Immunity, Vol. 17, 583–591, November, 2002, Copyright ©2002 by Cell Press

SOCS1/JAB Is a Negative Regulator of LPS-Induced Macrophage Activation

Ichiko Kinjyo,[1] Toshikatsu Hanada,[1] Kyoko Inagaki-Ohara,[1] Hiroyuki Mori,[1] Daisuke Aki,[1] Masanobu Ohishi,[1] Hiroki Yoshida, Masato Kubo,[2] and Akihiko Yoshimura[1,3]

[1]Division of Molecular and Cellular Immunology
Medical Institute of Bioregulation
Kyushu University
3-1-1 Maidashi, Higashi-ku
Fukuoka 812-8582

[2]Division of Immunobiology
Research Institute for Biological Sciences
Science University of Tokyo
Noda 278-0022
Japan

Summary

Bacterial lipopolysaccharide (LPS) triggers innate immune responses through Toll-like receptor (TLR) 4. We show here that the suppressor of cytokine-signaling-1 (SOCS1/JAB) is rapidly induced by LPS and negatively regulates LPS signaling. SOCS1$^{+/-}$ mice or SOCS1$^{-/-}$ mice with interferon-γ (IFNγ)-deficient background were more sensitive to LPS-induced lethal effects than were wild-type littermates. LPS-induced NO_2^- synthesis and TNFα production were augmented in SOCS1$^{-/-}$ macrophages. Furthermore, LPS tolerance, a protection mechanism against endotoxin shock, was also strikingly reduced in SOCS1$^{-/-}$ cells. LPS-induced I-κB and p38 phosphorylation was upregulated in SOCS1$^{-/-}$ macrophages, and forced expression of SOCS1 suppressed LPS-induced NF-κB activation. Thus, SOCS1 directly suppresses TLR4 signaling and modulates innate immunity.

Introduction

Bacterial LPS elicits a multitude of effects on the immune system and can cause a fatal pathological effect. The major target of LPS is macrophages and monocytes, and many of the biological effects of LPS are elicited by secondary mediators, in particular, proinflammatory agents such as tumor necrosis factor-α (TNFα) and reactive oxygen and nitrogen species (Beutler et al., 1985; Berczi., 1998; Tracey et al., 1987). Ligation of the TLR4-MD2 complex induced by LPS results in recruitment of the adaptor molecule, MyD88, to the receptor cytoplasmic domain, and then a serine/threonine kinase IL-1R-associated kinase (IRAK) that is subsequently recruited becomes phosphorylated, dissociates from the receptor complex, and associates with TNF receptor-associated factor (TRAF)-6 (Poltorak et al. 1998; Akira et al., 2001; Medzhitov and Janeway, 2000). This subsequently leads to the activation of two different pathways involving the c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein (MAP) kinase family and the Rel family transcription factor NF-κB (Akira et al., 2001; Medzhitov et al., 1998). However, since LPS still activates NF-κB and JNK/p38 in MyD88-deficient macrophages, the existence of a MyD88-independent pathway(s) that leads to NF-κB and JNK/p38 activation in TLR4 signaling has been proposed (Kawai et al., 2001).

Although the inflammatory response is critical to control the growth of pathogenic microorganisms (Cross et al., 1995; Eden et al., 1988; Hagberg et al., 1984; Shahin et al., 1987), excessive cytokine production is harmful to the host and can even be fatal (Beutler et al., 1985; Danner et al., 1991). Chronic repeated exposure to endotoxin (or LPS) causes a transient increase in the threshold of endotoxin challenge (Beeson, 1947; Greisman et al.,1966; Ziegler-Heitbrock, 1995). This endotoxin tolerance is a negative feedback mechanism for protection from endotoxic shock (Gustafson et al., 1995; Henricson et al., 1990; Salkowski et al., 1998). Several factors are involved in this process, including TLR4 downregulation (Nomura et al., 2000) and decreased NF-κB activation (Ziegler-Heitbrock et al., 1997; Goldring et al., 1998; Kastenbauer and Ziegler-Heitbrock, 1999). However, the underlying mechanisms are largely unknown. Recently, IRAK-M, which is restricted to monocytes/macrophages, is shown to be induced upon TLR stimulation and negatively regulate TLR signaling (Kobayashi et al., 2002).

Recently, cytokine signal suppressors SOCS1 and SOCS3 were found to be induced by LPS or CpG-DNA stimulation in macrophages (Stoiber et al., 1999; Crespo et al., 2000; Dalpke et al., 2001). We recently found that SOCS3 plays an important role in the antagonistic effect of IL-10 against LPS-induced macrophage activation (Berlato et al., 2002). SOCS1$^{-/-}$ mice have been shown to die within 3 weeks after birth by severe inflammation (Naka et al., 1998; Starr et al., 1998). However, in these mice, innate immunity was strongly enhanced, probably due to hypersensitivity of SOCS1$^{-/-}$ mice to interferon-γ (IFNγ) (Alexander et al., 1999). SOCS1 interacts with JAK tyrosine kinases and inhibits the kinase activity, thereby suppressing the cytokine signal transduction (Yasukawa et al., 2000). SOCS1 has been implicated in the hyporesponsiveness to cytokines such as IFNγ after exposure of LPS to macrophages (Crespo et al., 2002). However, since SOCS1 has been reported to inhibit several signaling pathways other than JAK/STATs, including TNFα, insulin, and Vav (Morita et al., 2000; Kawazoe et al., 2001; De Sepulveda et al., 2000) and SOCS1-deficient mice exhibited systemic inflammatory response syndrome/multiple organ dysfunction syndrome-like severe inflammation (Naka et al., 1998, 2001; Starr et al., 1998; Alexander et al., 1999; Marine et al., 1999), we investigated the possibility that SOCS1 directly downmodulates LPS signaling.

In this paper, we found that SOCS1-deficient mice were more sensitive to LPS shock than wild-type littermates. Furthermore, macrophages from SOCS1$^{-/-}$ mice were more sensitive to endotoxin stimulation than SOCS1$^{+/+}$ macrophages. SOCS1$^{-/-}$ cells have strikingly impaired endotoxin tolerance, showing that SOCS1 neg-

