Suppressor of cytokine signaling-1 Attenuates the Duration of Interferon γ Signal Transduction in Vitro and in Vivo*

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Suppressor of cytokine signaling-1 (SOCS-1) is a cytokine-inducible intracellular protein that functions to negatively regulate cytokine signal transduction pathways. Studies in vitro have shown that constitutive overexpression of SOCS-1 inhibits signaling in response to a range of cytokines, including interferons (IFN). Mice lacking SOCS-1 die from a complex disease characterized by liver degeneration and massive inflammation. Whereas there is clear evidence of increased IFNγ signaling in SOCS-1−/− mice, it is unclear to what extent this is due to increased IFNγ levels or to increased IFNγ sensitivity. Here we have used SOCS-1−/− IFNγ−/− mice, which remain healthy and produce no endogenous IFNγ, to demonstrate that in vitro and in vivo hepatocytes lacking SOCS-1 exhibit a prolonged response to IFNγ and that this correlates with a dramatically increased sensitivity to the toxic effects of IFNγ in vivo. Thus, SOCS-1 is required for the timely attenuation of IFNγ signaling in vivo.

SOCS-11 is an intracellular SH2 domain-containing protein that was initially isolated by screening a retroviral cDNA library for inhibitors of IL-6 signaling (1). It is also known as STAT-inducible STAT inhibitor-1 (SSI-1) (2) or JAK-binding protein (JAB) (3). SOCS-1 defines a family of eight proteins, SOCS-1 to -7 and CIS (cytokine-inducible SH2-containing protein), each of which contains a central SH2 domain and a C-terminal SOCS box (4).

In vitro studies have shown that the expression of SOCS-1, -2, -3, and CIS is inducible by a number of cytokines and that, when constitutively overexpressed, each SOCS protein can act to switch off cytokine-induced responses by inhibiting the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (1–3). The latter studies are difficult to interpret because not only are the SOCS proteins grossly overexpressed, but they are also expressed in a temporally inappropriate manner, being present prior to the onset of signaling rather than induced as a consequence of signal transduction, which occurs physiologically.

Although both SOCS-1 and SOCS-3 act to suppress signal transduction from a similarly broad spectrum of cytokines in vitro, including IL-6, IL-4, growth hormone, IFNα/β, and IFNγ (1–3, 5–8), analyses of mice deficient in either SOCS-1 or SOCS-3 have indicated that each may play a relatively specific role in vivo. Mice lacking SOCS-3 die during embryogenesis apparently from erythrocytosis, suggesting that SOCS-3 is critical in regulating fetal liver erythropoiesis (9). SOCS-1 deletion results in mice that die, before weaning, of a complex multorgan disease (10–12). Although the primary defect in mice lacking SOCS-3 has not been defined, the fatal neonatal disease characteristic of SOCS-1-deficient mice requires IFNγ, because this pathology does not develop in SOCS-1-deficient mice that also lack the IFNγ gene (13).

Interferons are fundamental components of the immune system. The predominant function of interferons is to confer cellular resistance to viral infections, although other immunomodulatory functions have also been described (14, 15). IFNγ is produced by activated T lymphocytes and natural killer cells during viral infections and initiates an antiviral program in target cells by up-regulating the expression of genes that mediate the anti-viral response, including those encoding MHC class I antigens (14, 15). Similar to other cytokines, interferons activate the JAK/STAT pathway (16), and studies in vivo have shown that STAT1 is critical for mediating biological responses to IFNγ (17, 18). Elevated levels of IFNγ can be toxic, and it is clear that both IFNγ production and responses to this cytokine must be tightly regulated in order to achieve a balance between beneficial and harmful effects (19–21). Other cytokines including IL-4, IL-10, and IL-13 contribute to some extent to this regulation by antagonizing IFNγ functions (22–24). In addition, negative regulators such as SOCS proteins act to limit signal transduction in response to IFNγ.

