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

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Summary

Suppressor of cytokine signaling-1 (SOCS-1), also known as STAT-induced STAT inhibitor-1 (SSI-1), is a negative feedback molecule for cytokine signaling, and its in vivo deletion induces fulminant hepatitis. However, elimination of the STAT1 or STAT6 gene or deletion of NKT cells substantially prevented severe hepatitis in SOCS-1-deficient mice, while administration of IFN-γ and IL-4 accelerated its development. SOCS-1 deficiency not only sustained IFN-γ/IL-4 signaling but also eliminated the cross-inhibitory action of IFN-γ on IL-4 signaling. These results suggest that SOCS-1 deficiency-induced persistent activation of STAT1 and STAT6, which would be inhibited by SOCS-1 under normal conditions, may induce abnormal activation of NKT cells, thus leading to lethal pathological changes in SOCS-1-deficient mice.

Introduction

Most hematopoietic cells are exposed to various cytokines and are systematically regulated by a complicated network of cytokines. Signaling through Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is one of the most important signal pathways activated immediately after cytokine stimulation (for a review, see Darnell et al., 1994; Kishimoto et al., 1994; Ihle, 1995). Suppressor of cytokine signaling-1 (SOCS-1), also known as STAT-induced STAT inhibitor-1 (SSI-1), is an intracellular protein that inhibits JAK-mediated cytokine signaling by binding to JAKs (Starr et al., 1997; Endo et al., 1997; Naka et al., 1997; for a review, see Naka et al., 1999; Krebs and Hilton, 2000; Chen et al., 2000; Nicola and Greenhalgh, 2000; Yasukawa et al., 2000; Naka et al., 2001; Starr, 2001). Although SOCS-1 can be induced by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-4, IL-2, and IL-6, the target molecules of SOCS-1 among members of the JAK family have not been precisely identified yet (Starr et

al., 1997; Endo et al., 1997; Naka et al., 1997; Helman et al., 1998; Narazaki et al., 1998; Yasukawa et al., 1999; Nicholson et al., 1999; Losman et al., 1999; Pezet et al., 1999; Morita et al., 2000; Fujimoto et al., 2000; for a review, see Naka et al., 1999; Krebs and Hilton, 2000; Chen et al., 2000; Nicola and Greenhalgh, 2000; Yasukawa et al., 2000).

SOCS-1-deficient mice (SOCS-1 KO mice) are born healthy but with growth disclose various kinds of abnormalities, including growth retardation, thymic atrophy, fulminant hepatitis, with serious fatty degeneration and lung injuries with infiltration of mononuclear cells, and all die within 3 weeks after birth (Starr et al., 1998; Naka et al., 1998). T cells from SOCS-1 KO mice show prolonged signaling in response to IL-4 or IFN-γ, demonstrating that SOCS-1 is an inhibitory factor required for the cessation of IL-4 or IFN-γ signaling in vivo (Starr et al., 1998; Naka et al., 1998). Recently, it has been reported that IFN-γ-deficient SOCS-1 KO mice are free from these severe pathological changes (Alexander et al., 1999; Marine et al., 1999), indicating that SOCS-1 indeed negatively regulates overshooting of IFN-γ signaling. This study provided a new insight into the essential role of the IFN-γ/SOCS-1 system in the development and maintenance of the immune system. However, they do not seem to imply that the lethal changes in SOCS-1 KO mice are solely attributable to oversignaling of IFN-γ, because SOCS-1 also negatively regulates various kinds of signalings (Naka et al., 1997, 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Adams et al., 1998; Hansen et al., 1999; Losman et al., 1999; Ram and Waxman, 1999; Pezel et al., 1999; Tomic et al., 1999; Morita et al., 2000; Kawazoe et al., 2001). Indeed, a recent study revealed that IFN-γ-induced SOCS-1 plays a critical role in inhibiting STAT6 activation in vitro (Dickensheets et al., 1999; Venkataraman, et al., 1999). Thus, without cross-talk inhibition via SOCS-1, IL-4 and IFN-γ signalings might be independently, occasionally at the same time, and persistently transduced to abnormally activate lymphocytes, causing injuries of immune organs and liver. It is therefore important to determine whether SOCS-1 deficiency induces pathological changes by abolishing a cross-talk inhibition of IFN-γ and IL-4 signalings or simply by amplifying one signal. In this study, we first tested whether deletion of the STAT1 gene instead of the IFN-γ gene results in similar prevention of fulminant hepatitis in SOCS-1 KO mice. Second, we analyzed whether deletion of the STAT6 gene also prevents these pathological changes, as we wished to clarify whether SOCS-1 deficiency facilitates simultaneous overshooting of IL-4, which, in combination with IFN-γ signaling, induces lethal pathologies. Lastly, we examined whether SOCS-1 in hepatic NKT cells acts as a pivotal regulating factor that limits their hepatocyte-killing action. This is the first in vivo study to show that SOCS-1 is a critical molecule for cross-talk inhibition of IFN-γ and IL-4 signalings, resulting in suppression of the overactivation of hepatocytotoxic NKT cells.

