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主論文

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.

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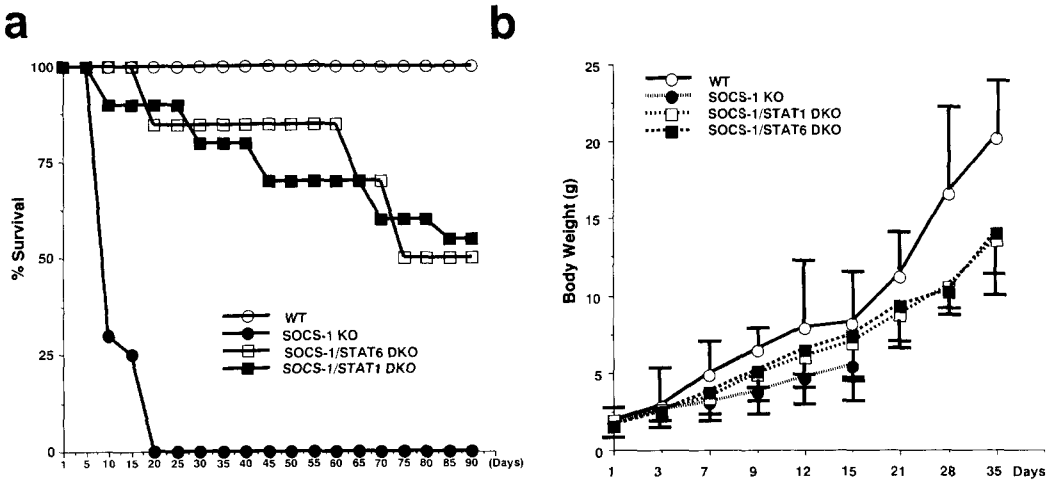

c

		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

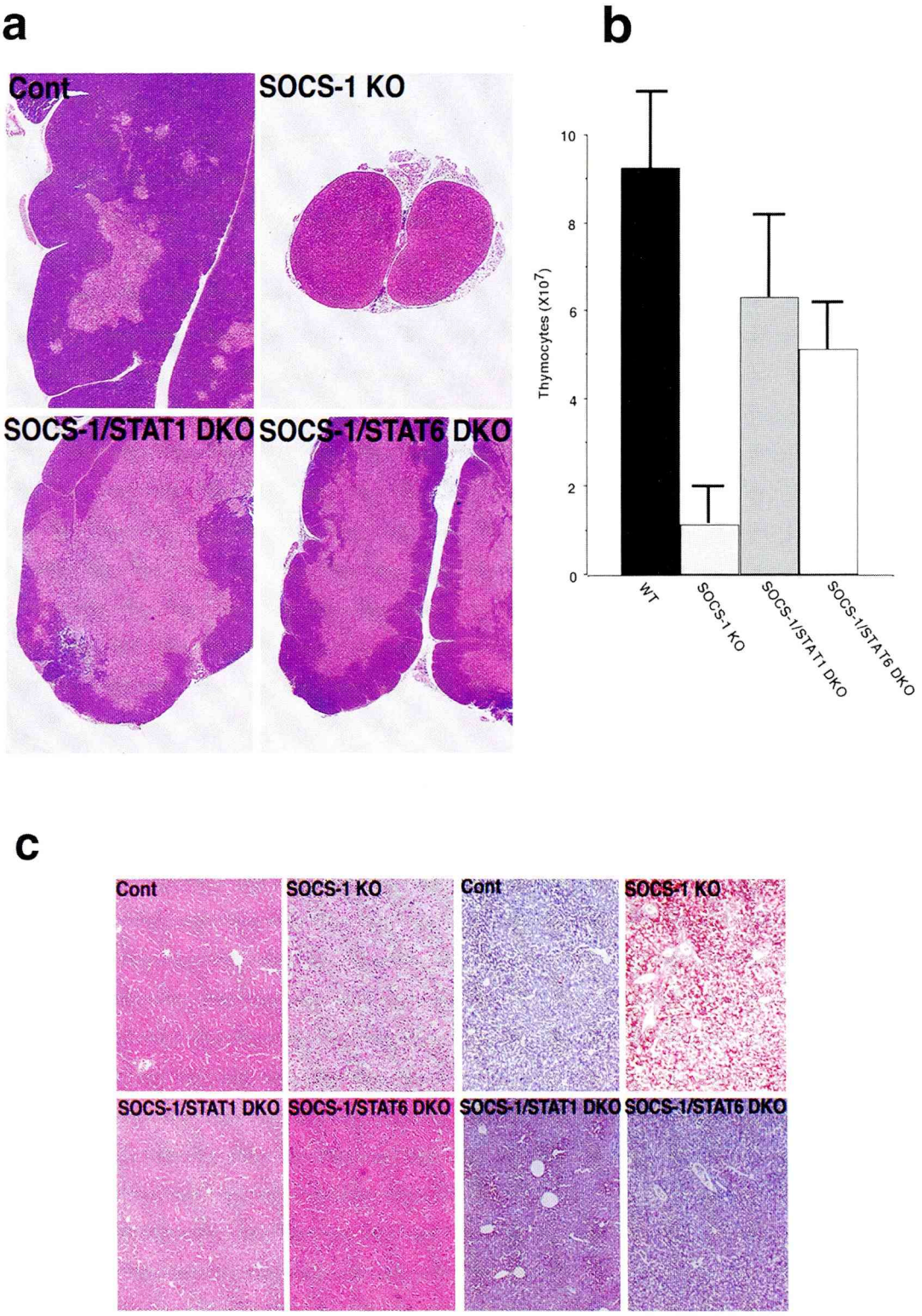

Figure 2. Improvement of Tissue Anomalies in SOCS-1 KO Mice by Elimination of STAT1 or STAT6

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.

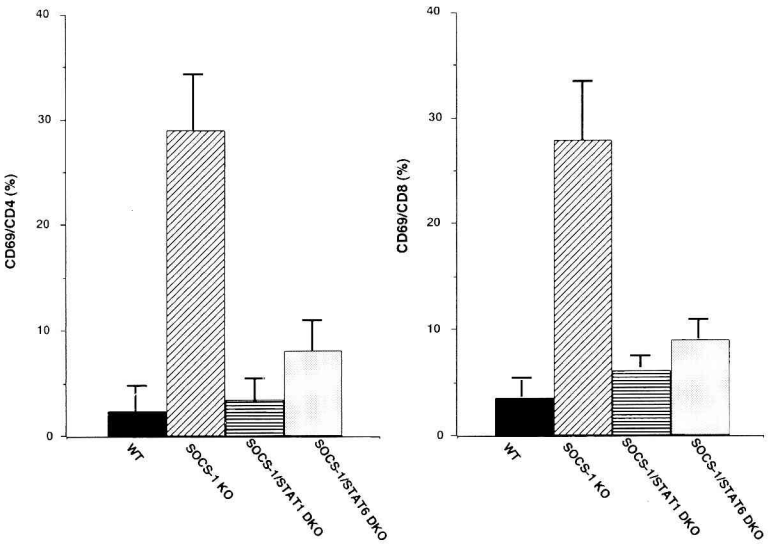

Figure 3. Disruption of STAT1 or STAT6 Restores Spontaneously Overactivated T Cells in SOCS-1 KO Mice

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

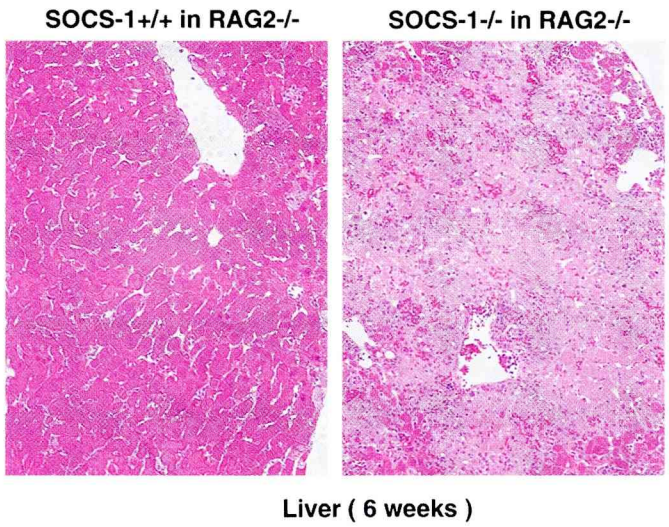

Figure 4. RAG2 KO Mice Reconstituted with SOCS-1-Deficient Lymphocytes Spontaneously Develop Fulminant Hepatitis

(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 ± 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: × 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.

