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SOCS1 Is a Critical Inhibitor of Interferon γ Signaling and Prevents the Potentially Fatal Neonatal Actions of this Cytokine

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Summary

Mice lacking suppressor of cytokine signaling-1 (SOCS1) develop a complex fatal neonatal disease. In this study, *SOCS1*$^{-/-}$ mice were shown to exhibit excessive responses typical of those induced by interferon γ (IFNγ), were hyperresponsive to viral infection, and yielded macrophages with an enhanced IFNγ-dependent capacity to kill *L. major* parasites. The complex disease in *SOCS1*$^{-/-}$ mice was prevented by administration of anti-IFNγ antibodies and did not occur in *SOCS1*$^{-/-}$ mice also lacking the *IFN*γ gene. Although IFNγ is essential for resistance to a variety of infections, the potential toxic action of IFNγ, particularly in neonatal mice, appears to require regulation. Our data indicate that SOCS1 is a key modulator of IFNγ action, allowing the protective effects of this cytokine to occur without the risk of associated pathological responses.

Introduction

Resistance to infections is dependent on the coordinated action of the cytokine network. Key contributions are made by interferons (IFN; Billiau, 1996; Boehm et al., 1997; De Maeyer and De Maeyer-Guignard, 1998), which comprise two broad groups. Type I interferons include the closely related forms of IFNα and a single form of IFNβ, whereas IFNγ is the sole type II interferon (De Maeyer and De Maeyer-Guignard, 1998). The IFNα proteins and IFNβ are produced by many cell types in response to viral infection (De Maeyer and De Maeyer-Guignard, 1998). In contrast, IFNγ is produced exclusively by activated T cells and natural killer (NK) cells (Billiau, 1996; Boehm et al., 1997; De Maeyer and De Maeyer-Guignard, 1998). IFNγ serves to upregulate expression of a wide variety of genes involved in antigen presentation, activation of macrophages, antiviral, and antiproliferative responses (reviewed by Tatake and Zeff, 1993; Boehm et al., 1997).

The major signal transduction pathway initiated by interferons has been elucidated by a series of elegant biochemical and genetic studies (Darnell et al., 1994; Ihle et al., 1994; Schindler and Darnell, 1995; Bach et al., 1997; Darnell, 1997; Leonard and O'Shea, 1998). IFNγ acts by binding to and inducing the multimerization of a cell surface receptor composed of the IFNGR1 and IFNGR2 chains (Hemmi et al., 1994; Novick et al., 1994). This results in juxtaposition of janus kinase 1 (JAK1) bound to IFNGR1 and JAK2 bound to IFNGR2, which cross-phosphorylate and activate each other (Muller et al., 1993a; Watling et al., 1993; Igarashi et al., 1994). Activated JAKs phosphorylate tyrosine residues within the cytoplasmic domains of the receptor subunits, which act as docking sites for signal transducer and activator of transcription 1 (STAT1; Greenlund et al., 1994; Heim et al., 1995). Phosphorylation of a C-terminal tyrosine (Y701) in STAT1 facilitates interaction with the SH2 domain of a second STAT1 molecule, mediating dimerization (Shuai et al., 1994). STAT1 dimers subsequently migrate to the nucleus, where they bind to gamma-activated sequence (GAS) elements contained within the promoters of IFNγ-inducible genes (Shuai et al., 1992; Muller et al., 1993b), including the genes for inducible nitric oxide synthase (iNOS) and the transcription factor interferon regulatory factor 1 (IRF1). The increased susceptibility to *Mycobacteria*, *Leishmania major*, and some viruses in mice or humans harboring mutations in the genes for IFNγ, its receptor, IRF1, or iNOS highlights the importance of this pathway in resistance to infection (Huang et al., 1993; Matsuyama et al., 1993; Kamijo et al., 1994; MacMicking et al., 1995; Durbin et al., 1996; Meraz et al., 1996; Newport et al., 1996; Lu et al., 1998).

The actions of IFNγ are not always beneficial, since infections may elicit a host response of sufficient magnitude to become life threatening, for example, morbidity associated with *Staphylococcus aureus* infection and the hepatotoxicity associated with hepatitis B infection (Billiau and Vandekerckhove, 1991; Ando et al., 1993; Matthys et al., 1995). The potentially toxic effects of IFNγ, including fatty degeneration of the liver, have also been demonstrated in experiments in which circulating IFNγ levels are experimentally elevated in neonatal mice (Gresser, 1982; Toyonaga et al., 1994). Regulatory mechanisms must therefore exist to maintain the fine balance between beneficial and detrimental responses to IFNγ. This is achieved in part through the production of interleukin-4 (IL-4), IL-10, and IL-13, which counteract the effects of IFNγ (Paul, 1991; Moore et al., 1993; Zurawski and de Vries, 1994), and by negative regulation of IFNγ signal transduction, for example, by the SH2 domain–containing phosphatase SHP1 (Massa and Wu, 1996).