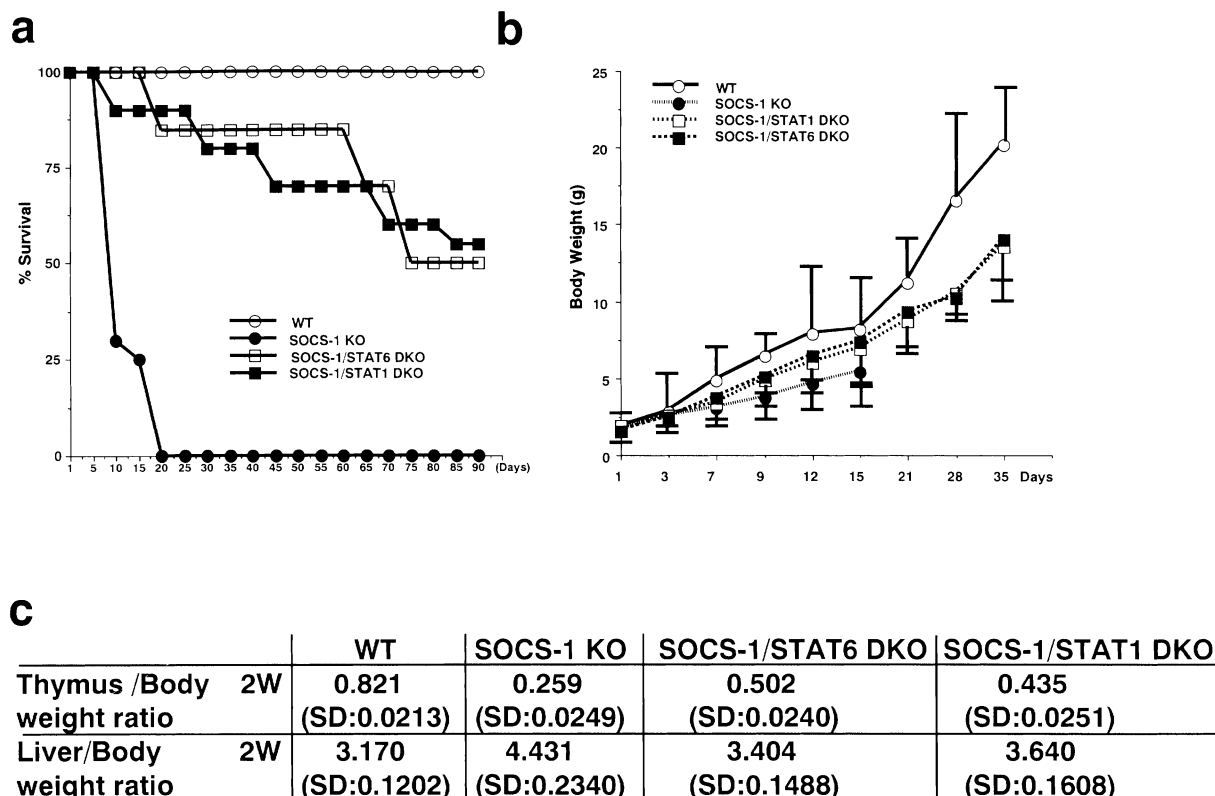[5] Correspondence: naka@imed3.med.osaka-u.ac.jp

a

b

c

		WT	SOCS-1 KO	SOCS-1/STAT6 DKO	SOCS-1/STAT1 DKO
Thymus /Body weight ratio	2W	0.821 (SD:0.0213)	0.259 (SD:0.0249)	0.502 (SD:0.0240)	0.435 (SD:0.0251)
Liver/Body weight ratio	2W	3.170 (SD:0.1202)	4.431 (SD:0.2340)	3.404 (SD:0.1488)	3.640 (SD:0.1608)

Figure 1. Disruption of STAT1 or STAT6 Prevents Perinatal Death of SOCS-1 KO Mice

Mice with various gene mutations were kept under SPF conditions. Survival rate (A) and body weight (B) were measured until 90 and 35 days after birth, respectively. Two weeks after birth, thymus and liver were sampled from various types of mice, and the ratios of the weight of these organs to body weight were calculated (C). Data in (A) are data of 20 mice in each experimental group and are representative of two independent experiments with similar results. Data in (B) and (C) show mean ± SD for five mice from each group and are representative of three independent experiments with similar results.

Results

STAT1 or STAT6 Deficiency Protects SOCS-1 KO Mice from Various Kinds of Abnormalities

We generated SOCS-1 KO mice lacking STAT1 (SOCS-1/STAT1 DKO mice) or SOCS-1 KO mice lacking STAT6 (SOCS-1/STAT6 DKO mice) by deletion of the STAT1 or STAT6 gene in SOCS-1 KO mice. All the SOCS-1 KO mice died within 3 weeks after birth. In contrast, mortality was significantly reduced in SOCS-1/STAT1 or SOCS-1/STAT6 DKO mice (Figure 1A). As also previously reported (Starr et al., 1998; Naka et al., 1998), SOCS-1 KO mice showed a marked growth retardation, while SOCS-1/STAT1 and SOCS-1/STAT6 DKO mice grew larger than SOCS-1 KO mice but were still smaller than wild-type (WT) mice (Figure 1B). Moreover, severe thymic atrophy was partly improved, and hepatomegaly was almost completely eliminated in SOCS-1/STAT1 and SOCS-1/STAT6 DKO mice (Figure 1C).

Elimination of either STAT1 or STAT6 led to improvement in pathological changes in various organs of SOCS-1 KO mice. Histological findings and determination of the numbers of various cells on day 10 after birth revealed that thymic atrophy had partly improved in SOCS-1/STAT1 or SOCS-1/STAT6 DKO mice (Figures 2A and 2B),and that complete histological restoration

had been achieved in the livers of both types of DKO mice (Figure 2C). SOCS-1 KO mice showed fulminant hepatitis characterized by severe fatty degeneration and necrosis with massive lymphocyte infiltration (Starr et al., 1998; Naka et al., 1998; Alexander et al., 1999). In contrast, the livers from SOCS-1/STAT1 DKO mice like those from SOCS-1/IFN-γ DKO mice (Alexander et al., 1999; Marine et al., 1999) remained almost intact except for infiltration by a small number of lymphocytes (Figure 2C), thus confirming the pathological role of IFN-γ in this liver injury. To our surprise, SOCS-1/STAT6 DKO mice also had almost intact liver tissue, which suggests that SOCS-1 might prevent development of fulminant hepatitis by inhibiting of simultaneous transduction of both IFN-γ and IL-4 signals.

Relevant Role of SOCS-1-Deficient Lymphocytes in Severe Hepatitis Observed in SOCS-1 KO Mice