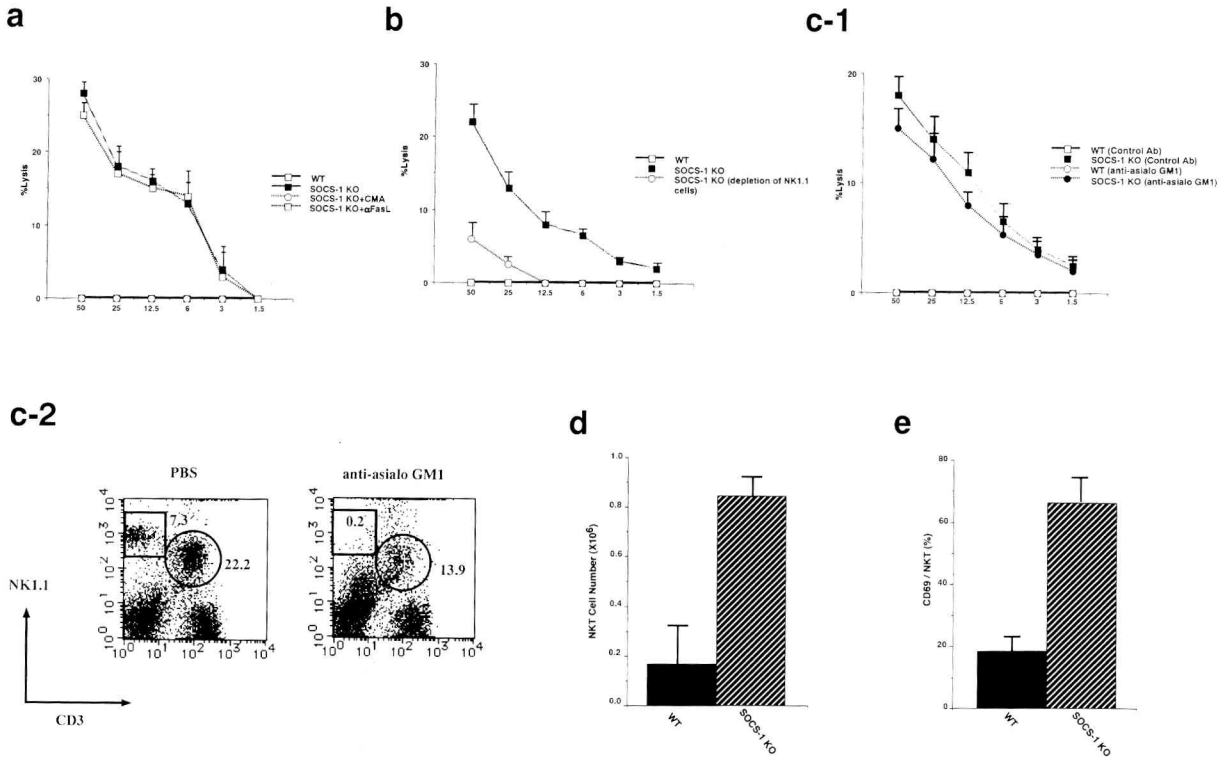

Figure 5. Hepatocytotoxicity of Hepatic NKT Cells in SOCS-1 KO Mice

(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

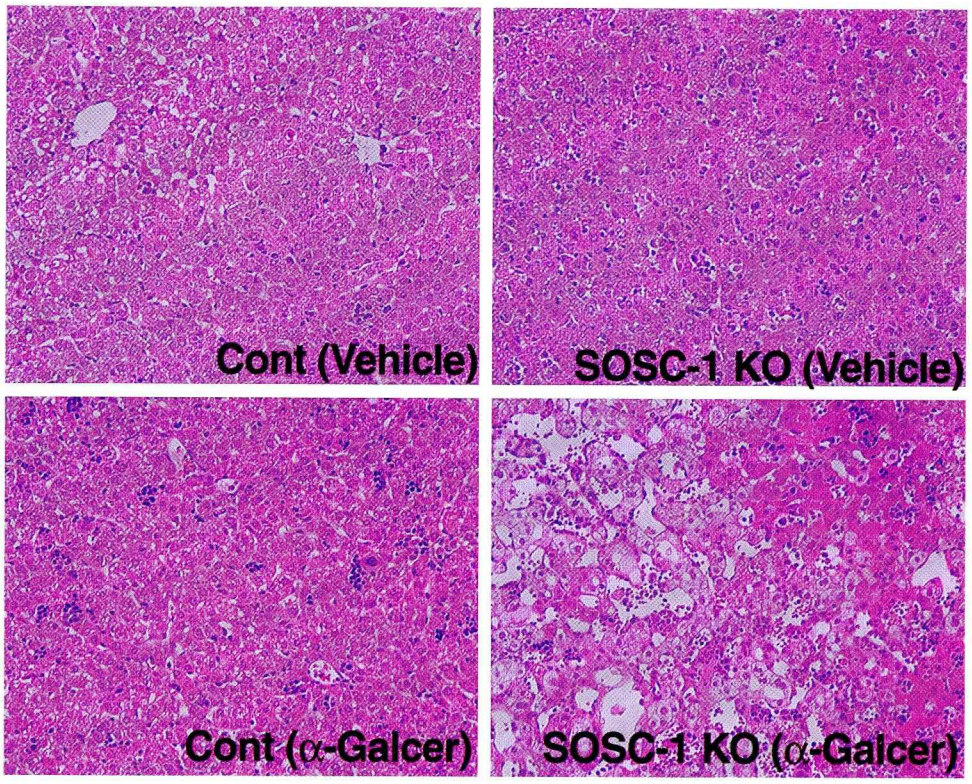

Figure 6. α-GalCer Was Administered to Pre-Onset SSI-1 KO Mice or WT Mice

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,

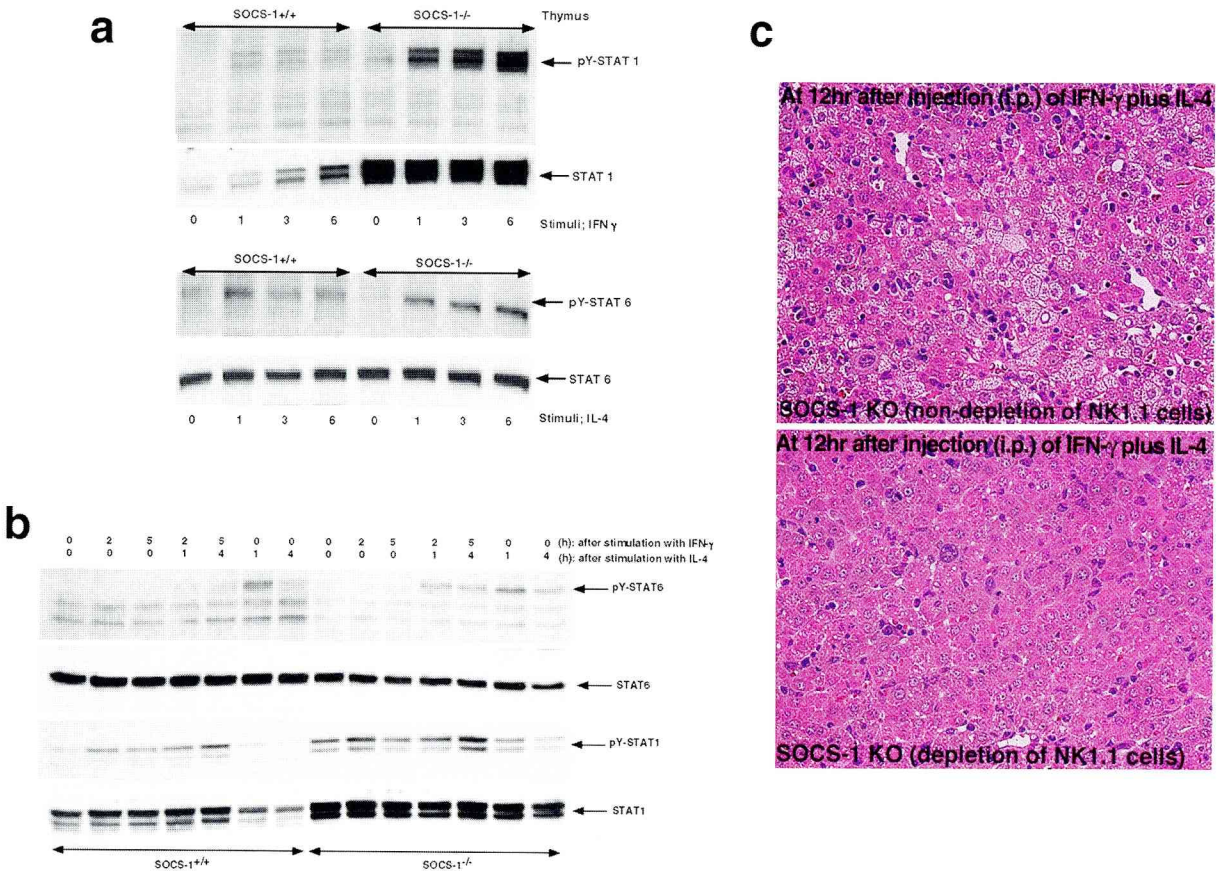

Figure 7. Involvement of Dysregulated Cross-Talk Inhibition of IFN-γ and IL-4 Signaling in SOCS-1-Deficient NKT Cells in Severe Hepatitis

(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_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA) 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_2VO_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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Structure and function of a new STAT-induced STAT inhibitor

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Structure and function of a new STAT-induced STAT inhibitor

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The signalling pathway that comprises JAK kinases and STAT proteins (for signal transducer and activator of transcription) is important for relaying signals from various cytokines outside the cell to the inside[1–3]. The feedback mechanism responsible for switching off the cytokine signal has not been elucidated. We now report the cloning and characterization of an inhibitor of STAT activation which we name SSI-1 (for STAT-induced STAT inhibitor-1). We found that SSI-1 messenger RNA was induced by the cytokines interleukins 4 and 6 (IL-4, IL-6), leukaemia-inhibitory factor (LIF), and granulocyte colony-stimulating factor (G-CSF). Stimulation by IL-6 or LIF of murine myeloid leukaemia cells (M1 cells) induced SSI-1 mRNA expression which was blocked by transfection of a dominant-negative mutant of Stat3, indicating that the SSI-1 gene is a target of Stat3 (refs 4–7). Forced overexpression of SSI-1 complementary DNA interfered with IL-6- and LIF-mediated apoptosis and macrophage differentiation of M1 cells, as well as IL-6 induced tyrosine-phosphorylation of a receptor glycoprotein component, gp130, and of Stat3. When SSI-1 is overexpressed in COS7 cells, it can associate with the kinases Jak2 and Tyk2. These findings indicate that SSI-1 is responsible for negative-feedback regulation of the JAK–STAT pathway induced by cytokine stimulation.

Most cytokine receptors consist of two or three polypeptide chains, namely a ligand-specific receptor chain and a signal transducer that is commonly used by various cytokines[8–11]. The nature of this receptor system explains the functional redundancy of cytokines. Homo- or heterodimerization of receptor components with ligands or ligand–receptor complexes stimulates a unique cytokine signalling cascade, the JAK–STAT pathway[1–3,12], which causes the activation of target genes in the cell nucleus; little is known about the direct targets of the STAT family. One feature of cytokines is the transient expression of their activity, which suggests that negative-feedback regulation operates in cytokine-signal transduction: possible candidates for exerting this control are the SH2-domain-containing phosphotyrosine phosphatase SHP-1, which associates with the tyrosine-phosphorylated IL-3 receptor β-chain and with the erythropoietin receptor (EPO-R)[13–15], and a cytokine-inducible SH2-containing protein (CIS) which binds to the Stat5-binding sites of EPO-R (refs 16, 17, and A. Yoshimura *et al.*, personal communication). We have now cloned a cDNA encoding an SH2-domain-containing protein that is inducible by Stat3 and inhibits Stat3 function.