[3]Correspondence: yakihiko@bioreg.kyushu-u.ac.jp

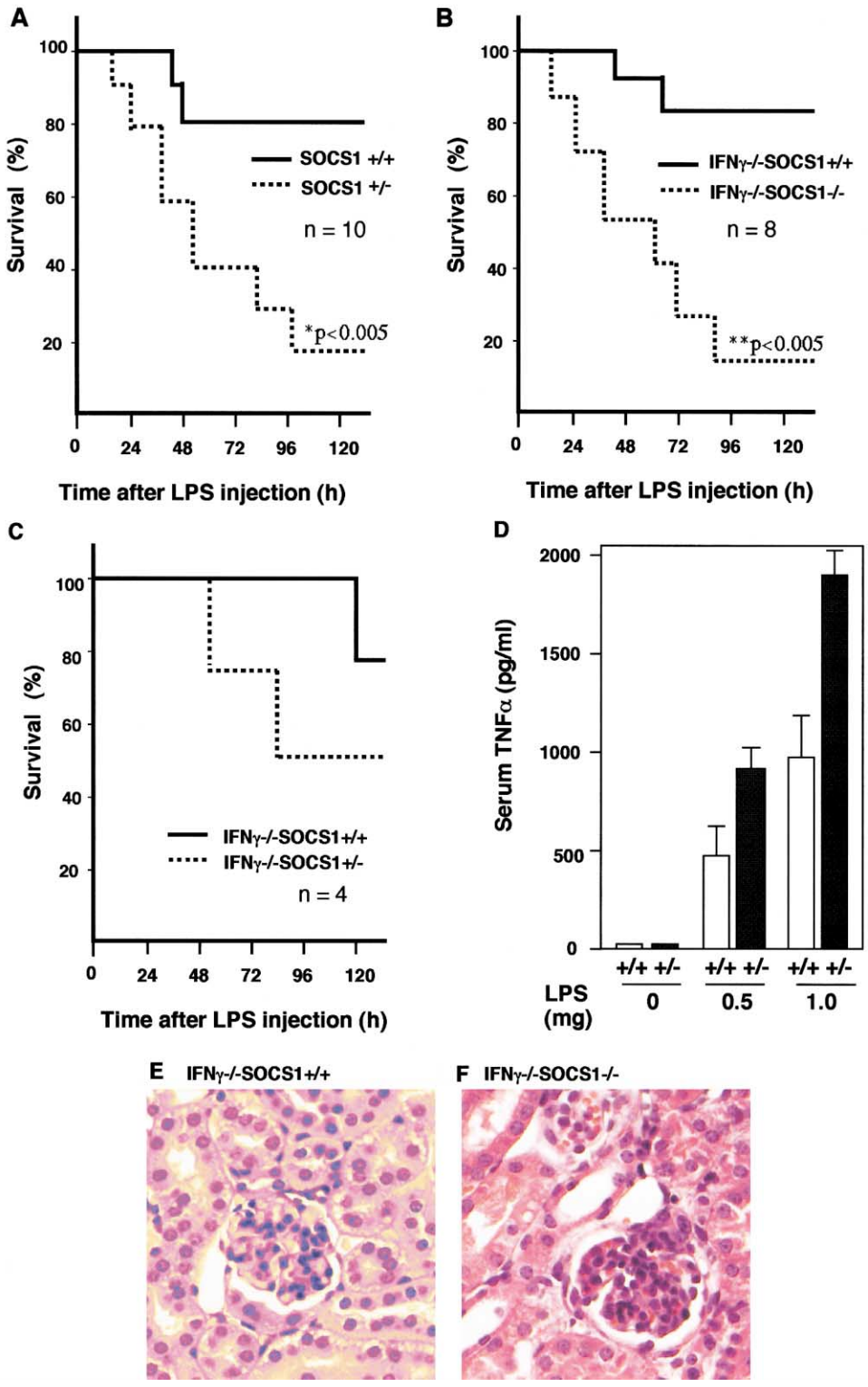

Figure 1. Hypersensitivity of SOCS-1 KO Mice to LPS In Vivo

(A) Eight-week-old SOCS1$^{+/-}$ and littermate SOCS1$^{+/+}$ mice were intraperitoneally injected with 2 mg LPS for female (n = 5) and 5 mg LPS for male (n = 5) mice. Lethality was observed over 120 hr after this challenge. The data are representative of two independent experiments with similar results.

(B) Five- to eight-week-old IFN$\gamma^{-/-}$SOCS1$^{+/+}$ or IFN$\gamma^{-/-}$SOCS1$^{-/-}$ mice (n = 8) were injected with 3 mg LPS, and lethality was observed. Statistical analysis was performed using the Student's t test, and the p values are shown.

(C) Five- to eight-week-old IFN$\gamma^{-/-}$SOCS1$^{+/+}$ or IFN$\gamma^{-/-}$SOCS1$^{+/-}$ mice (n = 4) were injected with 3 mg LPS, and lethality was observed.

(D) Serum from IFN$\gamma^{-/-}$SOCS1$^{+/+}$ or IFN$\gamma^{-/-}$SOCS1$^{+/-}$ mice injected with the indicated amounts of LPS was collected at 1.5 hr after the challenge, and TNFα levels were measured by ELISA. Data show mean ± SD for three mice from each group.

(E and F) Acute tubular necrosis in kidneys of IFN$\gamma^{-/-}$SOCS1$^{+/+}$ (E) and IFN$\gamma^{-/-}$SOCS1$^{-/-}$ (F) mice following challenge with LPS. Representative histological sections with hematoxylin and eosin staining of kidneys from surviving mice that were sacrificed at 96 hr after LPS administration. Magnification, ×400.

atively regulates TLR signaling. SOCS1 overexpression suppressed LPS-induced I-κB phosphorylation and NF-κB transcriptional activity. These data suggest that SOCS1 plays an important role in the downregulation of LPS signal transduction.

Results

Response of SOCS1-Deficient Mice to LPS

SOCS1 knockout (−/−) mice die within 3 weeks with severe inflammation in almost all organs (Naka et al., 1998; Starr et al., 1998). As shown previously, SOCS1$^{-/-}$Rag2$^{-/-}$ mice survived much longer, suggesting the role of T cells in the lethality of SOCS1-deficient mice (Marine et al., 1999). Consistent with these findings, we also found that SOCS1$^{-/-}$TCRα$^{-/-}$ double knockout mice also survived much longer than simple SOCS1 knockout mice in specific pathogen-free (SPF) conditions (data not shown). However, these mice die within 1 month with severe inflammation under conventional conditions, suggesting that the SOCS1$^{-/-}$ genetic background is much more sensitive to bacterial infection. To confirm the involvement of SOCS1 in the sensitivity to bacterial pathogen, we compared endotoxic shock between SOCS1$^{+/+}$ and SOCS1$^{+/-}$ mice. SOCS1$^{-/-}$ could not be used since they were already sick within 1–2 weeks after birth, and their macrophages might already be activated by increased IFNγ concentration associated with inflammation (Naka et al., 1998; Starr et al., 1998). Age- and sex-matched cohorts of mice were challenged intraperitoneally with two different amounts of LPS and observed over 120 hr. As shown in Figure 1A, SOCS1$^{+/-}$ mice were more sensitive to LPS-induced lethality than were SOCS1$^{+/+}$ mice. Since SOCS1$^{+/-}$ mice exhibited no signs of inflammation before LPS challenge, these data led to the notion that SOCS1 levels modify the LPS sensitivity.