At least in vitro, SOCS-1 expression is induced by IFNγ and overexpression of SOCS-1 inhibits IFNγ signaling (6, 7). Clearly, IFNγ is an essential mediator of the fatal disease seen in mice lacking SOCS-1 (13), but the precise mechanism is unclear. This report compares IFNγ signaling in wild-type and SOCS-1-deficient mice in vivo and finds that both biochemical and biological responses to IFNγ are attenuated by SOCS-1.

EXPERIMENTAL PROCEDURES

Maintenance of Mice and Injection of IFNγ—SOCS-1−/− IFNγ−/− mice were generated as described previously and were maintained on a mixed 129Sv and C57Bl/6 genetic background (10, 13). For biological studies, neonatal mice were given daily intraperitoneal injections of 3, 10, and 30 μg of recombinant IFNγ.
Intraperitoneal injections of IFN-γ from birth. Mice were administered either 3 μg of IFN-γ (top panel), 0.3 μg of IFN-γ (middle panel) or saline (bottom panel), and their health was monitored daily. n = number of mice/group.

Immunoprecipitation and Western Blotting of Liver Proteins—For biochemical studies, adult mice weighing an average of 29 g were given daily intraperitoneal injections of IFN-γ or saline containing 10 μg of IL-6 (a gift from Dr. Richard Simpson, Ludwig Institute for Cancer Research, Melbourne, Australia). Mice were sacrificed when moribund or at 21 days of age.

Electrophoretic Mobility Shift Assays—Adherent hepatocytes were harvested from 10-week-old mice as described previously (25). Briefly, livers were perfused with a rubber policeman and were washed once with cold phosphate-buffered saline containing 1 mM pervanadate. Cell pellets were snap frozen on dry ice, and high salt nuclear extracts were prepared as described (26). Electrophoretic mobility gel shift assays were performed on 10 μg of nuclear protein using the m67 oligonucleotide as described (27). To

[Fig. 1. Mice lacking SOCS-1 are more sensitive to the effects of exogenous IFN-γ. Neonatal mice were given daily intraperitoneal injections of IFN-γ from birth. Mice were administered either 3 μg of IFN-γ (top panel), 0.3 μg of IFN-γ (middle panel) or saline (bottom panel), and their health was monitored daily. n = number of mice/group.]

[Fig. 2. IFN-γ-induced STAT1 phosphorylation is prolonged in SOCS-1−/− IFN-γ−/− mice. SOCS-1−/− IFN-γ−/−, SOCS-1−/− IFN-γ−/− and SOCS-1−/− IFN-γ−/− mice were injected with 2 μg of IFN-γ (A) or 10 μg of IL-6 (B) and sacrificed at the times indicated. Liver lysates were prepared and STAT1 protein immunoprecipitated with an anti-STAT1 antibody. Duplicate blots were incubated with either an antibody specific for phosphorylated STAT1 (α-STAT1-P, left panels) or STAT1 (right panels).]
SOCS-1 Attenuates IFNγ Signal Transduction

**RESULTS**

**SOCS-1 Is Required for Regulating Responses to IFNγ in Vivo**—The neonatal mortality in SOCS-1 knockout mice has been shown to be dependent on IFNγ, because mice lacking functional genes for both SOCS-1 and IFNγ are viable and healthy (13). We wished to use SOCS-1−/− mice to assess the extent to which SOCS-1 regulates IFNγ signal transduction; however, such studies are complicated by the endogenous production of IFNγ in vitro and in vivo and compounded by the neonatal morbidity and mortality of SOCS-1−/− mice. To overcome these problems we took advantage of the observation that SOCS-1−/− IFNγ−/− mice, which fail to produce IFNγ, are healthy and survive to adulthood.