As SOCS-1 is strongly expressed in lymphoid organs, especially in T cells (Starr et al., 1997; Naka et al., 1997; Marine et al., 1999), we compared the proportions of splenic T cells expressing CD69, an activation marker, among mice with various genetic mutations. SOCS-1 KO mice showed an increase in the population of activated T cells, while SOCS-1/STAT1 or SOCS-1/STAT6 DKO mice
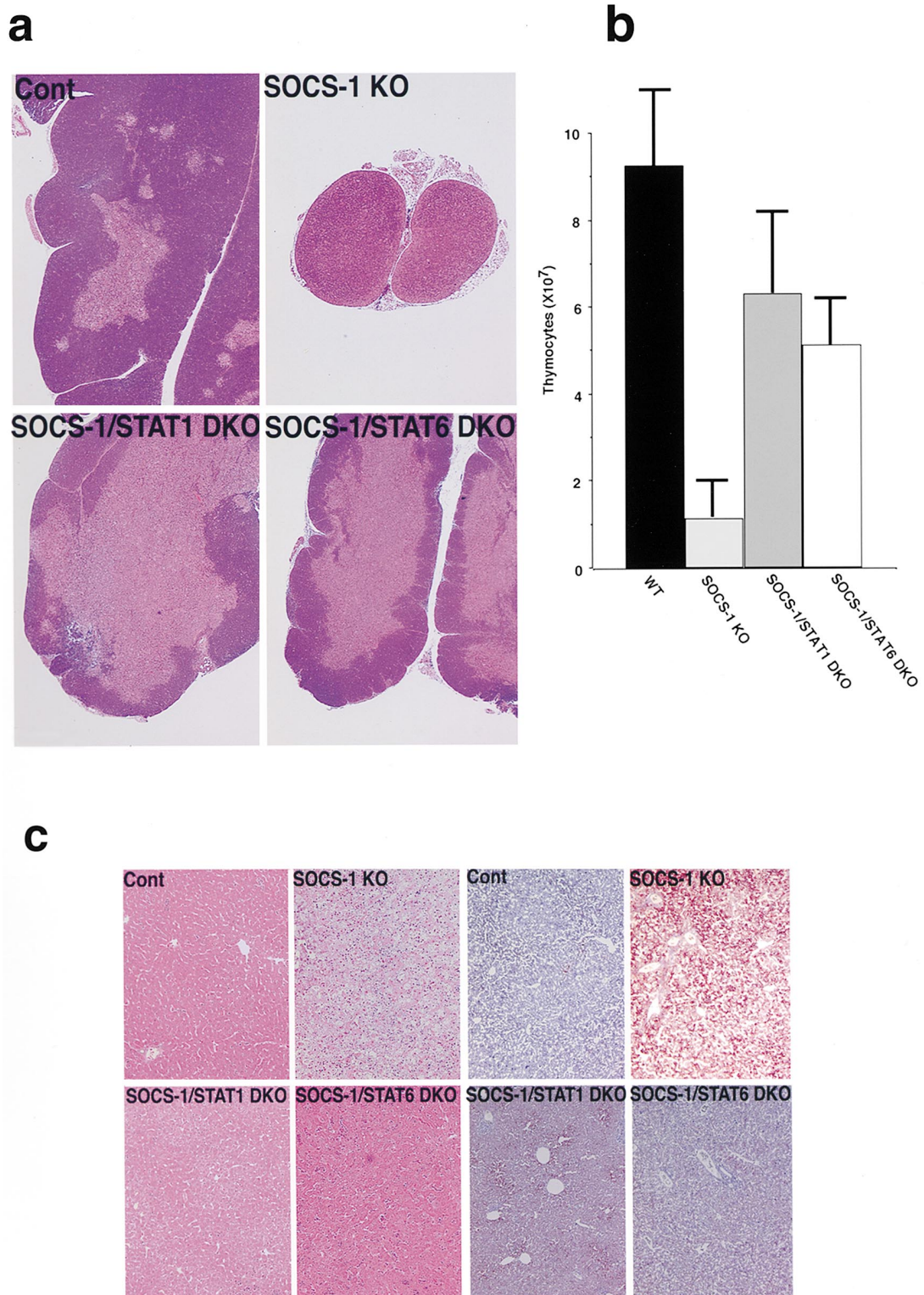
a

b

c

Figure 2. Improvement of Tissue Anomalies in SOCS-1 KO Mice by Elimination of STAT1 or STAT6

Thymus (A and B) and liver (C) were sampled from mice with various genotypes 2 weeks after birth for histological study (A and C) and cell count (B). Thymocytes were isolated, and the cells were counted (B). (A) Hematoxylin/eosin (HE) staining, original magnification: ×25. (C) HE staining (left four panels) and Sudan III staining (right four panels), original magnification: ×80. Data in (A) and (C) are representative of 10 independent experiments with similar results. Data in (B) show mean ± SD of five mice from each group and are representative of five independent experiments with similar results.
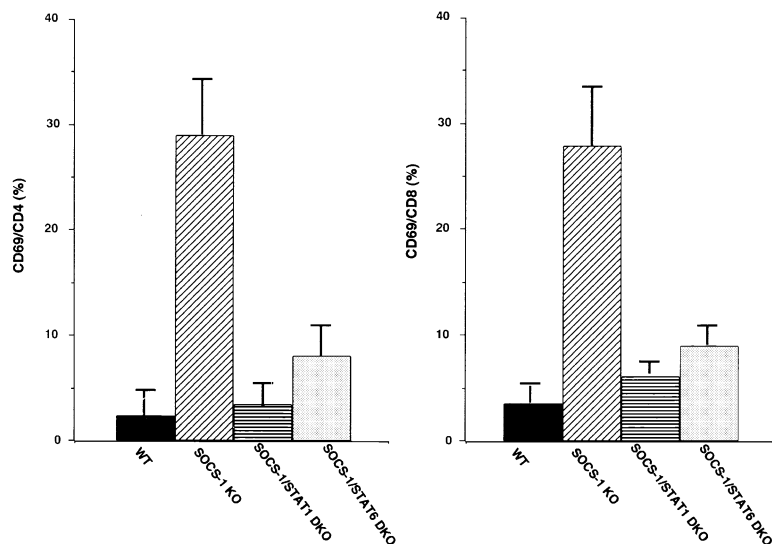
Figure 3. Disruption of STAT1 or STAT6 Restores Spontaneously Overactivated T Cells in SOCS-1 KO Mice

Splenic CD4^{+} cells (left panel) or CD8^{+} cells (right panel) were isolated from mice with various genotypes by MACS. The proportion of CD69 expression for each cell group was measured by FACS. Data are representative of 10 independent experiments with similar results.

contained a reduced but still higher proportion of spontaneously activated T cells in their spleen when compared with, respectively, the spleens from SOCS-1 KO and WT mice (Figure 3).

Next, we investigated the cellular mechanism underlying the pathological changes in SOCS-1 KO mice, with special attention to severe liver injuries. To clarify whether these lethal alterations are due to abnormalities in lymphocytes, in stromal cells sustaining lymphocyte maturation, or in parenchymal cells, we transferred hematopoietic progenitor cells from the SOCS-1 KO fetal livers (E16.5 days) into RAG2-deficient (RAG2 KO) mice. All the RAG2 KO mice reconstituted with SOCS-1-deficient lymphocytes died within 7 weeks after transplantation, while RAG2 KO mice reconstituted with WT lymphocytes survived normally. Furthermore, SOCS-1-deficient chimeric mice showed various characteristics similar to those of SOCS-1 KO mice, such as relative weight loss in the thymus and spleen and relative weight gain in the liver (Figure 4A). Indeed, histological findings for various tissues from SOCS-1-deficient chimeric mice obtained 6 weeks after transplantation revealed disappearance of the thymic cortex (data not shown) and severe liver injuries (Figure 4B). Moreover, the critical involvement of SOCS-1-deficient lymphocytes in the induction of these pathological changes is well documented by a recent study in which SOCS-1-deficient bone marrow cells, when used to reconstitute Jak3-deficient mice lacking T cells, B cells, and NK cells, replicated the phenotype seen in SOCS-1-deficient mice (Marine et al., 1999). In contrast, RAG2-deficient SOCS-1 KO mice that lacked T cells and B cells but contained NK cells showed healthy growth (Marine et al., 1999). These findings in combination with our results presented here strongly indicate that abnormal lymphocytes cause the multiple pathological changes in SOCS-1 KO mice.