To clone other members of the STAT family, we prepared a monoclonal antibody against a sequence motif (GTFLLRFS) found in the SH2 domain of Stat3 (refs 4, 5). Using this antibody, we screened a murine thymus cDNA library and isolated 20 unknown genes, two of which contained an SH2 domain. One was later identified as CIS[16] and the other was a new gene (named SSI-1). SSI-1 cDNA has a single open reading frame that encodes a 212-amino-acid polypeptide and contains an SH2 domain in the middle of its sequence (at codons 79–167). No other consensus motifs like SH3 domains were found in SSI-1 (Fig. 1a). The SH2 domain of SSI-1 was homologous to that of CIS 36% but showed no significant homology with those of Stat3 or Stat6 except at the phosphotyrosine recognition site[4,5,18,19] (Fig. 1b).

SSI-1 expression was examined in various murine tissues. SSI-1 mRNA was ubiquitously expressed, with expression being strong in lung, spleen and testis, and weak in all other tissues (Fig. 2a). We examined SSI-1 induction in several factor-dependent cell lines (Fig. 2b). Hybridoma MH60 and myeloid leukaemia M1 cells both expressed SSI-1 mRNA, with expression peaking 60–120 min after treatment with IL-6 plus soluble IL-6 receptor (sIL-6R). IL-4-dependent CT4S cells[20] and G-CSF-dependent NFS60 cells expressed SSI-1 mRNA in response to IL-4 and G-CSF, respectively. These results show that SSI-1 is induced not only by IL-6 and G-CSF, both of which activate Stat3 (refs 4, 21), but also by IL-4, a cytokine

a

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ggcccctcgagtaggatggtagcacgcaaccaggtggcagccgacaatgcgatctccc
                M  V  A  R  N  Q  V  A  A  D  N  A  I  S  P

cggcagcagagccccgacggcggtcagagccctcctcgtcctcgtcttcgtcctcgccag
 A  A  E  P  R  R  R  S  E  P  S  S  S  S  S  S  S  S  P  A

cggcccccgtgcgtccccggccctgcccgggggtcccagccccagcccctggcgacactc
 A  P  V  R  P  R  P  C  P  G  V  P  A  P  A  P  G  D  T  H

acttccgcaccttccgctcccactccgattaccggcgcatcacgcggaccagcgcgctcc
 F  R  T  F  R  S  H  S  D  Y  R  R  I  T  R  T  S  A  L  L

tggacgcctgcggcttctattggggacccctgagcgtgcacggggcgcacgagcggctgc
 D  A  C  G  F  Y  W  G  P  L  S  V  H  G  A  H  E  R  L  R

gtgccgagcccgtgggcaccttcttggtgcgcgacagtcgccaacggaactgcttcttcg
 A  E  P  V  G  T  F  L  V  R  D  S  R  Q  R  N  C  F  F  A

cgctcagcgtgaagatggcttcgggccccacgagcatccgcgtgcacttccaggccggcc
 L  S  V  K  M  A  S  G  P  T  S  I  R  V  H  F  Q  A  G  R

gcttccacttggacggcaaccgcgagaccttcgactgccttttcgagctgctggagcact
 F  H  L  D  G  N  R  E  T  F  D  C  L  F  E  L  L  E  H  Y

acgtggcggcgccgcgccgcatgttgggggccccgctgcgccagcgccgcgtgcggccgc
 V  A  A  P  R  R  M  L  G  A  P  L  R  Q  R  R  V  R  P  L

tgcaggagctgtgtcgccagcgcatcgtggccgccgtgggtcgcgagaacctggcgcgca
 Q  E  L  C  R  Q  R  I  V  A  A  V  G  R  E  N  L  A  R  I

tccctcttaacccggtactccgtgactacctgagttccttcccccttccagatctgaccgg
 P  L  N  P  V  L  R  D  Y  L  S  S  F  P  F  Q  I  *
ctgccgctgtgccgcagcattaagtgggggcgccttattatttcttattattaattatta

ttatttttctggaaccacgtgggagccctccccgcctgggtcggagggagtggttgtgga

gggtgagatgcctcccacttctggctggagacctcatcccacctctcaggggtgggggtg

ctcccctcctggtgctccctccgggtcccccctggttgtagcagcttgtgtctggggcca

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caggggtcggggagggtctctggcttcatttttctgctgtgcagaatatcctattttata

tttttacagccagtttaggtaataaactttattatgaaagtttttttttaaaagaaacaa

acaaagatt
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CIS       WYWGSITASEARQHLQKMPEGTFLVRDSTHPSYLFTLSVKTTRGPTNVRIEYADSSFRL
SSI-1     GFYWGPLSVHGAHERLRAEPVGTFLVRDSRQRNCFFALSVKMASGPTSIRVHFQAGRFHL
STAT3     G   G  S        L   P GTFLLRFS
STAT6         G  S        L   P GTFLLRFS                    S