SOCS1 has been characterized as a negative regulator of interferon-γ (IFNγ) which is a potent activator of macrophages (Alexander et al., 1999; Naka et al., 2001; Marine et al., 1999). To avoid the effect of interferon-γ, we used mice with IFNγ-deficient genetic background. As reported, IFNγ$^{-/-}$SOCS1$^{-/-}$ mice survived much longer than SOCS1$^{-/-}$ mice, and inflammatory lesions were nil in all organs when we used these mice in experiments (4–6 weeks postnatally) (Metcalf et al., 2002). As shown in Figures 1B and 1C, IFNγ$^{-/-}$SOCS1$^{-/-}$ mice (B) as well as IFNγ$^{-/-}$SOCS1$^{+/-}$ (C) were more sensitive to LPS than IFNγ$^{-/-}$SOCS1$^{+/+}$ mice. We also found that serum TNFα levels after LPS injection were two times higher in IFNγ$^{-/-}$SOCS1$^{+/-}$ mice than in IFNγ$^{-/-}$SOCS1$^{+/+}$ mice (Figure 1D). IFNγ$^{-/-}$SOCS1$^{-/-}$ mice exhibited similar acute tubular necrosis in the kidney as well as general signs of septic shock as found in IFNγ$^{-/-}$SOCS1$^{+/+}$ mice (Figures 1E and 1F). However, tissue damage was more profound in IFNγ$^{-/-}$SOCS1$^{-/-}$ mice than in IFNγ$^{-/-}$SOCS1$^{+/+}$ mice. Thus, SOCS1-deficient mice die not due to a specific abnormality caused by SOCS1 deficiency but simply due to higher sensitivity to LPS. These data suggest that SOCS1 defines LPS sensitivity to IFNγ independently.

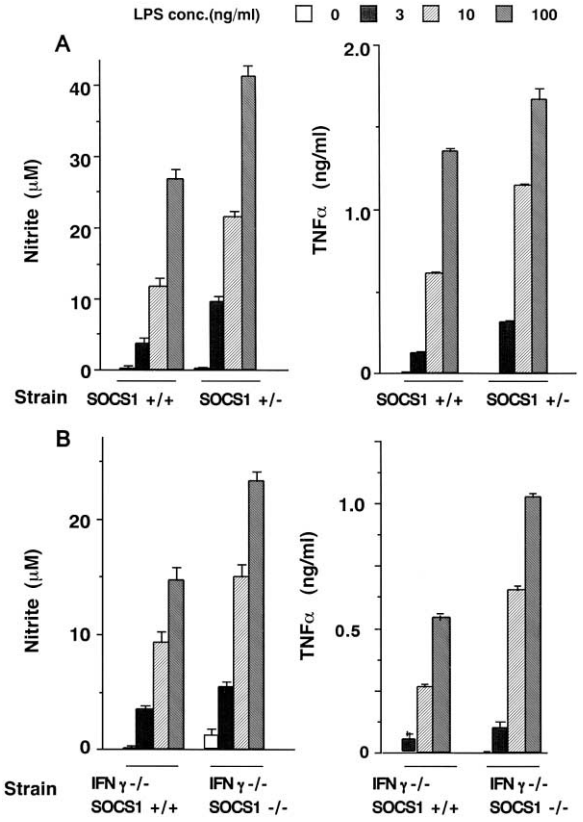

Figure 2. Hyperresponse to LPS of SOCS1-Deficient Macrophages

Resident peritoneal macrophages from SOCS1$^{+/+}$, SOCS1$^{+/-}$, IFNγ$^{-/-}$SOCS1$^{+/+}$, or IFNγ$^{-/-}$SOCS1$^{-/-}$ mice were stimulated with the indicated concentrations of LPS for 12 hr, and then the production of NO_2^- and TNFα was determined. Data are representative of three independent experiments, with similar results.

Enhanced LPS Signaling in SOCS1$^{-/-}$ Macrophages

Next, we examined LPS responses in macrophages from mice with various genetic backgrounds. As expected, macrophages from SOCS1$^{-/-}$ mice were already activated, and they produced nitric oxide (NO_2^-) even without LPS stimulation (data not shown). To avoid the effect of inflammation, we used SOCS1$^{+/-}$ (Figure 2A) and IFNγ$^{-/-}$SOCS1$^{-/-}$ (Figure 2B) mice. LPS-induced NO_2^- and TNFα synthesis in macrophages from SOCS1$^{+/-}$ and IFNγ$^{-/-}$SOCS1$^{-/-}$ mice were 1.5- to 2-fold higher than those from control mice. Maximum levels of LPS-induced NO_2^- and TNFα in SOCS1$^{-/-}$ macrophages were comparable to the levels of WT macrophages treated with both LPS and IFNγ (data not shown). Macrophages from SOCS1$^{-/-}$TCRα$^{-/-}$ mice were also more sensitive to LPS than were SOCS1$^{+/+}$TCRα$^{-/-}$ macrophages (data not shown). Production of IL-6 and IL-1β in response to LPS was also augmented in SOCS1$^{-/-}$ macrophages compared with SOCS1$^{+/+}$ macrophages (data not shown). SOCS1$^{-/-}$ macrophages also produced significantly higher levels of IL-6 and TNFα in response to CpG-DNA than did wild-type macrophages (see Figure 4).

To define the molecular mechanism of hyperresponsiveness to LPS in SOCS1$^{-/-}$IFNγ$^{-/-}$ mice, we examined the tyrosine phosphorylation of STAT1 and serine phos-

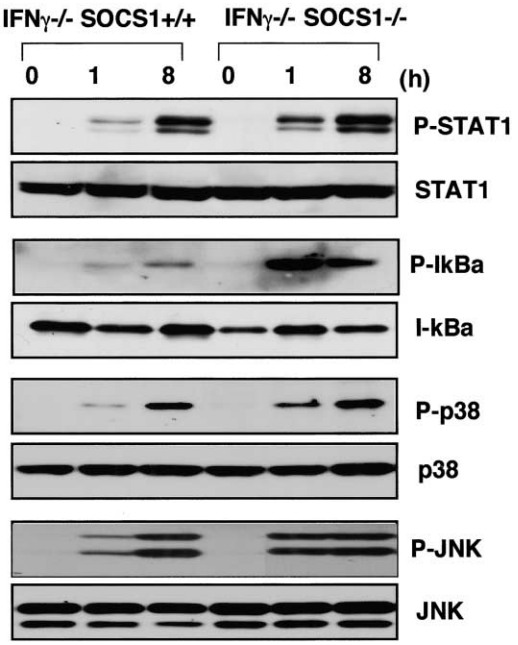

Figure 3. Hyperactivation of LPS Signaling Molecules in SOCS1-Deficient Peritoneal Macrophages