Spontaneously Activated Hepatic NKT Cells Cause Liver Injuries in SOCS-1 KO Mice

Next, we identified the cell types and effector molecules responsible for severe liver injuries in SOCS-1 KO mice.

Since RAG2-deficient SOCS-1 KO mice avoid severe liver injury (Marine et al., 1999), NK cells seem to be insignificant as hepatocytotoxic effector cells. As the liver has a unique immune system characterized by the presence of abundant NKT cells, a potent cytotoxic cell population (Kawamura et al., 1998), we focused on the role of hepatic NKT cells in this liver injury. Hepatic lymphocytes from SOCS-1 KO mice killed hepatocytes from syngeneic WT mice in a perforin-dependent but Fas/Fas ligand (Fas L)-independent manner (Figure 5A). The hepatocytotoxicity of SOCS-1-deficient hepatic lymphocytes was reduced after the deletion of NK1.1^{+} cells consisting of NK cells and NKT cells (Figure 5B). NK cells and NKT cells show different susceptibilities to in vivo treatment with anti-asialo GM1, possibly due to the difference in their expression of asialo GM1 (Sonoda, et al., 1999). By taking advantage of this difference, we could selectively deplete NK cells without affecting NKT cells (Figure 5C-2). This treatment did not eliminate the hepatocytotoxicity of SOCS-1-deficient hepatic lymphocytes (Figure 5C-1). These results suggest that NKT cells may belong to the effector cell populations killing hepatocytes. In fact, SOCS-1 was induced in WT NKT cells upon stimulation with IFN-γ (data not shown), and the number of NKT cells (CD3/NK1.1 double positive cells) was markedly elevated in the liver of SOCS-1 KO mice compared with that of WT mice (Figure 5D). Furthermore, more than 65% of SOCS-1-deficient NKT cells in the liver spontaneously expressed CD69, while only less than 20% of WT hepatic NKT cells did so (Figure 5E). In contrast, both SOCS-1/STAT1 and SOCS-1/STAT6 DKO mice contained comparable number of NKT cells and similar levels of their CD69 expression in their livers as compared to WT mice (data not shown).

To investigate whether SOCS-1-deficient NKT cells have the potential to cause such severe liver injury in vivo, we administered α-galactoceramide (α-GalCer), a selective activator of NKT cells to pre-onset SOCS-1 KO mice. As shown in Figure 5F, SOCS-1 KO mice but not WT mice suffered from fulminant hepatitis 12 hr

a

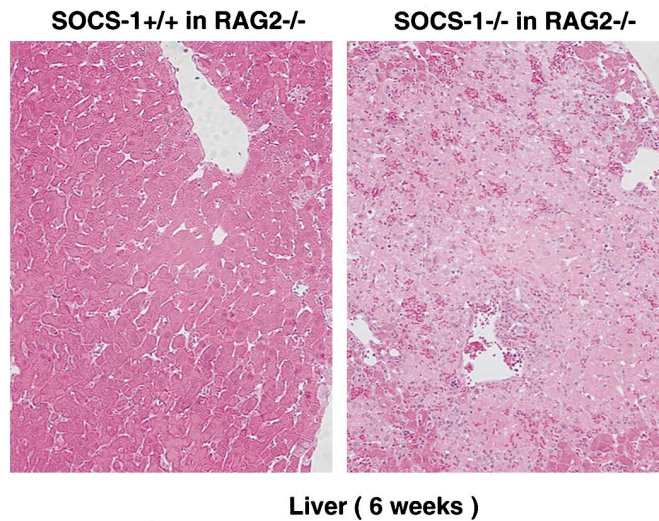|		SOCS-1$^{+/+}$ in RAG2$^{-/-}$	SOCS-1$^{-/-}$ in RAG2$^{-/-}$
Thymus / Body weight ratio	6W	0.227 (+/- 0.0188)	0.072 (+/- 0.0072)
Spleen / Body weight ratio	6W	1.427 (+/- 0.1577)	0.694 (+/- 0.0724)
Liver /Body weight ratio	6W	4.460 (+/- 0.2408)	6.749 (+/- 0.2646)

b

SOCS-1+/+ in RAG2-/-   **SOCS-1-/- in RAG2-/-**

Liver (6 weeks)

Figure 4. RAG2 KO Mice Reconstituted with SOCS-1-Deficient Lymphocytes Spontaneously Develop Fulminant Hepatitis

(A) RAG2 KO mice were reconstituted with fetal liver stem cells from SOCS-1 KO (SOCS-1$^{-/-}$ in RAG2$^{-/-}$) or WT mice (SOCS-1$^{+/+}$ in RAG2$^{-/-}$). Six weeks after reconstitution, thymus, spleen, and liver were sampled to measure their weight, and the ratios of the weight of these organs to body weight were calculated. Data show mean $\pm$ SD of 10 mice in each group and are representative of three independent experiments with similar results.

(B) Six weeks after reconstitution, liver was sampled for histological study. HE staining, original magnification: $\times$ 80. Data are representative of five independent experiments with similar results.