CIS       DSNCLSRPRILAFPDVVSLVQHYVASCAADTRSDSPD
SSI-1     DGN-----RE-TFDCLFELLEHYVAAPRRMLGAPL
STAT3                                A
STAT6     D                  L
```

Figure 1 a, Nucleotide and deduced amino-acid sequences of SSI-1 cDNA. The asterisk indicates the stop codon. The amino-acid sequence of the SH2 domain is shown in bold. **b**, Sequence alignment of the SH2 domain of SSI-1 with those of CIS, Stat3 and Stat6. Bold letters show amino-acid residues that are identical in SSI-1, CIS, Stat3 and Stat6.

that activates Stat6 (ref. 19). Consistent with these findings, the promoter region of the SSI-1 gene was found to contain Stat3 and Stat6 binding sequences[4,19,22] (data not shown).

We next examined SSI-1 induction in M1 cells transfected with wild-type Stat3 (M1/Stat3) and a Stat3 mutant (M1/Y705F) (Fig. 2c). Stat3(Y705F) is a dominant-negative form of Stat3, in which a tyrosine at residue 705 that is phosphorylated by a JAK kinase has been substituted by phenylalanine[6,7]. In this experiment, transfectants containing only the neomycin-resistance gene (M1/Neo) were used as controls. SSI-1 mRNA was more strongly induced by IL-6 plus sIL-6R or LIF in M1/Stat3 cells than in M1/Neo control cells, but was not induced in M1/Y705F cells. These results indicate that the SSI-1 gene is one of the target genes of Stat3 and is induced by the JAK–STAT pathway.

To test the effect of SSI-1 on the gp130-mediated signalling pathway, we established M1 transfectants that constitutively express wild-type SSI-1 (M1/SSI-1) or a mutant SSI-1 (M1/TR) that is truncated at the C-terminal region which includes the SH2 domain. As shown in Fig. 3a,b, M1/Neo and M1/TR underwent growth arrest and cell death after treatment with IL-6 plus sIL-6R or LIF, as did the parental M1 cells (M1). The dead cells showed features of apoptosis such as chromatin condensation and apoptotic bodies (Fig. 3c). In contrast, growth of M1/SSI-1 cells did not arrest following stimulation (Fig. 3a–c). Expression of the receptor for the immunoglobulin fragment Fcγ (FcγR) on days 0, 1, 2 and 3 was examined with flow cytometry in M1/Neo, M1/SSI-1 and M1/TR cells treated with IL-6 plus sIL-6R. Despite a partial inhibition of Stat3 phosphorylation (Fig. 4a), SSI-1 almost completely blocked IL-6-induced FcγR expression, which is a direct target gene of Stat3 in M1cells (Fig. 3d)[6]. These results suggest that SSI-1 inhibits the Stat3-mediated signalling pathway.

Stimulation of cytokines of the IL-6 family induces tyrosine-phosphorylation of the signal-transducing receptor component gp130 to activate Stat3 by JAK kinases[23–25]. To determine how SSI-1 exerts its inhibitory effect on the gp130-mediated signalling pathway, we measured the tyrosine-phosphorylation of gp130 and Stat3 in M1 cells after stimulation with IL-6 plus sIL-6R. As shown in Fig. 4a, tyrosine-phosphorylation of these molecules was much reduced in M1/SSI-1 cells compared with that in control M1/Neo and M1/TR cells. Next we tested whether SSI-1 interacts directly with JAK kinases. The cDNAs encoding SSI-1 and Jak2 or Tyk2 were co-transfected into COS7 cells; as shown in Fig. 4b, SSI-1 co-precipitated with Jak2 or Tyk2. These results indicate that the activity of JAK kinases is suppressed by SSI-1. To see whether the inhibitory effect of SSI-1 is specific to JAK kinases, we immunoblotted M1/Neo, M1/SSI-1 and M1/TR cell lysates before and after IL-6 plus sIL-6R stimulation with anti-phosphotyrosine. In the case of M1/SSI-1, the intensity of the bands corresponding to gp130 and Stat3 was significantly reduced compared with the same bands from control M1/Neo and M1/TR cells. The intensity of other bands was comparable among M1/Neo, M1/SSI-1 and M1/TR cell lysates. In addition, there was no difference in the phosphorylation of Flt-3 and the insulin-receptor-β among these cells after stimulation with the corresponding ligands. These results indicate that SSI-1 specifically inhibits the JAK–STAT signalling pathway (Fig. 4c).

Cytokine binding to the receptor induces homo- or hetero-dimerization of the receptor components and activates JAK kinases that are constitutively associated with the intracellular domains of the receptor components; once activated, these kinases tyrosine-phosphorylate receptor components. STATs then associate with phosphotyrosine residues on the receptor components through their SH2 domains and in turn are tyrosine-phosphorylated by JAK kinases[1–3,18,25]. Here we have cloned the cDNA encoding SSI-1, an SH2-domain-containing protein that is inducible by Stat3 and inhibits Stat3. It has been reported that CIS participates in negative-feedback regulation of the JAK–STAT signalling pathway[16,17]. CIS inhibits Stat5 function by directly associating with the Stat5-binding region of the EPO receptor, but the mechanism of SSI-mediated inhibition of the JAK–STAT pathway is quite different. SSI-1 reduces tyrosine-phosphorylation of Stat3 as well as of gp130; as tyrosine-phosphorylation of gp130 is a prerequisite for its association with SSI-1 at the Stat3-binding region, SSI-1 is unlikely to compete with Stat3 for binding to gp130. SSI-1 may block an earlier step in the JAK–STAT signalling pathway than CIS, probably by inhibiting JAK kinases. We have shown that SSI-1 associated with Jak2 and Tyk2, and this molecule has recently been cloned as a JAK-binding protein in a yeast two-hybrid system and also shown to bind Jak1 and Jak3 (A. Yoshimura *et al.*, personal communication). Another point is that SSI-1 is not induced by all STATs, ruling out a role as a general inhibitor of the JAK–STAT pathway.

We have shown that SSI-1 is induced in response to cytokines that act through gp130, and to IL-4. In our experiments, NFS60 and TF-1 cells did not express SSI-1 in response to IL-3 or EPO stimulation, respectively (data not shown). The physiological significance of the cytokine-specific induction of SSI-1 needs to be further investigated.

Note added in proof: Elsewhere in this issue, JAK inhibitors similar to SSI-1 are reported under the names SOCS[26] and JAB[27]. □

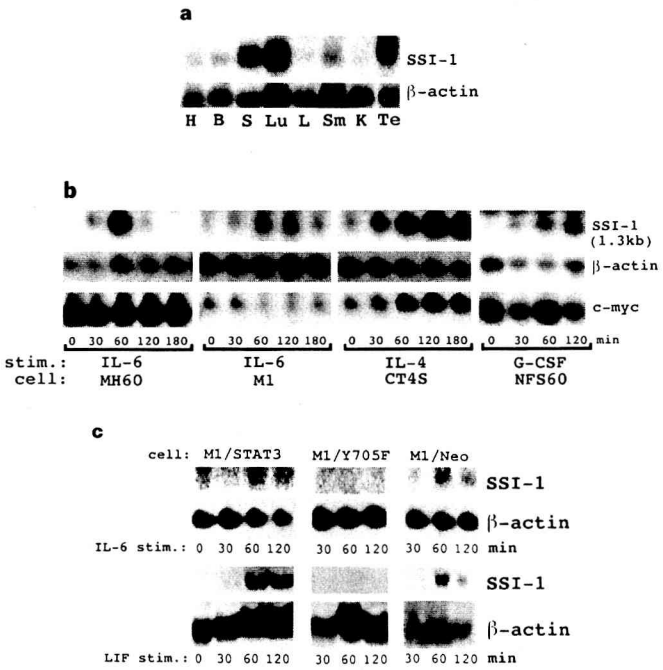


Figure 2 a, Expression of SSI-1 mRNA in murine tissues. H, heart; B, brain; S, spleen; Lu, lung; L, liver; Sm, skeletal muscle; K, kidney; Te, testis. **b**, Induction of SSI-1 mRNA in factor-dependent cells. MH60 and M1 cells were stimulated with IL-6 plus sIL-6R. CT4S and NFS60 cells were stimulated with IL-4 and G-CSF, respectively. *c-myc* mRNA is shown as a control for stimulation. **c**, Induction of SSI-1 mRNA in M1 cells transfected with the indicated Stat3 constructs. M1/Stat3: M1 cells with wild-type Stat3. M1/Y705F: M1 cells with a dominant-negative form of Stat3. M1/Neo: M1 cells with a neomycin-resistance gene alone. In **a–c**, β-Actin mRNA is included as a loading control.

