) cellulose (Amersham Pharmacia Biotech). Reverse transcription reaction was performed using random primer and Molony murine leukemia virus reverse transcriptase (Stratagene). PCR reaction consisted of 95°C for 1 min, 66°C for 1 min, 72 C for 1 min, ×38 cycle for both SOCS-1 and G3PDH. The primer pairs were SOCS-1, 5′CACTCACTTCCGCACCTTCC-3′ and 5′-CAGCCGGTCAGATCT GGAAG-3′; G3PDH, 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACC ACAGTCCATGCCATCAC-3′.

Activation of STAT1

Raw/Neo or Raw/SOCS-1 cells were incubated with 1 μg LPS for the indicated hours, and the proteins were electroblotted onto a nitrocellulose filter (Amersham Pharmacia Biotech). After blocking with 5% skim milk, the filter was incubated with anti-phospho Ser727 STAT1 Ab or anti-phospho Tyr701 STAT1 Ab (UBI) for 1 hr, and then with the horseradish peroxidase-linked second antibody (Amersham Pharmacia Biotech). ECL system (Amersham Pharmacia Biotech) was used to detect the signals.

Transfection with SOCS-1 cDNA

Raw cells were stably transfected with cDNA encoding SOCS-1 as shown previously (Naka et al., 1997; Morita et al., 2000).

Immunoprecipitation and Western Blotting

The day before transfection, 5×10^5 COS7 cells were plated on 60 mm dish. Cells were transfected with 3 μg pEF-BOS-SOCS-1 or 3 μg pEF-BOS-mutant SOCS-1, and 3 μg of pEF-BOS-IRAK by DEAE-dextran method. After incubation for 2 days, cells were lysed with lysis buffer (1% NP-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM Na_2VO_4, 0.5 mM DTT, 1/100 protease inhibitor cocktail). Immunoprecipitation was described previously (Kawazoe et al., 2001). Immunocomplex was resolved by 13.5% SDS-PAGE, and SOCS-1 was detected by mouse monoclonal anti-SOCS-1 antibody (first antibody, Kokusaishiyaku) and HRP-conjugated sheep anti-mouse Ig antibody (second antibody, Amersham Pharmacia). Signals were visualized by enhanced chemiluminescence reagent (Perkin Elmer).

Preparation of Kupffer Cells

Kupffer cells were isolated from WT or MyD88 KO mice according to the method described previously (Tsutsui et al., 1997) and were incubated with 1 μg LPS for the indicated hours.

Nitrite Oxidant Detection

Raw cells were plated at 1×10^5 cells/well in 96-well culture dishes with medium containing 5% FCS and were incubated for 4 hr before stimulation. After the cells were treated with LPS (2 μg/ml) for 42 hr, culture medium diluted ×4 was used for analysis by the Griess Reagent kit (Dojindo). Nitrite concentrations were determined by the measurement of the optical density at 570 nm.

Luciferase Assay

3×10^4 Raw cells were transfected with the indicated amounts of pEF-BOS-SOCS-1, and 0.2 μg of p55kB-Luc and 0.02 μg of pRL-TK (Promega) by Fugene (Roche). Luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was normalized with *Renilla* luciferase activity.

Statistics

Significance between control and experimental groups was examined with the Logrank (Mantel-Cox) test. A value of $p < 0.05$ was regarded as significant.

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