Phosphorylation of STAT1, I-κB, JNK, and p38 in macrophages in response to LPS. Resident peritoneal macrophages from knockout mice were stimulated with 100 ng/ml LPS for the indicated periods, and then cell extracts (2×10^5 cells) were immunoblotted with the indicated antibodies.

phorylation of I-κB, p38, and JNK (Figure 3). LPS activates IKKβ, resulting in the serine phosphorylation of I-κB, leading transcriptional activation of NF-κB. JNK and p38 are also activated through TRAF6. As an indirect effect, probably autocrine/paracrine secretion of cytokines, including IFNβ (Crespo et al., 2000; Toshchakov et al., 2002), activates the JAK/STAT pathway. We also found that STAT1 was rapidly tyrosine phosphorylated in macrophages in response to LPS (Figure 3). STAT1 phosphorylation was slightly enhanced, and the phosphorylation of I-κB, p38, and JNK was strongly enhanced in SOCS1$^{-/-}$ macrophages compared with control SOCS1$^{+/+}$ macrophages. These data strongly support our hypothesis that SOCS1 negatively regulates the LPS signaling pathway in macrophage activation through both direct and indirect mechanisms.

SOCS1 Is Required for Endotoxin Tolerance

Our results showing that SOCS1 is a negative regulator of TLR signaling suggested that SOCS1 might be involved in the induction of endotoxin tolerance. SOCS1 is also induced by TLR signaling (Dalpke et al., 2001 and Figure 5A). We then determined the ability of SOCS1$^{-/-}$ macrophages to develop endotoxin tolerance. SOCS1$^{-/-}$ macrophages (IFNγ$^{-/-}$ background) were first stimulated with 100 ng/ml of LPS or 1 μM CpG-DNA (primary LPS/CpG stimulation). CpG-DNA activates TLR9. After 24 hr incubation, cells were restimulated with 100 ng/ml of LPS or 1 μM CpG-DNA (second LPS/CpG stimulation), and IL-6 and TNFα production was examined after secondary stimulation (Figure 4). As shown by previous studies (Nomura et al., 2000), wild-type macrophages showed reduced cytokine production in accordance with a 24 hr incubation of LPS or CpG-DNA. Since primary LPS stimulation and CpG stimulation strongly reduced secondary CpG and LPS responses, respectively, crosstolerance between TLR4 and TLR9 apparently occurred in wild-type macrophages. However, SOCS1$^{-/-}$ macrophages showed almost a complete lack of LPS and CpG tolerance, and the cytokine levels produced upon secondary LPS/CpG restimulation were not decreased as much as in restimulated wild-type macrophages (Figure 4). Although similar impaired tolerance was observed in IRAK-M$^{-/-}$ macrophages (Kobayashi et al., 2002), it is striking that disruption of endotoxin tolerance was observed even after 24 hr primary stimulation in SOCS1$^{-/-}$ macrophages, while IRAK-M$^{-/-}$ macrophages showed recovery of tolerance after 24 hr primary stimulation (Kobayashi et al., 2002). Therefore, SOCS1 may be an important mechanism for the mediation of late endotoxin tolerance, which may operate after IRAK-M functions.

Effect of Forced Expression of SOCS1 on LPS-Induced NF-κB Activation

Next, the role of SOCS1 in LPS signaling was examined in a macrophage cell line, Raw264.7. SOCS-1 is rapidly induced by LPS as well as IFNγ treatment of Raw cells (Figure 5A). Pretreatment of Raw cells with IFNγ for 2 hr (data not shown) or for 12 hr reduced the levels of phosphorylation of I-κB induced by LPS (Figure 5B), which suggested that SOCS1-induced by IFNγ suppresses LPS signaling. Therefore, we then stably expressed SOCS1 in Raw cells by cDNA transfection. As shown in Figure 5C, overexpression of SOCS1 resulted in reduction of NO_2^- and TNFα synthesis in Raw cell transformants expressing SOCS1. LPS-induced I-κB phosphorylation was significantly reduced in these transformants (Figure 5D). These data suggest that SOCS1 inhibits LPS signaling by suppressing the TLR4-IKKβ-I-κB pathway.

Next, we examined the effect of transient overexpression of SOCS1 on LPS-induced NF-κB activation using a reporter gene assay. SOCS1 overexpression suppressed LPS-induced NF-κB transcriptional activity in Raw cells as well as in 293 fibroblastic cells expressing both TLR4 and MD2 (Figures 6A and 6B). However, SOCS1 did not inhibit TNFα-induced NF-κB activation in 293 cells (Figure 6A), suggesting that SOCS1 inhibits the TLR-specific but not TNFα-induced NF-κB activation pathway. As reported previously, both TLR4 and MD2 were required for LPS-induced NF-κB activation in 293 cells (Shimazu et al., 1999) (Figure 6B). Using various mutants that we created previously (Yasukawa et al., 1999), we found that the SH2 domain (R105E), KIR (kinase inhibitory region) (F59D), and C-terminal SOCS-box of SOCS1 (dC40) were required for full suppression of NF-κB (Figure 6C). Requirement of the SH2 domain and KIR of SOCS1 suggests that an unidentified tyrosine kinase pathway may be involved in the TLR4-mediated NF-κB activation pathway. Another possibility is that SOCS1 may be able to inhibit TRAF6-downstream serine/threonine kinase such as TAK1 through KIR (Nino-

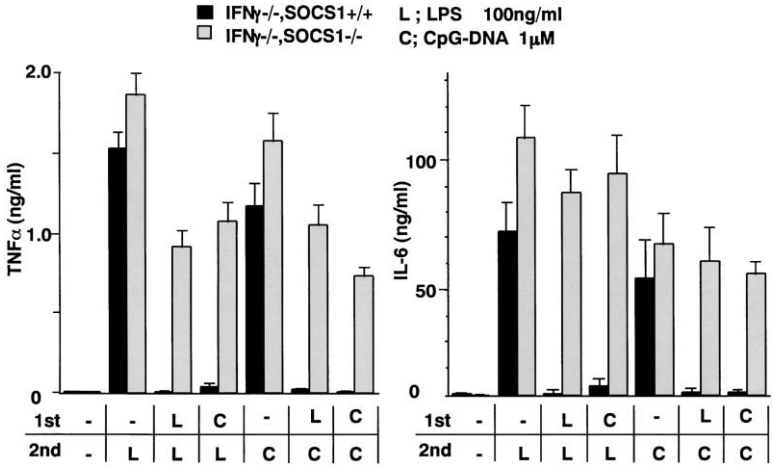

Figure 4. Perturbed Endotoxin Tolerance in SOCS1$^{-/-}$ Macrophages

Endotoxin tolerance was induced in resident peritoneal macrophages from IFN$\gamma^{-/-}$ SOCS1$^{+/+}$ or IFN$\gamma^{-/-}$SOCS1$^{-/-}$ mice by preactivation with 100 ng/ml of LPS (L) (1st LPS) or 1 μM CpG-DNA (C) (1st CpG). After 24 hr incubation, cells were washed and restimulated with 100 ng/ml of LPS (2nd LPS) or 1 μM CpG-DNA (2nd CpG) for 24 hr, and then IL-6 and TNFα levels were determined by ELISA. Examination of at least three different mice from each group showed similar findings.