Methods

Preparation of the monoclonal antibody. A hybridoma clone producing the monoclonal antibody (mAb) FL-238, which is reactive against the synthetic oligopeptide (Fmoc) GTFLLRFS (this sequence is highly conserved in the STAT family), was established by fusing spleen cells from BALB/c mice immunized with the synthetic oligopeptide TKPPGTFLLRFSESSKEG (amino acids 600 to 617 in the SH2 domain of Stat3) coupled to keyhole limpet haemocyanin with mouse myeloma cells P3-X63-Ag8-653. The mAb was purified by protein A affinity chromatography from the ascitic fluid of BALB/c mice.

Isolation of SSI-1 cDNA. Using the monoclonal antibody against the GTFLLRFS motif, SSI-1 cDNA was isolated from a murine thymus cDNA library (lambda ZAP, Stratagene) by using a PicoBlue Immunoscreening kit.

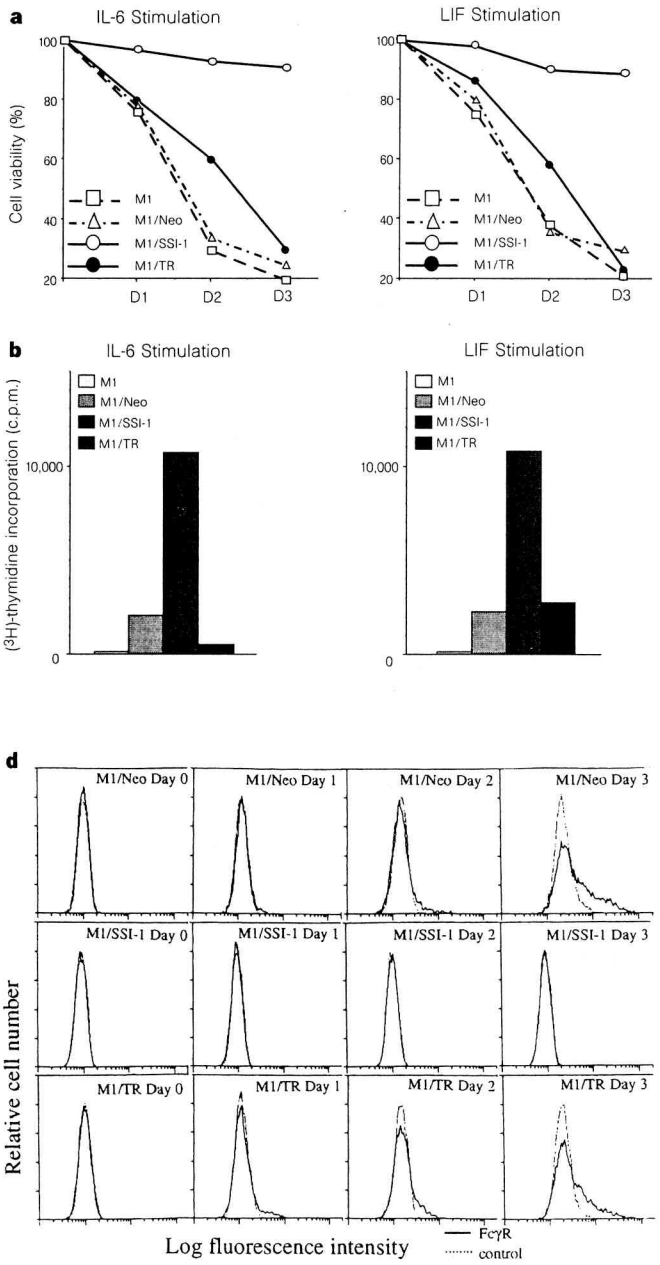
Cell culture. Myeloid leukaemia M1 cells were cultured in Eagle's minimal essential medium supplemented with double the normal concentrations of amino acids and vitamins and 10% (vol/vol) fetal calf serum (FCS). The IL-6-dependent myeloma MH60 cells were maintained in RPMI 1640 medium supplemented with IL-6 (5 ng ml^{-1}) and 10% FCS. IL-2/IL-4-dependent CT4S cells[20] were cultured in RPMI 1640 medium supplemented with IL-4 (10 U ml^{-1}) and 10% FCS. IL-3-dependent myeloid NFS60 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 10% conditioned medium from the WEHI-3B cell line as a source of IL-3. We had previously constructed dominant-negative forms of Stat3 and established MI cell lines that constitutively express them[6]. Transfectant M1 cells were cultured with 250 μg ml^{-1} Geneticin (Gibco).

Northern hybridization. Cells (except M1 cells) were factor-depleted for 4 h in RPM1 medium containing 1% BSA, and then all cells were stimulated with cytokines at the following concentrations for various periods: IL-2, 10 ng ml^{-1}; IL-3, 5 ng ml^{-1}; IL-4, 10 U ml^{-1}; IL-6, 100 ng ml^{-1}; sIL-6R, 50 ng ml^{-1}; G-CSF, 20 ng ml^{-1}; LIF, 1,000 U ml^{-1}. Cytoplasmic RNA was extracted using Iso-Gen (Nippon Gene). Total RNA (5 μg ml^{-1}) was electrophoresed on agarose gels and transferred to a nylon membrane (Hybond N^+, Amersham). The membrane was hybridized with radiolabelled cDNA probes.

Flow cytometry analysis. Transfectants (1×10^5) were cultured with IL-6 (300 ng ml^{-1}) plus sIL-6R (500 ng ml^{-1}) for 3 days. After collection, cells were incubated with 1 μg mouse IgG2a (Capel) for 20 min on ice. After washing with PBS, cells were incubated with fluroescein-isothiocyanate-conjugated anti-mouse IgG antibody (Zymed) for 20 min on ice. Cells were analysed in a fluorescence-activated cell sorter (Becton Dickinson FACS).

Plasmid construction and DNA transfection. Constructs were cloned into pEF-BOS, a mammalian expression vector. Briefly, SSI-1 cDNA was digested with the restriction enzymes *Xba*I and *Pvu*II and then inserted into the *Xba*I site of the pEF-BOS vector (pEF-BOS/SSI-1 (SSI-1)). For the construction of pEF-BOS/SSI-1 (TR), 360 bp of a *Bss*HII-digested fragment was deleted. cDNAs encoding Jak2 and Tyk2 were inserted into the *Xba*I site of the pEF-BOS vector.

M1 cells were transfected by electroporation with the SSI-1(SSI-1/TR) expression vector and pSV2 Neo at a 20 : 1 ratio, and neomycin-resistant clones



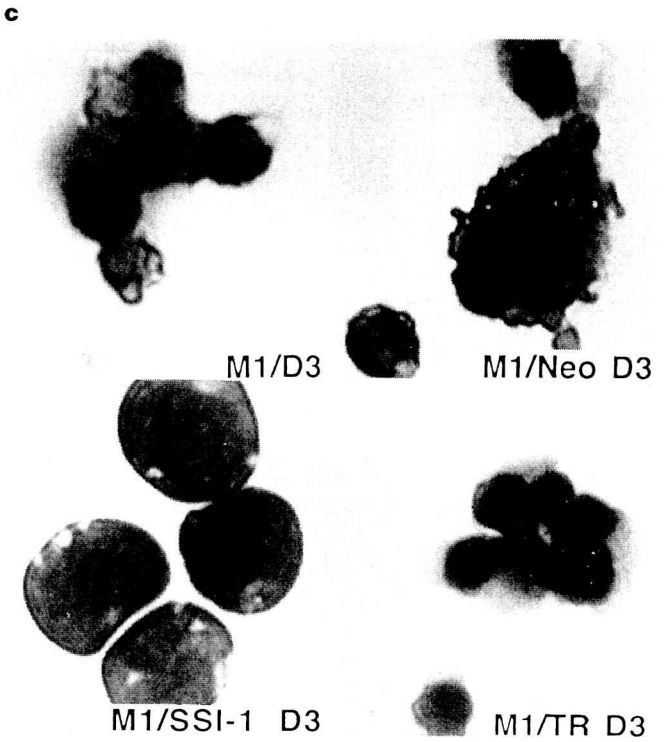
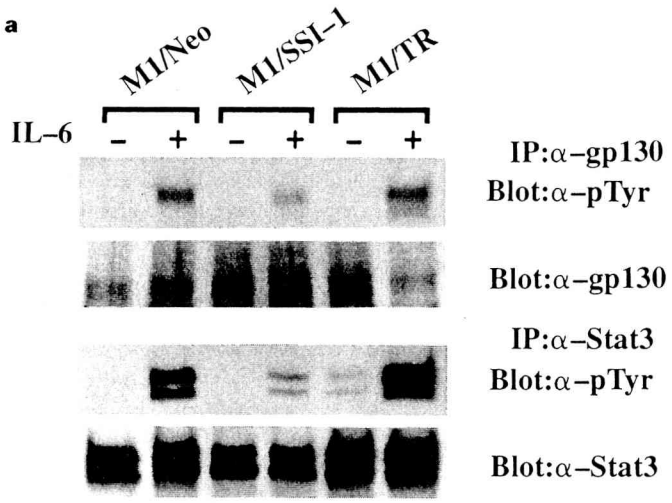
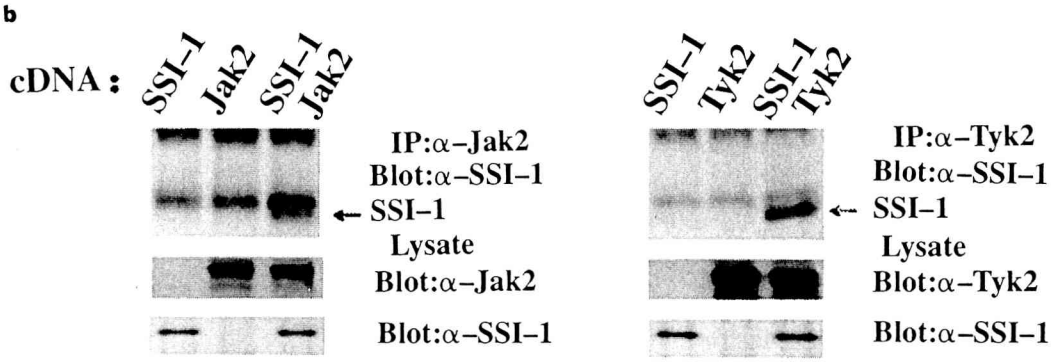


Figure 3 IL-6- or LIF-induced growth arrest and apoptosis of M1 clones. M1, parental M1 cells; M1/Neo, M1 cells carrying a neomycin-resistance gene; M1/SSI-1, M1 cells with wild-type SSI-1; M1/TR, M1 cells carrying mutant SSI-1. **a**, Parental and transfected M1 cells were seeded with IL-6 (300 ng ml^{-1}) plus sIL-6R (500 ng ml^{-1}) or LIF (1,000 U ml^{-1}) at day 0, and the viability of the cells was determined on days (D) 1, 2 and 3. **b**, [^{3}H]thymidine incorporation was measured on day 1 after stimulation. Each column represents the mean of three experiments. **c**, May-Grunwald-Giemsa staining of parental and transfected M1 cells on day 3. **d**, IL-6-induced expression of the Fcγ-receptor, as determined by flow cytometry.





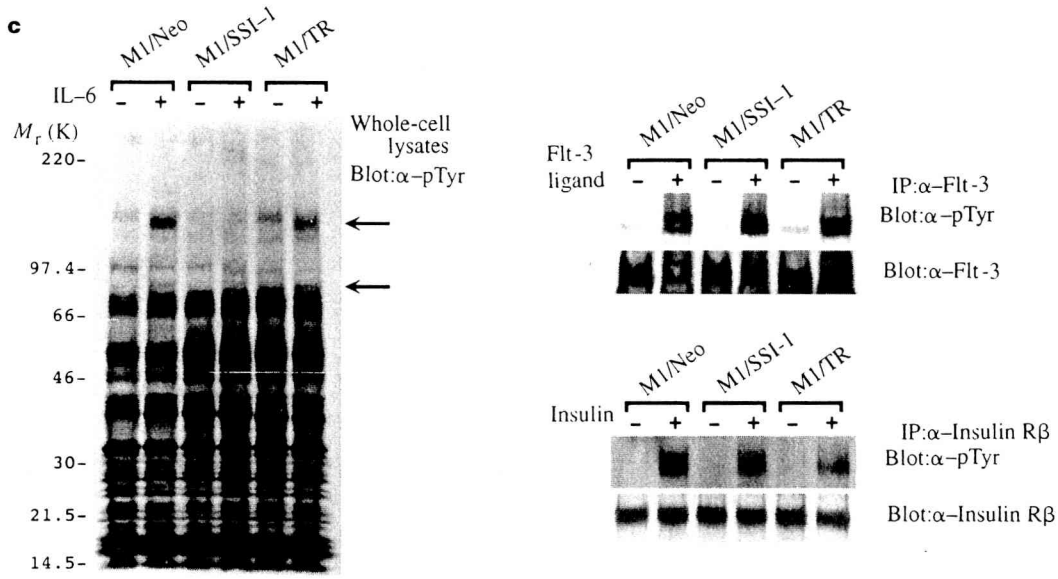


Figure 4 a, IL-6 induced tyrosine-phosphorylationof gp130 and Stat3 in M1 transfectants. Cells were either unstimulated or stimulated with IL-6 plus sIL-6R. Immunoprecipitated (IP) gp130 and Stat3 were immunoblotted with anti-phosphotyrosine antibody (α-pTyr). Blots were reprobed with the respective antibodies. **b**, Association of SSI-1 with Jak2 and Tyk2. COS7 cells were transfected with the indicated combinations of plasmids encoding SSI-1, Jak2 and Tyk2. Immunoprecipitates with anti-Jak2 or anti-Tyk2 antibody were immunoblotted with anti-SSI-1 antibody. The expression of these proteins was examined by immunoblotting the cell lysates. **c**, The effect of SSI-1 on IL-6, Flt-3 ligand and insulin-induced tyrosine-phosphorylation. Left: whole-cell lysates, before and after IL-6 stimulation, were immunoblotted with anti-phosphotyrosine antibody. Right: cells were either unstimulated or stimulated with Flt-3 ligand or insulin for 5 min. Immunoprecipitated Flt3 and insulin-receptor-β were immunoblotted with anti-phosphotyrosine antibody and reprobed with the respective antibodies.

were selected in growth medium containing Geneticin (Gibco) at 750 $\mu g\ ml^{-1}$.

Western blot analysis. Cells were treated with IL-6 (300 $ng\ ml^{-1}$) plus sIL-6R (500 $ng\ ml^{-1}$), Flt-3 ligand (300 $ng\ ml^{-1}$; Genzyme) or insulin (1 μM; Becton Dickinson) for 5 min or were untreated, and were solubilized with lysis buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_2VO_4) containing protease inhibitors. Immunoprecipitates obtained with anti-gp130 (Upstate Biotechnology), anti-Stat3 (ref. 4), anti-Flt-3 (Santa Cruz Biotechnology) or anti-insulin-receptor-β antibody (Santa Cruz Biotechnology) were resolved by SDS–PAGE under reducing conditions and transferred to nitrocellulose. Filters were probed with anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology) and reprobed with anti-gp130, anti-Stat3, anti-Flt-3 or anti-insulin-receptor-β antibody, respectively, after stripping the first blots. Blots were visualized with the ECL detection system (Amersham). COS7 cells (1×10^6) were transfected with the designated plasmids encoding SSI-1 (2 μg), Jak2 (20 μg), or Tyk2 (20 μg) by using the DEAE–dextran method. After 48 h, cells were solubilized in lysis buffer and the lysates used for western blot analysis.

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Immunology

Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice

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Contributed by Tadamitsu Kishimoto, October 19, 1998

ABSTRACT **Growth, differentiation, and programmed cell death (apoptosis) are mainly controlled by cytokines. The Janus kinase–signal transducers and activators of transcription (JAK-STAT) signal pathway is an important component of cytokine signaling. We have previously shown that STAT3 induces a molecule designated as SSI-1, which inhibits STAT3 functions. To clarify the physiological roles of SSI-1 *in vivo*, we generated, here, mice lacking SSI-1. These SSI-1−/− mice displayed growth retardation and died within 3 weeks after birth. Lymphocytes in the thymus and spleen of the SSI-1−/− mice exhibited accelerated apoptosis with aging, and their number was 20–25% of that in SSI-1+/+ mice at 10 days of age. However, the differentiation of lymphocytes lacking SSI-1 appeared to be normal. Among various pro- and anti-apoptotic molecules examined, an up-regulation of Bax was found in lymphocytes of the spleen and thymus of SSI-1−/− mice. These findings suggest that SSI-1 prevents apoptosis by inhibiting the expression of Bax.**

The homeostatic regulation of cell populations is controlled by a balance among proliferation, growth arrest, and apoptosis, and this balance is mainly controlled by cytokines and growth factors. Cytokines act by binding to receptors expressed on the surfaces of responsive cells, which are associated with one or more members of the Janus kinase (JAK) family of cytoplasmic tyrosine kinases. The JAK–signal transducers and activators of transcription (STAT) signal pathway plays an important role in cytokine signaling (1–3), and is unique in that it features a direct linkage of receptor–ligand interaction on the cell surface to gene expression in the nucleus (4–6). However, the mechanism of negative control of cytokine actions involved in limiting their signal transductions is comparatively less well characterized. In 1997, the molecules that were expressed by stimulation of cytokine such as interleukin 6 (IL-6) and inhibited cytokine signal transmission by binding to JAK were isolated [STAT-induced STAT inhibitor-1 (SSI-1), suppressor of cytokine signaling (SOCS-1), Jak-binding protein (JAB)] (7–9). Subsequently, SSI-1 was found to form a family consisting of at least eight molecules, which were structurally characterized by an SH2 domain and a C-terminal conserved region (SC-motif/SOCS-box/CH-domain) (10–12), and it was recently known that SSI-1 inhibits not only IL-6 signaling but also interferon (IFN)-γ, IL-2, IL-3, and growth hormone signaling *in vitro* (13). It is expected that further study of SSI family molecules engaged in the negative feedback mechanism of cytokines will clarify the control mechanism of cytokines, which have remained obscure.

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MATERIALS AND METHODS

Generation of SSI-1-Deficient Mice. A 129/G mouse genomic library (Stratagene) containing the SSI-1 gene was screened, subcloned into the pBluescript vector, and characterized by restriction endonuclease mapping and DNA sequencing. A targeting vector was designed to replace the SSI-1 intron with phosphoglycerate kinase neo. This targeting vector was flanked by a 5.5-kb fragment at the 5′ end and a 0.8-kb fragment at the 3′ end and contained a PGK-tk cassette at the 5′ end of the vector. It was linearized with *Ban*III and electroporated into embryonic day 14.1 embryonic stem cells. Clones resistant to G418 and gancyclovir were screened for homologous recombination by PCR with the primers shown in Fig. 1 and confirmed by means of Southern blot analysis with the probe (*Acc*I and *Kpn*I fragment) shown in Fig. 1. The homologous recombinant embryonic stem clones were injected into blastocysts of C57B1/6J mice and transferred into the uteri of pseudopregnant C57B1/6J females. The resulting chimaeric mice were backcrossed to C57B1/6J mice, and heterozygous mutants were identified by means of PCR and confirmed by using Southern blotting from tail DNA. Brother–sister matings of heterozygous mice resulted in homozygous mutants. All animals were housed under specific pathogen-free conditions.

Northern Blot Analysis. Total RNA was extracted from the lung tissues of SSI-1−/− and SSI-1+/+ mice with RNA ZOL B (Tel-Test, Friendswood, TX), and was electrophoresed, transferred to nylon membrane, and hybridized with ^{32}P-labeled mouse SSI-1 cDNA.

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Dye Conversion. Cell viability and number were assessed by MTT staining essentially as described by Mosmann (14).

Flow Cytometry. Thymocytes and splenocytes were prepared from age-matched SSI-1+/+ or SSI-1−/− mice. Red cell-depleted splenocytes were obtained after treatment with Ack buffer (0.15 M NH_4Cl/1.0 mM $KHCO_3$/0.1 mM Na_2 EDTA. After a washing with PBS, they were counted and resuspended in a staining medium. A total of 10^6 cells was stained as described in the protocols by using the following antibodies (Abs): anti-CD4, CD8, CD3, B220, IgM, Mac-1, and Gra-1 antibody (PharMingen). The stained cells were analyzed by means of double-color flow cytometry on a FACScalibur (Becton Dickinson) by using CELLQUEST software (Becton Dickinson).

Abbreviations: STAT, signal transducers and activators of transcription; SSI-1, STAT-induced STAT inhibitor-1; SOCS-1, supressor of cytokine signaling; JAB, Jak-binding protein; Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma/leukemia-2; JAK, Janus kinase; IL, interleukin; INF, interferon; TUNEL, terminal deoxynucleotidyl-transferase-mediated UTP end labeling; Ab, antibody.

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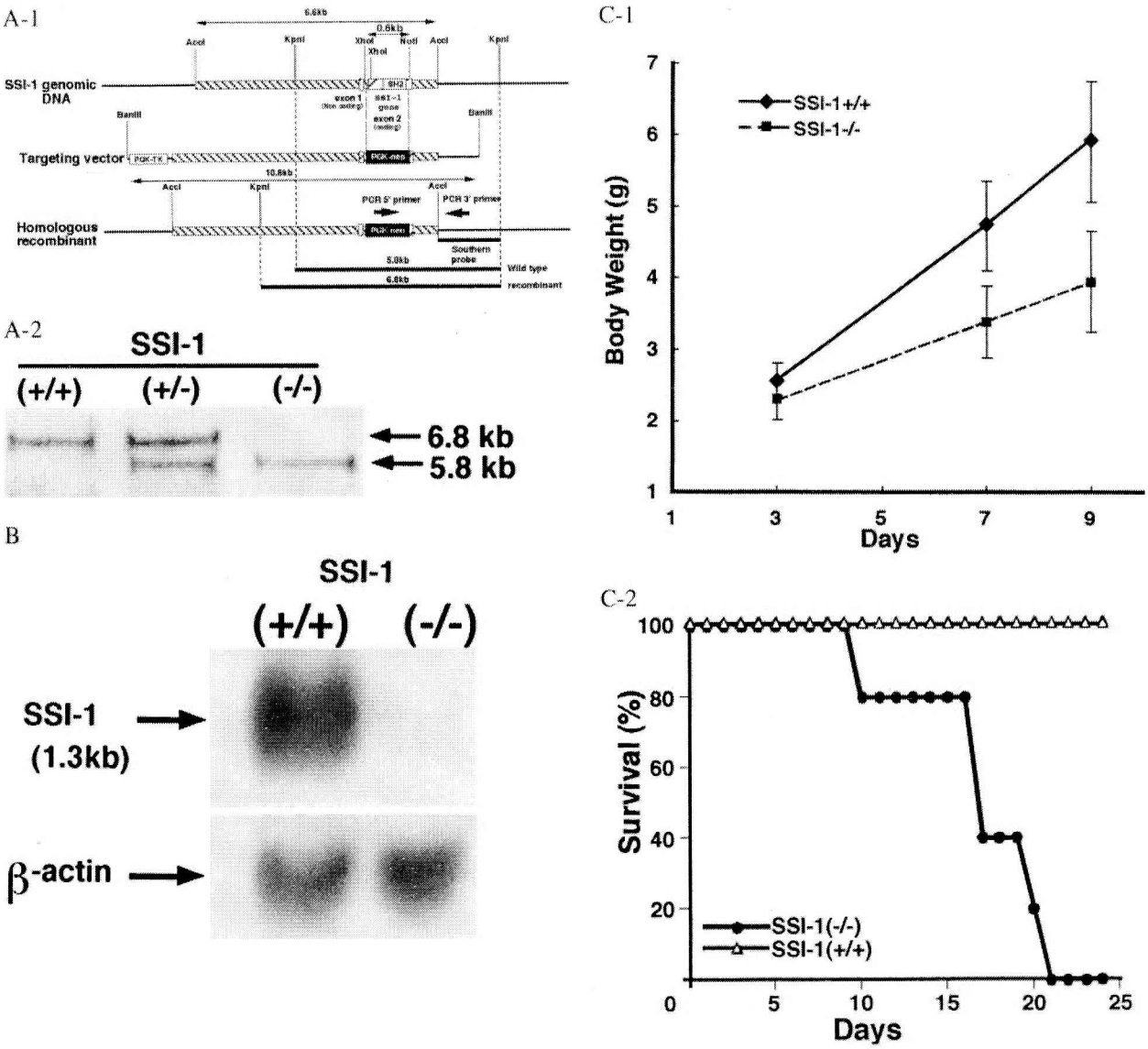

FIG. 1. (*A*) Homologous recombination of the SSI-1 locus. (*Aa*) Restriction map of the 129/G murine SSI-1 genomic clone, targeting vector, and homologous recombinant. PGK-neo replaced a 0.6-kb *Xho*I–*Not*I fragment containing the great part of exon 2. A herpes simplex virus thymidine kinase (HSV-TK) with a PGK promoter cassette was present on the 5′ flank of the vector. (*Ab*) Representative southern analysis of progeny from +/− × +/− mating. Tail biopsy genomic DNA was digested with *Kpn*I and probed with a southern probe. The wild-type allele is 5.8 kb, and the homologous recombinant exhibits the predicted 6.8-kb band. (*B*) SSI-1−/− mice showed no evidence of SSI-1 mRNA. Northern blot analysis of lung tissue lysates from SSI-1+/+ mice with the SSI-1 cDNA probe exhibited expression of 1.3-kb SSI-1 mRNA but SSI-1−/− mice lacked this product. β-actin mRNA was included as a loading control. (*Ca*) SSI-1 mice showed an ≈40% decrease in body weight by 9 days of age. The mean weight of SSI-1+/+ is shown by the solid line and that of SSI-1−/− by the broken line. Each line represents the mean of seven experiments. (*Cb*) SSI-1 mice all died between 2 and 3 weeks after birth. Percentage survivals of SSI-1+/+ are shown by △ and of SSI-1−/− by ●.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Staining. For detection of DNA fragmentation *in situ*, paraffin-embedded sections were tested by the TUNEL method as described elsewhere (15). Terminal deoxynucleotidyltransferase (TdT) was used to incorporate Biotin-16-dUTP (Boehringer Mannheim) into the ends of DNA fragments. TUNEL signals were detected with the aid of Texas Red-conjugated anti-avidin Ab (1:200, Biomeda, Foster City, CA).