Previous studies have documented the toxicity of IFNγ when administered to newborn mice (19). Further, the pathology induced by IFNγ treatment of wild-type mice is similar to that seen in SOCS-1−/− mice (13). The phenotype of SOCS-1−/− mice may be due to increased circulating levels of IFNγ, increased sensitivity of cells to the effects of IFNγ because of unregulated signaling, or a combination of both. To determine whether mice are more sensitive to IFNγ in the absence of SOCS-1, newborn SOCS-1−/− IFNγ−/− and SOCS-1+/− IFNγ−/− mice received daily intraperitoneal injections of IFNγ. These mice were monitored and were sacrificed when moribund. Mice lacking SOCS-1 were substantially more sensitive to the toxicity of IFNγ. Injections of 3 µg of IFNγ induced morbidity within 2 days in SOCS-1−/− IFNγ−/− mice, whereas this dose was tolerated for ~3 weeks in SOCS-1+/− IFNγ−/− mice (Fig. 1). Differences in sensitivity were also seen in response to the administration of 0.3 µg of IFNγ. In contrast to SOCS-1−/− IFNγ−/− mice, which were all moribund by 3 weeks of age and developed the same pathological features as SOCS-1−/− mice (Fig. 1 and data not shown), SOCS-1+/− IFNγ−/− mice all remained healthy after receiving this dose of IFNγ (Fig. 1).

**IFNγ-induced STAT Activation Is Prolonged in Mice Lacking SOCS-1**—To assess whether SOCS-1 regulates the intensity or duration of responses to IFNγ, the activation of downstream signaling molecules was assessed in mice lacking SOCS-1. The organ we chose to examine in these studies was the liver because it is severely affected both in SOCS-1−/− mice and in mice injected with IFNγ. SOCS-1−/− IFNγ−/−, SOCS-1+/− IFNγ−/−, and SOCS-1−/− IFNγ+/− mice were given intraperitoneal injections of IFNγ and the subsequent activation of STAT1 in the liver was monitored at various times thereafter. STAT1 phosphorylation was evident in all mice within 15 min of IFNγ injection, with the response continuing for at least 2 h but declining to basal levels by 4 h in SOCS-1−/− IFNγ−/− and SOCS-1−/− IFNγ+/− mice (Fig. 2A). However, phosphorylation of STAT1 was prolonged in mice lacking SOCS-1 and was maintained until 8 h after injection. This prolonged response was specific to IFNγ, because there was no difference in the kinetics of STAT1 or STAT3 phosphorylation induced by IL-6 in mice lacking SOCS-1 (Fig. 2B and data not shown). SOCS-1 appeared to be important for regulating the duration of the response to IFNγ, but not the intensity, because peak levels of STAT1 phosphorylation did not differ substantially between mice of different genotypes.

**SOCS-1 Is Required for Regulating Responses to IFNγ in Hepatocytes in Vitro**—The in vivo experiments described above indicate a role for SOCS-1 in regulating IFNγ-induced responses in the liver but may be complicated by factors such as clearance of IFNγ from the circulation. We therefore wished to examine IFNγ responses in vitro using primary hepatocyte cultures. To assess the sensitivity of hepatocytes to IFNγ, primary hepatocyte cultures were established from SOCS-1−/− IFNγ−/− and SOCS-1−/− IFNγ−/− mice. Hepatocytes were stimulated for 10 min with a pulse of IFNγ, after which time cytokine was washed from the cells, and the response was allowed to decay over varying periods. Gel shift analysis was used to monitor the DNA binding activity of STAT dimers throughout the time course. In both cell types, the response to IFNγ peaked at 30 min after stimulation. The response decayed rapidly in SOCS-1−/− IFNγ−/− cells and was virtually undetectable at 2 h poststimulation (Fig. 3A). In contrast, the activity of STAT dimers was prolonged in SOCS-1−/− IFNγ−/− cells, persisting beyond 3 h after IFNγ treatment (Fig. 3B).