after the α-GalCer challenge (Figure 6). Thus, SOCS-1-deficient NKT cells that are highly susceptible to the stimulation with α-GalCer might have the capacity to cause severe liver injury upon the appropriate stimuli, including cytokines, in vivo, although it is still elusive that α-GalCer reactive SOCS-1-deficient T cells solely mediate the hepatic pathological changes in SOCS-1 KO mice. These results suggest that liver injuries observed in SOCS-1 KO mice might be due to the anomalously activated NKT cells.
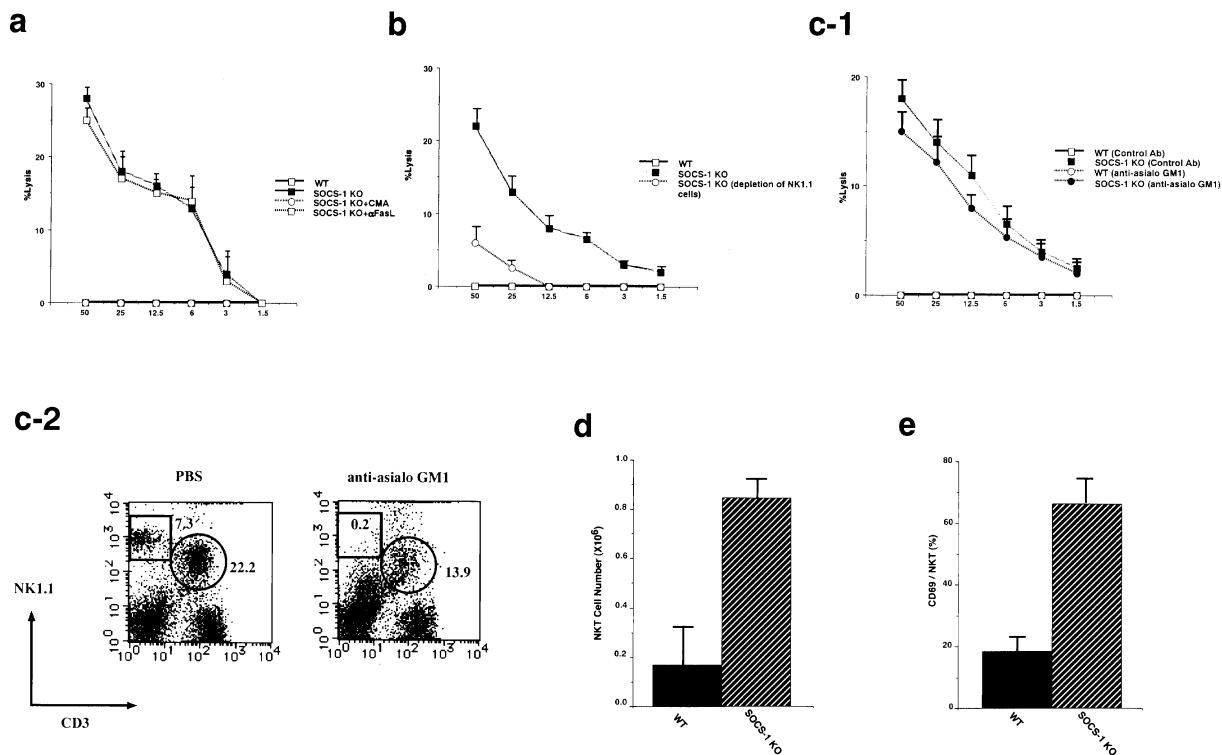
Figure 5. Hepatocytotoxicity of Hepatic NKT Cells in SOCS-1 KO Mice

(A) Hepatic lymphocytes were isolated from WT or SOCS-1 KO mice and were incubated with 100 nM concanamycin A (CMA) or 20 μg/ml of anti-murine Fas L for 1 hr, and their hepatocytotoxicity was determined by 4 h-[^{51}Cr] release assay.

(B) NK1.1$^+$ cells were removed from hepatic lymphocytes by MACS and their hepatocytotoxicity was determined.

(C-1) Hepatic lymphocytes were treated twice with anti-asialo GM1 and complement for depletion of NK cells, and their hepatocytotoxicity was determined. (C-2) Surface phenotypes of the cells before and after the treatment were determined by FACS.

(D) Hepatic lymphocytes from WT or SOCS-1 KO mice were incubated with biotinylated anti-NK1.1 followed by Cy-streptavidin and FITC-conjugated anti-CD3, and the NKT cell proportion (NK1.1$^+$CD3$^+$ cell population) was calculated.

(E) Hepatic lymphocytes were incubated with biotinylated anti-NK1.1 followed by Cy-streptavidin, FITC-conjugated anti-CD3, and PE-conjugated anti-CD69. The proportion of CD69$^+$ cells gated on NK1.1$^+$CD3$^+$ cells was calculated. Data show mean ± SD of triplicate and are representative of three independent experiments with similar results (A, B, and C-1). Data are representative of five independent experiments with similar results (C-2, D, and E).

Involvement of Simultaneous Signaling of IFN-γ/IL-4 in NKT Cells in Severe Liver Injuries

As previously reported, treatment of thymocytes from SOCS-1 KO mice with IL-4 resulted in long-term tyrosine phosphorylation of STAT6, indicating that SOCS-1 can inhibit STAT6 tyrosine phosphorylation in vivo (Naka et al., 1998). This is also the case for STAT1 signaling. As shown in Figure 7A, tyrosine phosphorylation of STAT1 was abnormally extended in SOCS-1-deficient thymocytes as compared with WT (Figure 7A), indicating that disruption of SOCS-1 sustains activation of both STAT1- and STAT6-mediated signalings.

Next, we investigated whether IFN-γ-induced SOCS-1 is essential for negative regulation of IL-4 signaling as well as its own signaling. To test this possibility, we cultured splenic T cells with IFN-γ followed by stimulation with IL-4 and determined the tyrosine phosphorylation of STAT6. As shown in Figure 7B, IFN-γ-prestimulated SOCS-1-deficient T cells did show tyrosine phosphorylation of STAT6 in response to IL-4, whereas the subsequent stimulation did not induce STAT6 tyrosine phosphorylation in WT T cells. An attempt to make the same comparison by using hepatic NKT cells failed

because of the difficulty in obtaining a sufficient number of NKT cells from WT livers. Although we performed the reverse procedure, we could not obtain the same results (data not shown). These results suggest that SOCS-1 induced by IL-4 might be insufficient to inhibit STAT1 phosphorylation and/or signaling suppressor(s) other than SOCS-1 might negatively regulate the IL-4-induced inhibition of STAT1 phosphorylation.