Immunohistochemical Staining. Paraffin-embedded sections were immunostained by a method described elsewhere (16). Sections were treated with anti-Bcl-2 Ab (1:100, Biomol, Plymouth Meeting, PA) or anti-Bax Ab (1:100) and then incubated with fluorescein isothiocyanate-conjugated goat anti-Hamster IgG (1:300, PharMingen) or fluorescein isothiocyanate-conjugated goat anti-Rabbit IgG (1:200, Seikagaku, Kogyo, Tokyo).

***In Vitro* Thymocyte Culture.** Fresh thymocytes were plated at 2×10^5 cells per 100 μl in a 96-well plate in medium containing 5% fetal calf serum and stimulated with anti-CD3 (1 μg/ml) Ab, anti-CD3 Ab plus IL-2 (20 ng/ml), anti-CD3 Ab plus IL-4 (20 ng/ml), or not stimulated. Cell viability was assayed by means of MTT dye conversion. For each day, triplicate cultures were counted and averaged.

Western Blotting. Thymocytes were stimulated with 1 μg/ml anti-CD3 Ab (PharMingen) plus 20 ng/ml IL-4 (Pepro Tech EC, Rocky Hill, NJ) at 37°C for 1 hr or 3 hr, or not stimulated. Cells were solubilized with lysis buffer (0.5% Nonidet P-40/10 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/1 mM Na_2PO_4) containing protease inhibitors. Immunoprecipitates obtained with anti-STAT6 Ab (R & D Systems) were blotted with anti-phosphotyrosine Ab (4G10; Upstate Biotechnology) and reblotted with anti-STAT6 Ab after stripping the first blot. Whole cell lysates were blotted with anti-Bax Ab or anti-Bcl-2 Ab (both Santa Cruz Biotechnology).

RESULTS

To clarify the functional and developmental roles of SSI-1, we used homologous recombination in embryonic stem cells to generate mice lacking SSI-1. The disrupted SSI-1 allele was ultimately transmitted through the germ line, as confirmed by Southern blot (DNA) analysis (Fig. 1*A*). Northern blots confirmed the loss of SSI-1 mRNA in lung tissues from SSI-1−/− mice (Fig. 1*B*). Homologous mutant mice (SSI-1−/−) were healthy and normal at birth and were initially indistinguishable from control littermates (SSI-1+/+). Although the body weight of SSI-1−/− mice did not differ significantly from that of SSI-1+/+ mice on postnatal day 3, an approximately 40% decrease in body weight was observed on postnatal day 9 (Fig. 1*C*). Moreover, SSI-1−/− mice all died within 3 weeks after birth (Fig. 1*C*).

SSI-1 has been shown *in vitro* to be a negative feedback factor in the JAK-STAT signal pathway. We therefore carried out the following experiment to determine whether lymphocytes lacking SSI-1 exhibited prolonged activation of STAT. We stimulated the thymocytes of 10-day-old mice with anti-CD3 Ab plus IL-4 and examined phosphorylation of STAT6 at 0, 1, and 3 hr after the stimulation. In the thymocytes lacking SSI-1, the phosphorylation of STAT6 was not transient and still detected 3 hr after the stimulation (Fig. 2*A*). The thymocytes of 10-day-old mice were then stimulated similarly with anti-CD3 Ab, anti-CD3 Ab plus IL-2, and anti-CD3 Ab plus IL-4 and survival of thymocytes on days 1, 2, and 3 was examined by using MTT dye conversion, respectively (14) (Fig. 2*B*). Without stimulation or with anti-CD3 Ab alone, apoptosis occurred in thymocytes from both SSI-1+/+ and −/− mice, but the addition of IL-2 or IL-4 reduced apoptosis and the number of living thymocytes increased. In the thymocytes of SSI-1−/− mice, IL-2- or IL-4-induced lymphocyte proliferation was significantly augmented compared with SSI-1+/+ mice. These findings suggested that this increase in proliferation could be due to the impaired negative feedback mechanism of cytokine signals.

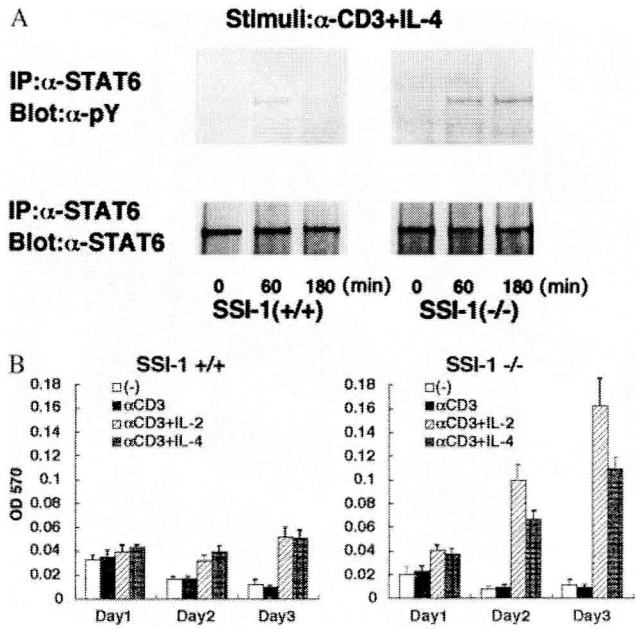

FIG. 2. (*A*) The activation of STAT6 continued after stimulation by IL-4 in thymocytes of SSI-1−/− mice. Cells were either nonstimulated or stimulated with IL-4 plus α-CD3. Immunoprecipitated (IP) STAT6 and STAT6 were immunoblotted with anti-phosphotyrosine Ab (α-pY). Blots were reprobed with α-STAT6 Ab. (*B*) MTT staining was measured on days 1, 2, and 3 after stimulation. Each column represents the mean of seven experiments.

When the spleen and thymus of SSI-1−/− mice were examined histologically by means of hematoxylin/eosin staining, little difference was seen between the number of lymphocytes in SSI-1−/− and SSI-1+/+ mice on postnatal day 1, whereas the numbers of lymphocytes in the spleen and thymus lacking SSI-1 had decreased markedly at 10 days of age. The spleen of SSI-1−/− mice at 10 days of age was distorted in shape and exhibited partial atrophy of the white pulp. In the thymus, moreover, a borderline between cortex and medulla was not visible (Fig. 3*A*). The number of total thymocytes and splenic lymphoid cells of SSI-1−/− mice markedly decreased and at 10 days of age, approximately 75–80% of lymphocytes had disappeared as compared with SSI-1+/+ (Fig. 3*B*). However, the number of bone marrow cells did not significantly differ between SSI-1+/+ and SSI-1−/− mice (data not shown).

Next, to examine lymphocyte differentiation in the spleen, thymus, and bone marrow, the splenocytes, thymocytes, and bone marrow cells of SSI-1−/− mice at 10 days of age were stained for two-color flow cytofluorometric analysis with Abs to B220, CD3, CD4, CD8, Mac-1, Gra-1, and IgM (Fig. 3*C*). In the spleen, the number of $B220^+$ or $CD3^+$ cells in SSI-1−/− mice were markedly decreased compared with those in SSI-1+/+ mice. However, the ratio of B220/CD3 splenocytes remained unaffected despite a massive decrease in the number of splenic lymphoid cells in SSI-1−/− mice. The number of $Mac\text{-}1^+$ and $Gra\text{-}1^+$ splenocytes did not significantly differ between SSI-1−/− and SSI-1+/+ mice (data not shown). The number of thymocytes in SSI-1−/− mice was only 25% of that of control thymocytes (Fig. 3*B*). However, the percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^+$ cells did not differ between SSI-1+/+ and SSI-1−/− mice. Similarly, the percentages of IgM^+ and $B220^+$ cells in the bone marrow did not significantly differ between SSI-1+/+ and SSI-1−/− mice. In SSI-1−/− mice, the spleen and thymus appeared to exhibit normal differentiation of lymphocytes despite a massive decrease of the number of lymphocytes. These findings suggested that SSI-1 is not essential for the generation and differentiation of lymphocytes but that it may be important for the survival of lymphocytes *in vivo*.