Supershift analyses (Fig. 3C) identified STAT1 homodimers as the predominant STAT dimers formed in response to IFNγ (Fig. 3, A and B, lower band), with a smaller amount of STAT1/STAT3 homodimers also being activated (Fig. 3, A and B, upper band). Similar to the in vivo studies, only the duration of the response to IFNγ was altered in SOCS-1−/− cells and not the intensity. Other experiments were performed in which hepato-
cytes were exposed to the stimulus for the entire time course rather than just a pulse. Active STAT dimers persisted for longer in these experiments but did not decay in cells lacking SOCS-1 throughout an 8-h culture period, whereas STAT dimer formation in SOCS-1−/− IFNγ−/− cells had decayed to a baseline level by 5 h of culture (data not shown).

**DISCUSSION**

IFNγ is critical for mediating the multiorgan pathology of SOCS-1−/− mice, because mice lacking both SOCS-1 and IFNγ are viable and healthy (13). However, the specific relationship between SOCS-1 and IFNγ has yet to be defined. Is the phenotype of SOCS-1−/− mice due to increased sensitivity of cells to IFNγ in the absence of SOCS-1 or to increased production of IFNγ? Both appear to contribute to the phenotype. A previous study has shown that circulating levels of IFNγ are increased in mice lacking SOCS-1 (12). The data presented here clearly establish that in the absence of SOCS-1, mice and cells isolated from them are more sensitive to the effects of IFNγ. Neonatal mice lacking SOCS-1 were substantially more sensitive to the toxic effects of administered IFNγ. Further, we have directly established a biochemical basis for this hypersensitivity and have shown that SOCS-1 attenuates signal transduction pathways in vivo, consistent with in vitro data. STAT1 activation was prolonged in the liver of SOCS-1−/− IFNγ−/− mice after injection of IFNγ. This increased sensitivity in vivo was paralleled in vitro by prolonged STAT1 activation in primary hepatocytes in response to IFNγ.

Although forced expression of SOCS-1 has been shown to limit the intensity of STAT activation in response to cytokine (1–3), SOCS-1 expression is temporally inappropriate in these studies. In the present study, which relies on more physiological expression, SOCS-1 appears to be important for limiting the duration of responses to cytokine and not the magnitude of the response. This result is consistent with the current model of SOCS action in which SOCS expression is induced by cytokine and then functions to switch off the cytokine-induced signal (1). The relative contributions to the SOCS-1−/− phenotype of increased IFNγ production and prolonged IFNγ responses are difficult to delineate. In situations in which IFNγ is limiting, for instance in SOCS-1−/− IFNγ−/− mice, pathological changes including uncontrolled macrophage activation are still evident and may result predominantly from inappropriate and prolonged signaling in response to more normal levels of circulating IFNγ (28). Further, serum IFNγ is undetectable in some SOCS-1−/− mice (data not shown). Our data suggest that hypersensitivity to IFNγ may be driving the typical SOCS-1−/− phenotype seen in these mice.

Although the JAK/STAT pathway has been well characterized using both primary and continuous cell lines, the kinetics of JAK/STAT activation in vivo following cytokine stimulation has not been comprehensively studied. In the course of this study we have developed assays to monitor the phosphorylation of STATs from mouse liver, resulting in a detailed study of the kinetics of IFNγ signaling in vivo.

The phenotype of SOCS-1−/− mice demonstrates a specific non-redundant role for SOCS-1 in determining the rate at which interferon signaling is attenuated. The lack of effect on IL-6 signaling in vivo may indicate that other SOCS proteins can compensate for the absence of SOCS-1 in other cytokine pathways. Whereas IFNγ signaling is clearly prolonged by several hours in SOCS1−/− mice, STAT1 activation is still switched off, albeit later than normal. This residual negative regulation of signal transduction may be mediated by other members of the SOCS family or by other inhibitory mechanisms such as phosphatases (29) and the protein inhibitor of activated STAT (PIAS) (30). The results presented in this study using wild-type and SOCS-1-deficient cells provide a benchmark against which cells from other compound-knockout mice can be compared.

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**REFERENCES**