Finally, we investigated whether SOCS-1-deficient NKT cells directly cause severe liver injury via dysregulated signalings of IFN-γ and IL-4. Pre-onset SOCS-1 KO mice administered with IFN-γ and IL-4 resulted in severe liver injuries similar to those seen in onset SOCS-1 KO mice (Figure 7C). In contrast, NK/NKT cell-depleted pre-onset SOCS-1 KO mice were resistant to such treatment (Figure 7C). Pre-onset SOCS-1 KO mice that had been pretreated with anti-asialo GM1 Ab lacked NK cells but maintained NKT cells in their livers as in the results of in vitro treatment shown in Figure 5C (unpublished data). They remained sensitive to a challenge with IFN-γ plus IL-4 (data not shown). Pre-onset SOCS-1 KO mice did not suffer from severe liver injuries at 24 hr after challenge with IFN-γ or IL-4 alone (data not shown). All
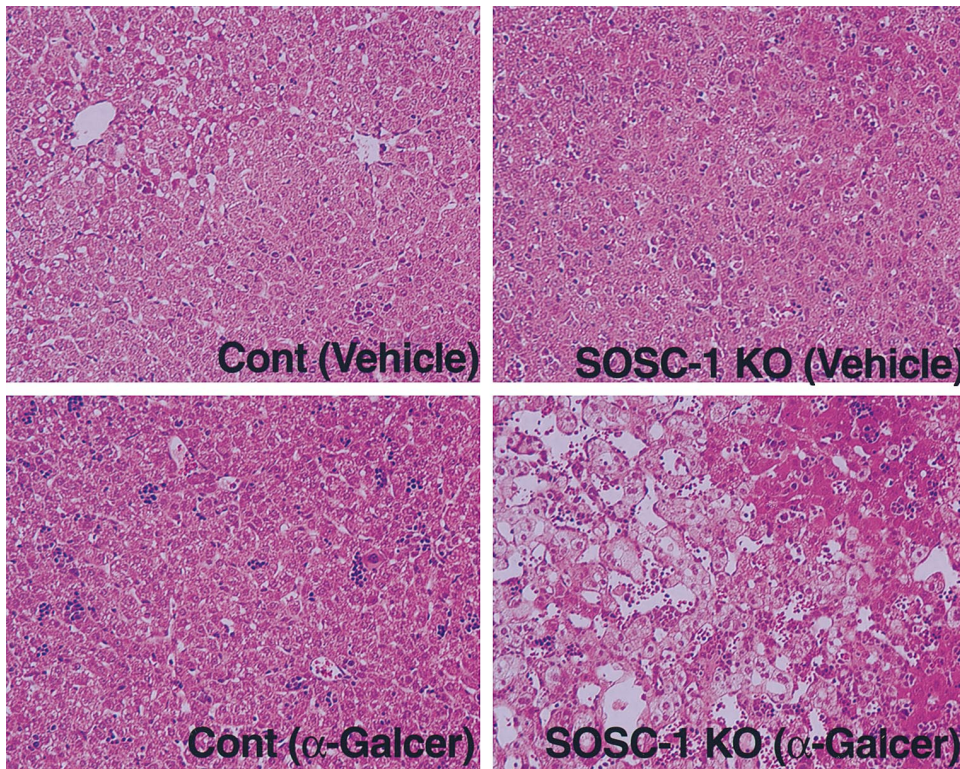
Figure 6. α-GalCer Was Administered to Pre-Onset SSI-1 KO Mice or WT Mice

At 12 hr, the liver specimens were sampled for histological study. (HE staining, original magnification: × 200) Data are representative of five independent experiments with similar results.

these findings together suggest that SOCS-1 may play an essential role in regulating cytokine signalings in vivo and that depletion of SOCS-1 may facilitate the concurrent transmission of multiple cytokine signalings to produce abnormally activated T cells, including hepatic NKT cells, thus leading to severe liver injuries.

Discussion

IFN-γ-deficient SOCS-1 KO mice have been shown to avoid various abnormalities observed in SOCS-1 KO mice (Alexander et al., 1999; Marine et al., 1999). This result suggested the possibility that SOCS-1 might be a selective inhibitor of IFN-γ signaling and that IFN-γ is principally responsible for inducing these pathological changes in vivo. However, previous studies have not clarified the mechanisms by which SOCS-1 selectively inhibits IFN-γ signaling. Rather, many in vivo and in vitro studies have demonstrated that SOCS-1 inhibits a wide range of signalings of cytokines/hormones, including IL-4, IL-6, IFN-γ, thrombopoietin, leukemia inhibitory factor, prolactin, insulin, growth hormone, and stem cell factor (Starr et al., 1997; Naka et al., 1997; Losman et al., 1999; Adams et al., 1998; Hansen et al., 1999; Ram and Waxman, 1999; Pezet et al., 1999; Tomic et al., 1999; Sakamoto et al., 1998; Song and Shuai, 1998; Morita et al., 2000; Kawazoe et al., 2001) and that SOCS-1 has the capacity to bind to all JAKs (Starr et al., 1997; Endo et al., 1997; Naka et al., 1997; Fujimoto et al., 2000; Losman et al., 1999; Helman et al., 1998; Pezet et al.,

1999; Narazaki et al., 1998; Yasukawa et al., 1999; Nicholson et al., 1999). Alternatively, SOCS-1 shows no specificity for cytokines or JAKs (for a review, see Naka et al., 1999; Krebs and Hilton., 2000; Chen et al., 2000; Nicola and Greenhalgh, 2000). In addition, a previous study of ours demonstrated that SOCS-1 is capable of inhibiting activation of STAT6 and the action of IL-4 in vivo (Naka et al., 1998). Moreover, various lesions observed in SOCS-1 KO mice were eliminated not only in SOCS-1/STAT1 DKO but also in SOCS-1/STAT6 DKO mice (Figures 1 and 2). On the basis of these findings, it can be assumed that SOCS-1 is a molecule acting downstream not only on IFN-γ but also on other cytokines, including IL-4 in vivo.