miya-Tsuji et al., 1999). It has been demonstrated that C-terminal SOCS-box is not required for the cytokine-dependent STATs activation in vitro (Yasukawa et al., 1999) but is necessary for full activity in vivo (Zhang et al., 2001). Therefore, requirement of the SOCS-box suggests that the mechanism for LPS signal suppression by SOCS1 is different from that of cytokine-dependent JAK/STAT pathway. And SOCS1 also inhibited NF-κB activation by IRAK and TRAF6^{-} but not by IKKβ when these molecules were overexpressed in 293 cells (Figure 6D). These data suggested that SOCS1 inhibits downstream of TRAF6 and upstream of IKKβ.

We examined the binding of SOCS1 with TRAF6, IRAK, IKK, and TAK1 by using Raw cells expressing Myc-tagged SOCS1. However, we could not detect endogenous molecules in the immunoprecipitates of Myc-SOCS1. Since SOCS1 has an SH2 domain which should interact with tyrosine-phosphorylated molecules, there may be unknown modulatory pathways of LPS signal transduction in which tyrosine kinases may be involved. Identification of the SOCS1 target molecule may reveal a novel regulatory pathway of TLR4.

Discussion

In this study, we have identified SOCS1 as a negative regulator of TLR signaling in macrophage. It has been demonstrated that LPS as well as CpG-oligonucleotide induces SOCS1 and SOCS3 mRNA in macrophages and SOCS molecules postulated to inhibit further activation of macrophages by IFNγ and/or other cytokines (Crespo et al., 2000, 2002; Dalpke et al., 2001). In the present paper, we describe evidence that SOCS1 can also directly inhibit the LPS to NF-κB signaling pathway in macrophages. Therefore, SOCS1 functions dually as a negative feedback regulator of TLR4.

There seem to be direct and indirect pathways for macrophage activation by LPS; one is the MyD88-IRAK-TRAF6-TAK1-IKKβ mediated phosphorylation of I-κB and TRAF6-mediated JNK, p38 activation, while the other is an indirect activation of the JAK/STAT pathway through autocrine/paracrine mechanisms (Crespo et al., 2000; Toshchakov et al., 2002). Apparently, these two pathways are important for the macrophage activation and pathological effect of LPS. For example, NO_2^- synthesis from STAT1$^{-/-}$ as well as MyD88$^{-/-}$ macrophages was reduced compared with those in wild-type macrophages (Ohmori and Hamilton, 2001; Kawai et al., 1999). The promoter of inducible NO synthase (iNOS) has been shown to possess STAT1 and NF-κB binding sites, and both seem to be required for full activation of this promoter (Ganster et al., 2001). Our previous and present results indicate that SOCS1 can negatively regulate both STAT and NF-κB pathways; one of the mechanisms of macrophage inactivation by SOCS1 could be suppression of the JAK/STAT pathway indirectly activated by the autocrine/paracrine mechanism. The other inhibitory mechanism seems to be a direct effect on the TLR4 to IKKβ pathway, since SOCS1 suppressed LPS- and TRAF6-induced NF-κB activation, while phosphorylation of I-κB, JNK, and p38 was enhanced in SOCS1$^{-/-}$ macrophages. Our findings demonstrate, at the molecular level, that SOCS1 is an important negative regulator of LPS-induced macrophage activation, although we have not identified the target molecule of SOCS1 in the TLR signaling pathways.

We have shown that SOCS1 plays an important role in the establishment of endotoxin tolerance (Figure 4). SOCS1 is induced by TLR stimulation and required for not only LPS but also CpG-DNA tolerance, indicating that SOCS1 is a key component of a feedback regulatory system of innate immunity. SOCS1, therefore, plays a critical role in the maintenance of homeostasis of the innate immune system. The molecular mechanism of endotoxin tolerance is complicated, and probably multiple mechanisms are involved. Simple overexpression of SOCS1 in the macrophage cell line could not completely suppress LPS-induced TNFa production and I-κB phosphorylation as shown in Figure 5. Therefore, we suspect that SOCS1 may interact with an unidentified modulator which is necessary for maintenance of LPS tolerance as well as LPS/CpG crosstolerance.

Recently, Takayanagi et al. reported that IFNγ induced ubiquitin-dependent rapid degradation of TRAF6, which results in strong inhibition of the RANKL-induced activation of NF-κB and JNK (Takayanagi et al., 2000). This study shows that there is crosstalk between the TRAF6-IKK pathway and the JAK/STAT pathway. Our present study raises the possibility of a role of SOCS1 in this