We examined the spleen and thymus of 10-day-old mice immunohistochemically by means of TUNEL staining (15) to determine the cause of the decrease in the number of lymphocytes in SSI-1−/− mice. In the spleen and thymus of SSI-1−/− mice, there were more cells labeled with TUNEL than in those of SSI-1+/+ mice (Fig. 4*A*). In addition, electron microscopic examination detected a larger number of apoptotic cells with the typical condensation of nuclear chromatin and apoptic bodies in SSI-1−/− mice (Fig. 4*B*). The spleen and thymus of 10-day-old mice were then stained immunohistologically (16) with anti-Bax Ab or anti-Bcl-2 Ab to clarify the mechanism of accelerated apoptosis of lymphocytes. Compared with SSI-1+/+ mice, the cells expressing Bax had markedly increased in the spleen and thymus of SSI-1−/− mice. However, there was no significant difference in Bcl-2 expression between SSI-1+/+ and SSI-1−/− mice (Fig. 4*C*). Moreover, no difference in the expression of molecules inducing apoptosis such as Fas, Fas ligand, tumor necrosis factor-α, and glucocortisol, could be detected between SSI-1+/+ and SSI-1−/− mice (data not shown). These findings suggested that accelerated apoptosis of lymphocytes in SSI-1−/− mice may be due to the augmented expression of Bax, resulting in a reduction in the lymphocytes.

DISCUSSION

The cytokine signal pathways that include Ras–MAP and JAK-STAT signal cascades are known to be involved in the generation of an anti-apoptotic signal, most likely through the induction of the Bcl-2 (17–19). Our results showed that in

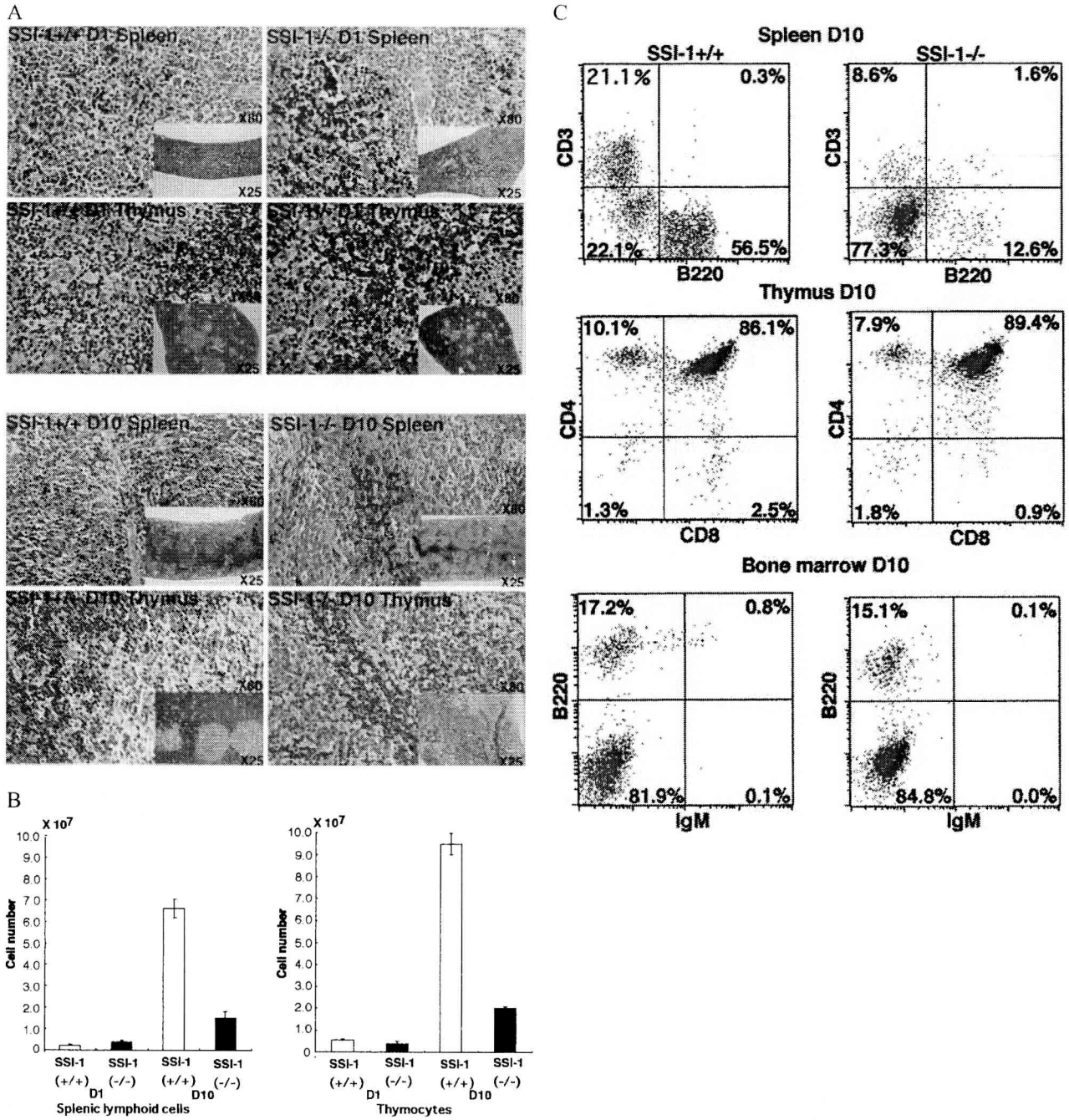

FIG. 3. (*A*) Histological appearances of the spleen and thymus in SSI-1−/− mice at 1 and 10 days of age. Hematoxylin/eosin stained sections of the spleen and thymus from SSI-1−/− mice 10-days-old reveal decreased lymphocytes (X80). The low magnification (X25) reveals a distorted shape and partial atrophy of white pulp in the spleen and an indistinct borderline between cortex and medulla in the thymus of SSI-1−/− mice. (*B*) Total thymocytes and splenic lymphoid cell numbers in SSI-1+/+ mice and −/− mice. The means and standard deviations for 10 +/+ and 10 −/− mice are shown. Numbers of total thymocytes and splenic lymphoid cells for SSI-1+/+ are shown by empty bars, and for SSI-1−/− by filled bars. (*C*) Flow cytometric analysis of splenocytes, thymocytes and bone marrow cells from SSI-1+/+ and SSI-1−/− mice 10-days-old.

thymocytes lacking SSI-1, cytokine-induced proliferation was augmented by prolonged activation of STAT *in vitro* (Fig. 2*A* and *B*). This finding indicates that, as expected, SSI-1 plays an important role in the negative feedback mechanism of the cytokine. However, in the thymus and spleen, a large number of lymphocytes lacking SSI-1 showed evidence of apoptosis with aging, which involved an increase in Bax expression *in vivo* (Fig. 4*A–C*). These seemingly contradictory findings may indicate the possibility that SSI-1 inhibits not only the JAK-STAT signal pathways, but also other signal pathways, which promote apoptosis.

The balance of interactions between pro- and anti-apoptosis members of the Bcl-2 gene family are believed to regulate apoptosis (20). Bax is one of the genes in this family. A predominance of Bax has been found to accelerate apoptosis in response to cytokine removal (20) and the expression of the Bax transgene in T cells leads to a large reduction in the numbers of mature T cells *in vivo* (21). In contrast, overexpression of Bcl-2 strongly reduces apoptosis (22). The ratio of Bcl-2 to Bax determines whether a cell will respond to apoptotic stimuli. Although the embryonic development of Bcl-2−/− mice is normal, these mice display growth retardation, shortened lifespan and massive apoptosis in the spleen and thymus (23, 24). These findings are similar to those for SSI-1−/− mice. Nevertheless, the present study did not show any significant difference in the level of Bcl-2 protein between

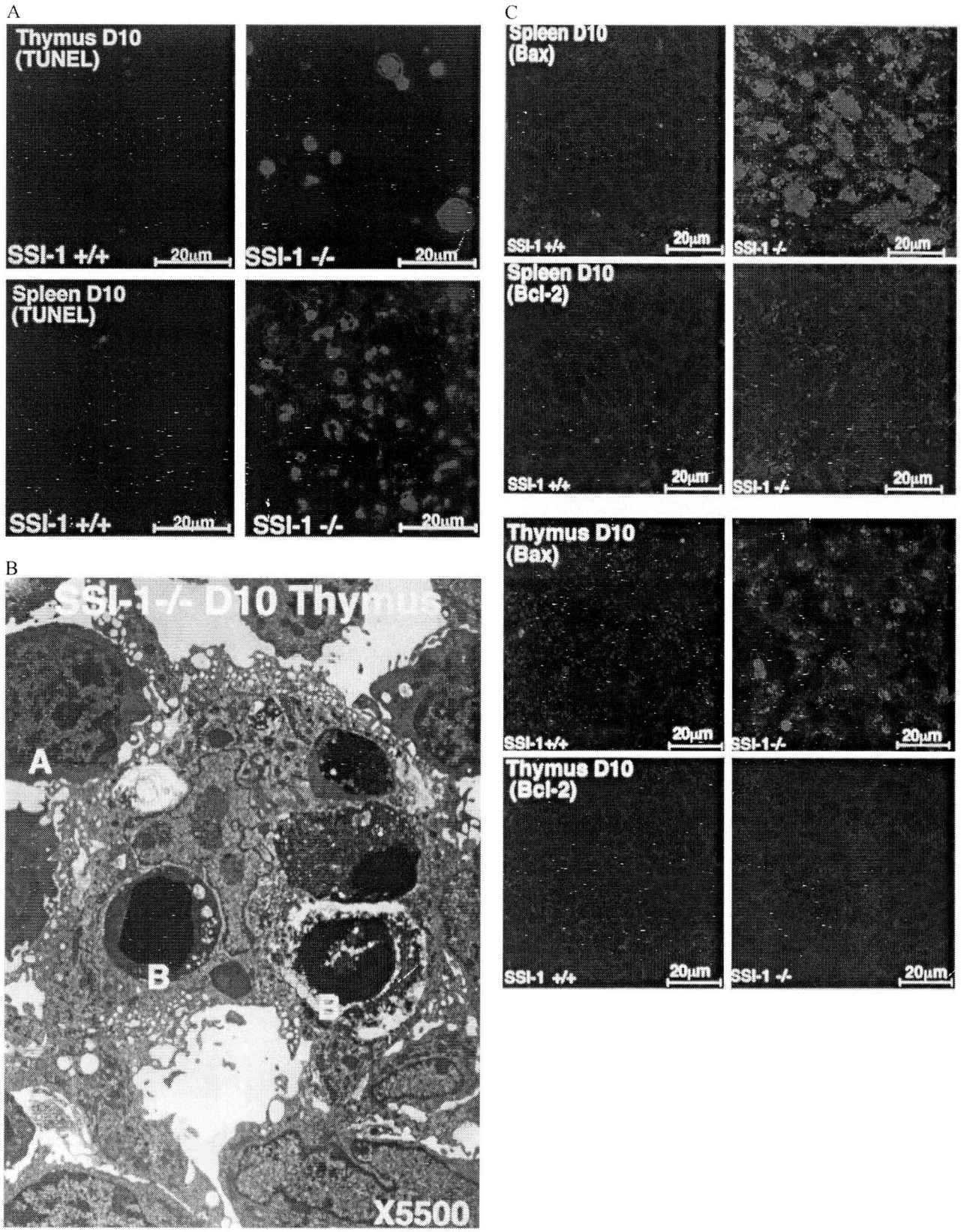

FIG. 4. (*A*) Numerous apoptotic splenocytes and thymocytes in the SSI-1−/− mice 10 days old are demonstrated by TUNEL staining. (*B*) Electron micrograph of the thymus of SSI-1−/− mice at 10 days old (×5500). Cells (*A*) are still relatively normal, but the apoptotic cells (*B*) have lost cell membrane microvilli and clearly exhibit chromatin condensation and apoptic bodies. (*C*) Immunohistochemical staining of the spleen and thymus. Comparisons of Bax expression in SSI-1+/+ mice (*Top Left*) and that in −/− mice (*Top Right*), and of Bcl-2 expression in +/+ mice (*Bottom Left*) and that in −/− mice (*Bottom Right*).

SSI-1−/− and SSI-1+/+ mice (Fig. 4*C*). Then, it has been recently demonstrated that a death signal generates the activation of Bax, which in turn induces homodimerization, translocation into mitochondrial membrane, mitochondrial dysfunction, and apoptosis (25). SSI-1−/− mice showed an increased level of Bax protein in the spleen and thymus (Fig.

4*C*). Thus, the apoptosis of lymphocytes lacking SSI-1 may be due to this increase in Bax expression. These findings suggest that, although the relation between Bax expression and the JAK-STAT signal pathway has not been clarified yet, SSI-1 may directly or indirectly inhibit the other signal pathways, except JAK-STAT signal cascades, which induce Bax expression and thus prevent Bax-induced apoptosis *in vivo*. However, further analysis is required to determine which signal pathway in the expression of Bax is inhibited by SSI-1 and whether SSI-1 inhibits apoptosis through inhibition of JAK activation.

Whereas SSI-1 is strongly expressed in thymus, spleen, and peripheral blood leukocytes, any expression of SSI-2 or -3 is hardly observed in them (3, 9). This difference in distribution suggests that other members of the SSI-family may not able to compensate for the lack of SSI-1 in lymphocytes. Although our analysis mainly focused on the immune system of SSI-1−/− mice in this study, SSI-1−/− mice at 10 days of age surprisingly showed apparent severe cardiac hypertrophy and histological degeneration of the hepatocytes (our unpublished data). The physiological roles of SSI-1 in other tissues have not been clarified for the present. Future analysis will be necessary to clarify them or to determine whether SSI-1 inhibits Bax expression similarly in tissues other than lymphocytes.

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