Our present data, that SOCS-1/STAT1 DKO avoided liver injury (Figures 1 and 2), have further substantiated the previous conclusion (Alexander et al., 1999; Marine et al., 1999). To our surprise, however, deletion of the STAT6 gene similarly prevented these pathological changes (Figures 1 and 2). These results suggest that persistent activation of both STAT1 and STAT6, but not of STAT1 alone, is responsible for inducing these pathological changes. IFN-γ and IL-4 were found to inhibit overactivation of STAT1 and STAT6 signalings, respectively, via SOCS-1 (Figure 7A). Moreover, STAT1-induced SOCS-1 inhibited STAT6 activation (Figure 7B). Thus, IFN-γ inhibits not only its own signal but also IL-4 signals via SOCS-1. Deletion of the STAT1 gene in SOCS-1 KO mice allows persistent activation of STAT6 but not of STAT1 and deletion of STAT6 gene vice versa,
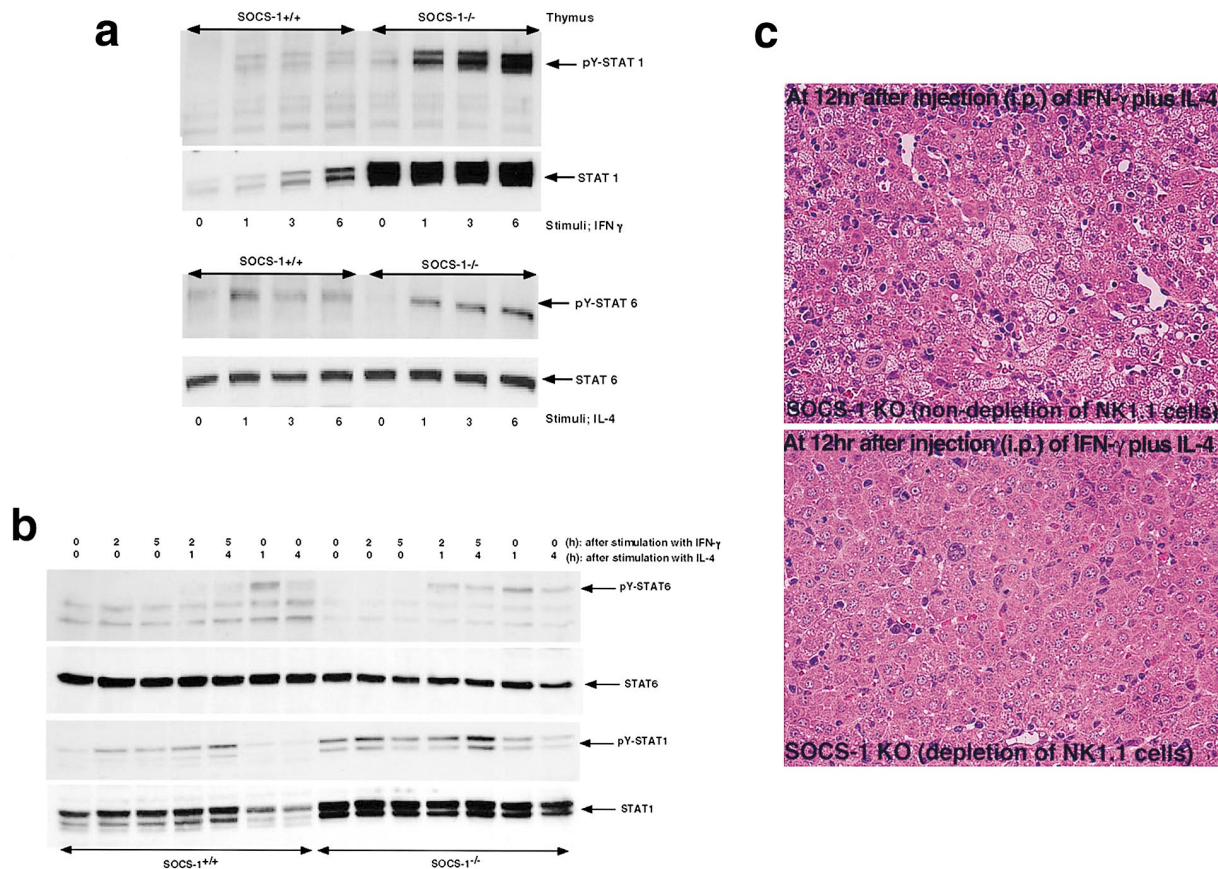
Figure 7. Involvement of Dysregulated Cross-Talk Inhibition of IFN-γ and IL-4 Signaling in SOCS-1-Deficient NKT Cells in Severe Hepatitis

(A) Thymocytes were incubated with IFN-γ or IL-4, and their STAT1 and STAT6 phosphorylation was determined by means of immunoblotting using anti-phosphorylated STAT1 and anti-phosphorylated STAT6, respectively.

(B) Splenic T cells were incubated with or without IFN-γ followed by additional incubation with IL-4 for the indicated time. The phosphorylated STAT1 and STAT6 were determined with the same procedure as shown in (A).

(C) SOCS-1 KO mice were administered with anti-NK1.1 mAb or control IgG, and 2 days later were challenged with IFN-γ plus IL-4. At 24 hr, the liver specimens were sampled for histological study. (HE staining, original magnification: × 200). Data are representative of five independent experiments with similar results.

resulting in avoiding severe pathological changes. Thus, activated STAT1 and STAT6, when collaborated in the absence of SOCS-1, mediate severe liver injury. Transgenic mice overexpressing IFN-γ or IL-4 in their lymphocytes or hepatocytes have been reported to display spontaneous thymic atrophy with relatively early death although they remain entirely free of fulminant hepatitis (Lewis et al., 1991; Tepper et al., 1990; Erb et al., 1997; Young et al., 1997; Toyonaga et al., 1994). These findings and the results of our study suggest that thymic and splenic changes observed in SOCS-1/STAT1 and SOCS-1/STAT6 DKO mice could be due to oversignaling of residual, endogenous IL-4, and IFN-γ, respectively. However, the severe liver injuries seem to require the participation in simultaneous signal transduction of multiple cytokines, at least of both IFN-γ and IL-4. Although IFN-γ-deficient SOCS-1 KO mice completely recovered from the complex disease observed in SOCS-1 KO mice (Alexander et al., 1999; Marine et al., 1999), the STAT1-deficient SOCS-1 KO mice in our study did not show complete recovery in some organs, such as thymus and spleen. This discrepancy may be due to the involvement

of another signal pathway besides JAK/STAT signal transduction in IFN-γ signaling.

Concanavalin A (Con A)-induced hepatitis is a T cell-mediated fulminant hepatitis model in mice (Tiegs et al., 1992; Lohr et al., 1994). Many factors are involved in Con A-induced hepatitis. Since IFN-γ-deficient mice are resistant to Con A-induced hepatitis, and pretreatment with neutralizing anti-IL-4 protects them against this form of hepatitis, both IFN-γ and IL-4 seem to be involved in this liver injury (Kaneko et al., 2000; Tagawa et al., 1997; Toyabe et al., 1997; Chen and Paul, 1997). Furthermore, Vα14- deficient mice lacking NKT cells are resistant to Con A hepatitis (Kaneko et al., 2000), which suggests an essential role for NKT cells in this disease. The liver contains a much higher proportion of NKT cells than do other tissues. Moreover, NKT cells possess receptors for both IL-4 and IFN-γ, so that they can receive simultaneous signaling from both IFN-γ and IL-4. It is also known that the cytotoxicity of NKT cells is enhanced by IL-12 (Kawamura et al., 1998) and IL-4 via upregulation of their expression of FasL and Granzyme B (Kaneko, et al., 2000). As shown in our study, this is also

the case for fulminant hepatitis in SOCS-1 KO mice. SOCS-1-deficient hepatic lymphocytes were intrinsically cytotoxic against syngeneic hepatocytes (Figure 5) and lost their hepatocytotoxicity after deletion of NKT cells (Figure 5). Furthermore, in vivo deletion of NKT cells rescued pre-onset SOCS-1 KO mice from IFN-γ plus IL-4-induced liver injuries (Figure 7C), while control IgG-administered pre-onset SOCS-1 KO mice suffered from liver injuries similar to those observed in onset SOCS-1 KO mice after administration of IFN-γ plus IL-4 (Figure 7C).