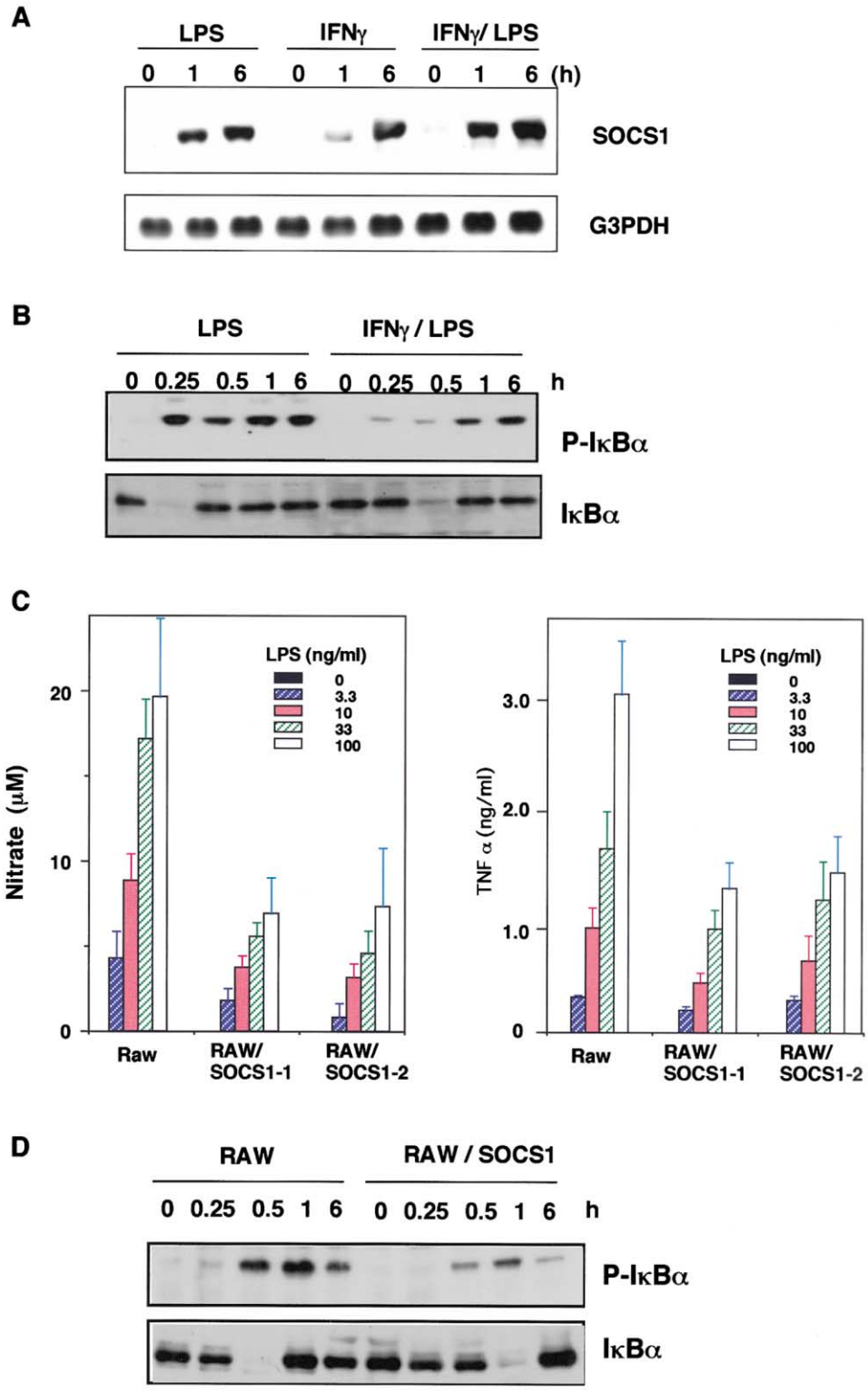

Figure 5. Suppression of LPS Response by SOCS1 in Raw Cells

(A) Raw cells were treated with 100 ng/ml LPS, 1000 unit/ml IFNγ, or both for indicated periods. Total RNA was isolated, and Northern hybridization with SOCS1 or control G3PDH probes were carried out.

(B) Raw cells were pretreated with (IFNγ/LPS) or without (LPS) 1000 unit/ml IFNγ for 12 hr, then stimulated with 100 ng/ml LPS or 1000 unit/ml IFNγ for the indicated periods. Phosphorylation of I-κB was determined by immunoblotting with anti-phospho I-κB specific antibody.

(C) NO_2^- and TNFα production in response to LPS in parental Raw cells (Raw) and two independent transformations (Raw/SOCS1-1 and -2) stably expressing SOCS1.

(D) Phosphorylation of LPS-induced I-κB in Raw cell transformants. Raw cells and SOCS1 transformants (Raw/SOCS1-2) were stimulated with 100 ng/ml LPS for the indicated periods. I-κB phosphorylation was measured by immunoblotting. Data are representative of three independent experiments with similar results.

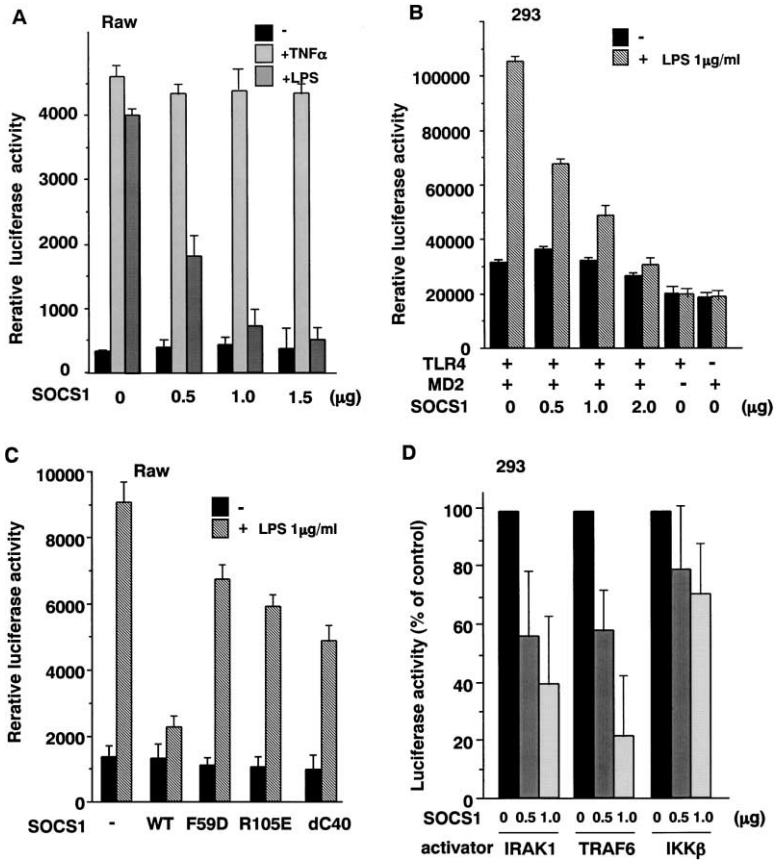

Figure 6. Suppression of LPS-Induced NF-κB Reporter Gene Activation by SOCS1 Overexpression

(A) Raw cells were transfected with the indicated amounts (μg) of SOCS1 plasmid and NF-κB reporter plasmid (2.0 μg), and control lacZ plasmid (2.0 μg). After stimulation with 1 μg/ml LPS for 12 hr, luciferase activity and β-galactosidase was measured. Relative luciferase activity normalized with lacZ activity is shown.

(B) 293 cells were transfected without (−) or with (+) TLR4 and MD2 (0.2 μg each), control lacZ plasmid (0.1 μg), and the indicated amounts of SOCS1 plasmid. After stimulation with (+) or without (−) 1 μg/ml LPS for 12 hr, luciferase activity was measured.

(C) Raw cells were transfected without (−) or with wild-type (WT) and the indicated mutant SOCS1 plasmids (2.0 μg), and NF-κB reporter plasmid. After stimulation with (+) or without (−) LPS for 12 hr, luciferase activity was measured. Equal expression levels of each SOCS1 construct were confirmed by immunoblotting.

(D) 293 cells were transfected with indicated TLR4 downstream molecules (0.5 μg IRAK, 0.1 μg TRAF6, and 0.5 μg IKKβ) without (–) or with the indicated amounts of SOCS1 plasmid. After 36 hr of transfection, luciferase activity was measured and normalized with the luciferase activity in the absence of SOCS1 plasmid.

crosstalk. Interestingly, IKK activation by TRAF6 has been shown to be dependent on the TRAF6-mediated ubiquitination system (Deng et al., 2000). Since SOCS1 contains the SOCS-box which recruits the Elongin B,C-Rbx1-Cul2 ubiquitination system (Kamizono et al., 2001), ubiquitination may be involved in SOCS1-mediated suppression of TRAF6-dependent IKK activation. Further study is under way to define the molecular mechanism responsible for the suppression of TRAF6 by SOCS1.

Experimental Procedures

Mice and Peritoneal Macrophages

SOCS1-deficient mice have been described elsewhere (Marine et al., 1999). SOCS1$^{-/-}$IFNγ$^{-/-}$ mice were generated by crossbreeding SOCS$^{-/-}$ mice and mice with C57BL6 background homozygous inactivation of the IFNγ gene, which were obtained from the Jackson Laboratory. All mice were housed in clean animal rooms with SPF conditions. SOCS1$^{+/-}$ and SOCS1$^{+/+}$ littermate mice were challenged with LPS (*Escherichia coli* serotype O55:B5, Sigma) interperitoneally (IP), and the resulting lethality was observed until 120 hr after this challenge. Five male and five female mice were in each experimental group.

Resident peritoneal macrophages were obtained by peritoneal lavage with 5 ml of cold PBS. Cells were incubated in plastic dishes overnight and then washed with PBS to eliminate nonadherent cells. Adherent cells were dispersed in each well of a 24-well tissue culture plate in DMEM containing 10% fetal bovine serum (FBS). Following stimulation with LPS for 24 hr, the production of NO_2^- and TNFα was determined; NO_2^- was measured as nitrite using the Griess reagent, and TNFα was measured using ELISA kits (Biosource, CA). Phosphorothioate-stabilized CpG-oligo DNA (TCC ATG ACG TTC CTG ACG TT) were purchased from GeneNet (Fukuoka, Japan).

Cell Culture and Stable Transfection

RAW264.7 macrophages (obtained from RIKEN Cell Bank, Japan) were cultured in 10 cm dishes containing Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS). Approximately 6×10^5 cells/well were seeded into 6-well plates and transfected 24 hr later with the pcDNA3-myc-SOCS-1 and its mutant constructs (Yasukawa et al., 1999) using Fugene (Roche) according to the manufacturer's instructions. Stable Raw cell transformants were selected with 0.8 mg/ml G418. Different clones were then isolated and cultured in the presence of 0.8 mg/ml G418. In all the experiments, cells were stimulated with 100 ng/ml of LPS. Transfection to 293 cells and luciferase assay were as described (Yasukawa et al., 1999). The NF-κB-responsive reporter plasmid (p55Igκ-luc), a generous gift from Dr. T. Fujita (The Tokyo Metropolitan Institute of Medical Science, Japan), contains three copies of an NF-κB binding site from the κ light chain immunoglobulin enhancer (Fujita et al., 1993). Recombinant plasmids for expression of TRAF6 and IKKβ were kindly provided by Dr. J. Inoue (Waseda University, Japan). IRAK cDNAs were a generous gift from Dr. T. Naka (Osaka University, Japan). Immunoblotting to detect phosphorylated STAT1 and I-κB was performed as described (Yasukawa et al., 1999). Anti-phospho-specific STAT1 antibody and I-κB were purchased from Cell Signaling Co.

Acknowledgments

We thank Y. Kawabata for technical assistance, Dr. T. Naka for critical discussion and comments, and M. Ohara for language assistance. This work was supported by Grants-in-Aid (for Y.A.) from the Ministry of Education, Science, Technology, Sports and Culture of Japan, the Japan Health Science Foundation, the Human Frontier Science Program, the Mochida Memorial Foundation, the Haraguchi Memorial Foundation, and the Japan Research Foundation for Clinical Pharmacology.

Received: May 24, 2002
Revised: September 10, 2002

References

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptor: critical proteins linking innate and acquired immunity. Nat. Immunol. *2*, 675–680.

Alexander, W.S., Starr, R., Fenner, J.E., Scott, C.L., Handman, E., Sprigg, N., Corbin, J.E., Cornish, A.L., Darwiche, R., Owczarek, C.M., et al. (1999). SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell *98,* 597–608.

Beeson, P.B. (1947). Tolerance to bacterial pyrogens. I. Factors influencing its development. J. Exp. Med. *86*, 29–38.

Berczi, I. (1998). Neurohormonal host defense in endotoxin shock. Ann. NY Acad. Sci. *840*, 787–802.

Berlato, C., Cassatella, M.A., Kinjyo, I., Gatto, L., Yoshimura, A., and Bazzonia, F. (2002). J. Immunol. *168*, 6404–6411.

Beutler, B., Milsark, I.W., and Cerami, A.C. (1985). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science *229*, 869–871.

Crespo, A., Filla, M.B., Russell, S.W., and Murphy, W.J. (2000). Indirect induction of suppressor of cytokine signaling-1 in macrophages stimulated with bacterial lipopolysaccharide: partial role of autocrine/paracrine interferon-β. Biochem. J. *349*, 99–104.

Crespo, A., Filla, M., and Murphy, W. (2002). Low responsiveness to IFN-gamma, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. Eur. J. Immunol. *32*, 710–719.

Cross, A., Asher, L., Seguin, M., Yuan, L., Kelly, N., Hammack, C., Sadoff, J., and Gemski, P., Jr. (1995). The importance of a lipopolysaccharide-initiated, cytokine-mediated host defense mechanism in mice against extraintestinally invasive Escherichia coli. J. Clin. Invest. *96*, 676–686.

Dalpke, A.H., Opper, S., Zimmermann, S., and Heeg, K. (2001). Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. J. Immunol. *166*, 7082–7089.

Danner, R.L., Elin, R.J., Hosseini, J.M., Wesley, R.A., Reilly, J.M., and Parillo, J.E. (1991). Endotoxemia in human septic shock. Chest *99*, 169–175.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000). Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell *103*, 351–361.

De Sepulveda, P., Ilangumaran, S., and Rottapel, R. (2000). Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation. J. Biol. Chem. *275*, 14005–14008.

Eden, C.S., Shahin, R., and Briles, D. (1988). Host resistance to mucosal gram-negative infection. Susceptibility of lipopolysaccharide nonresponder mice. J. Immunol. *140*, 3180–3185.

Fujita, T., Nolan, G.P., Liou, H.C., Scott, M., and Baltimore, D. (1993). The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. *7*, 1354–1363.

Ganster, R.W., Taylor, B.S., Shao, L., and Geller, D.A. (2001). Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B. Proc. Natl. Acad. Sci. USA *98*, 8638–8643.