We observed spontaneous development of fulminant hepatitis in SOCS-1 KO mice. At present, we do not know whether this change is induced intrinsically or extrinsically, but we succeeded in shortening the period required for development of the lethal pathological changes by administration of anti-CD3 (data not shown) or α-GalCer (Figure 6). Thus, we can assume that SOCS-1 KO mice may be hypersensitive to potentially contaminating pathogens even under SPF conditions.

The study presented here demonstrated that IFN-γ-induced SOCS-1 is essential for the inhibition of IL-4 signaling in vivo and that the disturbance of this cross-talk inhibition abnormally activates NKT cells, particularly their hepatocytotoxic activity against self-driven cells, consequently leading to fulminant hepatitis. However, these results cannot exclude the possibility that other cells besides NKT cells are involved. SOCS-1/CD1 double KO mice will be expected to provide direct evidence of the essential role of NKT cells in liver injuries of SOCS-1 KO mice. Our present study allows us to investigate the possible involvement of SOCS-1 dysfunction in autoimmunity and infection-induced intractable organ failures. Further analysis of SOCS-1 might lead to new therapies even for cancer by focusing on strengthening the cytotoxic action of NKT cells. We are now clarifying how SOCS-1 takes part in cross-talk inhibition for other cytokines and what roles dysregulated SOCS-1 plays in other tissue damage.

Experimental Procedures

Mice
STAT1 (Meraz et al., 1996), STAT6 (Takeda et al., 1996), and SOCS-1 KO mice (Naka et al., 1998) were established as previously described. All SOCS-1 KO mice were on the C57BL/6 background (backcrossed more than seven generations).

Cell Preparation and Cell Counts
Single-cell suspensions were obtained from thymi and spleens after the cells had been passed through mesh filters. Spleen cells were also treated with Ack buffer (0.15 M NH$_4$Cl, 1 mM KHCO$_3$, and 0.1 mM Na$_2$EDTA) to lyse RBC. The total number of cells was determined by microscopic observation of trypan blue-stained cells and using hemocytometers

Flow Cytometric Analysis
Cells were stained with the following monoclonal Abs (mAbs): FITC-, PE-, or APC-conjugated anti-CD3ϵ, anti-CD4, anti-CD8, anti-CD69, and anti-B220 (PharMingen, San Diego CA). Stained cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). Live lymphocytes were gated according to their forward and side scatter profiles.

Western Blot Analysis
Splenic T cells, which were sorted by using anti-B220, anti-Gr1, and anti-Mac1 Ab mAbs (Miltenyi Biotec, Bergisch Gladbach, Germany),

were treated with 10 μg/ml of IFN-γ, 10 μg/ml of IL-4, or 10 μg/ml of IFN-γ followed by 10 μg/ml of IL-4 for the times indicated. The cells were lysed with an NP-40 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 10 mM Na$_2$VO$_4$, 0.5 mM dithiothreitol, 1 μg/ml Pepstatin A, 1 μg/ml Leupeptin, and 1 mM PMSF) and clarified by centrifugation. The supernatants collected were subjected to an 8% SDS-PAGE. The proteins were transferred onto a nitrocellulose filter (Schleier & Schuell, Dassel, Germany). After blocking with 5% skim milk, the filter was incubated with anti-phospho-specific STAT1 or phospho-specific STAT6 Abs (New England Biolab, MA, USA), and then with the horseradish peroxidase-conjugated anti-rabbit IgG Ab. The signals were detected with an ECL system (Amersham, Bukinghamshire, England). After the Abs had been stripped, the filter was reprobed with anti-STAT1 (Transduction Labolatry, KY, USA) or anti-STAT6 Abs (Santa Cruz, CA, USA).

Assay for Hepatocytotoxicity
Hepatocytotoxicity was determined by the method described elsewhere with some modification (Tsutsui et al., 1992). In brief, liver parenchymal cells (1 × 10^5/ml) prepared from WT C57BL/6 mice were incubated in a 96-well-collagen type I-coated plate overnight, washed twice with the culture medium kept at 37°C, and labeled with [^{51}Cr]-sodium chromate for 1 hr. Hepatic lymphocytes from mice with various mutants were incubated with 20 μg/ml of anti-murine Fas L or 100 nM concanamycin A (Wako, Osaka, Japan) for 1 hr and incubated with the labeled liver parenchymal cells at various effector-target cell ratios for 4 hr. In some experiments, we depleted NK1.1$^+$ cells from hepatic lymphocytes by MACS after incubation with biotinylated anti-NK1.1 (PharMingen) followed by additional incubation with streptavidin-microbeads (Miltenyi Biotec). We also depleted the NK cell population by incubating the cells twice with 100 μg/ml of anti-asialo GM1 (Wako) plus complement for 30 min, followed by determination of their hepatocytotoxicity. Hepatocytotoxicity was calculated as previously reported (Tsutsui et al., 1992). Spontaneous release of hepatocytes was less than 8% of their maximum release.

Depletion of NKT Cells and In Vivo Stimulation of IFN-γ Plus IL-4
Mice at 3 days after birth were injected intraperitoneally with 0.1 ml PBS containing 2.5 mg anti-NK1.1 mAb (PK136). Two days after treatment with NK1.1 mAb, these mice were injected intraperitoneally with 0.1 ml PBS containing both 3 μg IFN-γ and 1 μg IL-4.

Administration of α-GalCer
Mice (3 days after birth) were injected intraperitoneally with 0.1 ml PBS containing either 1 μg α-GalCer or control vehicle, which were provided by KIRIN (Tokyo, Japan).

Adoptive Transfer
Recipient RAG2 KO mice, kindly provided by Dr. Itoh at CIEA (Kanagawa, Japan), were irradiated with 700 rad. Donor cells from embryonic day 16.5 (E16.5) fetal liver of SOCS-1 KO or WT mice were suspended in DMEM supplemented with 2% fetal calf serum (2 × 10^7/ml). Recipient animals were injected i.v. with 2 × 10^6 donor cells.

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