Goldring, C.E., Reveneau, S., Pinard, D., and Jeannin, J.F. (1998). Hyporesponsiveness to lipopolysaccharide alters the composition of NF-kappaB binding to the regulatory regions of inducible nitric oxide synthase gene. Eur. J. Immunol. *28*, 2960–2970.

Greisman, S.E., Young, E.J., and Woodward, W.E. (1966). Mechanisms of endotoxin tolerance. IV. Specificity of the pyrogenic refractory state during continuous intravenous infusions of endotoxin. J. Exp. Med. *124*, 983–1000.

Gustafson, G.L., Rhodes, M.J., and Hegel, T. (1995). Monophosphoryl lipid A as a prophylactic for sepsis and septic shock. Prog. Clin. Biol. Res. *392*, 567–579.

Hagberg, L., Hull, R., Hull, S., McGhee, J.R., Michalek, S.M., and Svanborg Eden, C. (1984). Difference in susceptibility to gram-negative urinary tract infection between C3H/HeJ and C3H/HeN mice. Infect. Immun. *46*, 839–844.

Henricson, B.E., Benjamin, W.R., and Vogel, S.N. (1990). Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. Infect. Immun. *58*, 2429–2437.

Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Morita, S., Kitamura, T., Kato, H., et al. (2001). The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. J. Biol. Chem. *276*, 12530–12538.

Kastenbauer, S., and Ziegler-Heitbrock, H.W. (1999). NF-kappaB1 (p50) is upregulated in lipopolysaccharide tolerance and can block tumor necrosis factor gene expression. Infect. Immun. *67*, 1553–1559.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 115–122.

Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P.F., Sato, S., Hoshino, K., and Akira, S. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. *167*, 5887–5894.

Kawazoe, Y., Naka, T., Fujimoto, M., Kohzaki, H., Morita, Y., Narazaki, M., Okumura, K., Saitoh, H., Nakagawa, R., Uchiyama, Y., et al. (2001). SSI-1/SOCS-1 family proteins inhibit insulin signal transduction pathway through modulating IRS-1 phosphorylation. J. Exp. Med. *193*, 263–269.

Kobayashi, K., Hernandez, L.D., Galan, J.E., Janeway, C.A., Jr., Medzhitov, R., and Flavell, R.A. (2002). IRAK-M is a negative regulator of Toll-like receptor signaling. Cell *110*, 191–202.

Marine, J.C., Topham, D.J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., and Ihle, J.N. (1999). SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. Cell *98*, 609–616.

Medzhitov, R., and Janeway, C., Jr. (2000). Innate immunity. N. Engl. J. Med. *343*, 338–344.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C.A., Jr. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell *2*, 253–258.

Metcalf, D., Mifsud, S., Di Rago, L., Nicola, N.A., Hilton, D.J., and Alexander, W.S. (2002). Polycystic kidneys and chronic inflammatory lesions are the delayed consequences of loss of the suppressor of cytokine signaling-1 (SOCS-1). Proc. Natl. Acad. Sci. USA *99*, 943–948.

Morita, Y., Naka, T., Kawazoe, Y., Fujimoto, M., Narazaki, M., Nakagawa, R., Fukuyama, H., Nagata, S., and Kishimoto, T. (2000). SSI/SOCS-1 suppresses TNF a-induced cell death by regulation of p38 MAP kinase signal in fibroblast. Proc. Natl. Acad. Sci. USA *97*, 5405–5410.

Naka, T., Matsumoto, T., Narazaki, M., Fujimoto, M., Morita, Y., Ohsawa, Y., Saito, H., Nagasawa, T., Uchiyama, Y., and Kishimoto, T. (1998). Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor) deficient mice. Proc. Natl. Acad. Sci. USA *95*, 15575–15582.

Naka, T., Tsutsui, H., Fujimoto, M., Kawazoe, Y., Kohzaki, H., Morita, Y., Nakagawa, R., Narazaki, M., Adachi, K., Yoshimoto, T., et al. (2001). SOCS-1/SSI-1-deficient NKT cells participate in severe hepatitis through dysregulated cross-talk inhibition of IFN-γ and IL-4 signaling in vivo. Immunity *14*, 535–545.

Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999). The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature *398*, 252–256.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., and Akira, S. (2000). Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J. Immunol. *164*, 3476–3479.

Ohmori, Y., and Hamilton, T.A. (2001). Requirement for STAT1 in LPS-induced gene expression in macrophages. J. Leukoc. Biol. *69*, 598–604.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Salkowski, C.A., Detore, G., Franks, A., Falk, M.C., and Vogel, S.N. (1998). Pulmonary and hepatic gene expression following cecal ligation and puncture: monophosphoryl lipid A prophylaxis attenuates sepsis-induced cytokine and chemokine expression and neutrophil infiltration. Infect. Immun. *66*, 3569–3578.

Shahin, R.D., Engberg, I., Hagberg, L., and Svanborg Eden, C. (1987). Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local gram-negative infection. J. Immunol. *138*, 3475–3480.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J. Exp. Med. *189*, 1777–1782.

Starr, R., Willson, T.A., Viney, E.M., Murray, L.J.L., Rayner, J.R., Jenkins, B.J., Alexander, W.S., Metcalf, D., Nicola, N.A., and Hilton, D.J. (1998). Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc. Natl. Acad. Sci. USA *95*, 14395–14399.

Stoiber, D., Kovarik, P., Cohney, S., Johnston, J.A., Steinlein, P., and Decker, T. (1999). Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. J. Immunol. *163*, 2640–2647.

Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K., et al. (2000). RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature *408*, 600–605.

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., and Cerami, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature *330*, 662–664.

Toshchakov, V., Jones, B.W., Perera, P.-Y., Thomas, K., Cody, M.J., Zhang, S., Williams, B.R.G., Major, J., Hamilton, T.A., Fenton, M.J., and Vog, S.N. (2002). TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat. Immunol. *3*, 392–398.

Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J.N., and Yoshimura, A. (1999). The JAK-binding protein JAB inhibits *Janus* tyrosine kinase activity through binding in the activation loop. EMBO J. *18*, 1309–1320.

Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000). Negative regulation of cytokine signaling pathways. Annu. Rev. Immunol. *18*, 143–164.

Zhang, J.G., Metcalf, D., Rakar, S., Asimakis, M., Greenhalgh, C.J., Willson, T.A., Starr, R., Nicholson, S.E., Carter, W., Alexander, W.S., et al. (2001). The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo. Proc. Natl. Acad. Sci. USA *98*, 13261–13265.

Ziegler-Heitbrock, H.W.L. (1995). Molecular mechanism in tolerance to lipopolysaccharide. J. Inflamm. *45*, 13–26.

Ziegler-Heitbrock, H.W., Petersmann, I., and Frankenberger, M. (1997). p50 (NF-kappa B1) is upregulated in LPS tolerant P388D1 murine macrophages. Immunobiology *198*, 73–80.