In vitro studies have also implicated a family of SH2-containing proteins, the suppressors of cytokine signaling (SOCS) proteins, in the negative regulation of cytokine signal transduction. Of the eight SOCS proteins (SOCS1 to 7 and CIS), SOCS1 and SOCS3 appear to be

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the most potent inhibitors of cytokine signaling (Nicholson et al., 1999). SOCS1 was initially identified in a functional screen for proteins capable of inhibiting IL-6 signaling (Starr et al., 1997). Subsequent studies have shown that STAT activation in response to many cytokines results in increased transcription of the *SOCS1* and *SOCS3* genes and that, when overexpressed, SOCS1 and SOCS3 inhibit the biological effect of cytokines, including IFNγ, that act through the JAK/STAT pathway (Wang et al., 1996; Endo et al., 1997; Matsumoto et al., 1997; Naka et al., 1997; Starr et al., 1997; Adams et al., 1998; Auernhammer et al., 1998; Bjorbaek et al., 1998; Hilton et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999). Consistent with the observation that SOCS1 can inhibit the action of a broad range of cytokines, SOCS1 appears to bind to all four members of the JAK family and inhibit their catalytic activity (Endo et al., 1997; Naka et al., 1997; Nicholson et al., 1999). These studies have led to the view that SOCS1 may be part of a general negative feedback loop regulating cytokine action.

The importance of this regulatory pathway was revealed in mice lacking a functional *SOCS1* gene (*SOCS1*$^{-/-}$ mice). These mice die between 2 and 3 weeks of age of a disease that involves fatty degeneration and necrosis of the liver, macrophage infiltration of several organs, and multiple hematopoietic abnormalities, including severe lymphopenia (Starr et al., 1998; Metcalf et al., 1999). In this report, we establish that *SOCS1*$^{-/-}$ mice are hypersensitive to IFNγ and that the complex multiorgan disease and premature death that develops in these mice can be prevented by administration of neutralizing anti-IFNγ antibodies and is absent in mice lacking both functional *SOCS1* and *IFN*γ genes. We conclude that SOCS1 is a critical regulator of cellular sensitivity to interferon-γ, balancing the beneficial immunological activities with the fatal neonatal effects of this cytokine.

Results

Aberrant IFNγ Signaling in *SOCS1*$^{-/-}$ Mice

The capacity of SOCS1 to inhibit IFNγ signaling in vitro (Starr et al., 1997; Sakamoto et al., 1998; Song and Shuai, 1998) and the observation that administration of this cytokine to neonatal mice induces pathology similar to that observed in *SOCS1*$^{-/-}$ mice (Gresser et al., 1981) suggested that the *SOCS1*-deficient disease may be mediated by IFNγ. To directly compare the effects of IFNγ administration with *SOCS1*$^{-/-}$ pathology, neonatal C57BL/6 mice were injected daily for 14 days with 3 μg of IFNγ. Like *SOCS1*$^{-/-}$ mice, the injected mice died in the second and third weeks of life, and histological examination showed that in addition to hepatic changes, their lungs, heart, and pancreas were infiltrated with macrophages. Moreover, the hematopoietic abnormalities observed, including severe lymphopenia and moderate granulocytosis, were also similar to those seen in *SOCS1*$^{-/-}$ mice (data not shown).

To determine more directly whether IFNγ plays a role in the development of pathology in *SOCS1*$^{-/-}$ mice, we examined them for the presence of a dysregulated signal transduction response to IFNγ. Using an oligonucleotide probe capable of binding to STAT1 and STAT3 in electromobility shift assays, activation of STAT1, a key step in IFNγ signal transduction, was readily detected in the livers of 14-day-old *SOCS1*$^{-/-}$ mice but not *SOCS1*$^{+/-}$ or wild-type mice (Figure 1A). We found, however, no evidence of STAT3 activation (Figure 1A; data not shown), suggesting that dysregulated signaling mediated by other inflammatory cytokines such as leukemia inhibitory factor (LIF), IL-6, and oncostatin M (OSM) that utilize STAT3 was not occurring in *SOCS1*$^{-/-}$ animals.

In addition, expression of mRNA for the IFNγ-inducible genes *IRF1* and *iNOS* was elevated in tissues of *SOCS1*$^{-/-}$ mice (Figure 1B). Likewise, class I MHC expression was markedly elevated in the liver (Figure 1C) and in hematopoietic cells, including thymic and splenic T cells, bone marrow and splenic B cells, and monocytes of *SOCS1*$^{-/-}$ mice (Figure 1D and data not shown). Elevation of class I MHC expression in the thymus, bone marrow, and spleen cells was observed at birth, prior to the development of overt disease.

IFNγ Is Essential for Disease Development and Premature Death in Neonatal *SOCS1*$^{-/-}$ Mice

To directly assess the role of IFNγ in the onset and development of disease in *SOCS1*$^{-/-}$ mice, three litters of mice (29 in total) from *SOCS1*$^{+/-}$ parents were injected twice weekly from birth with a neutralizing anti-IFNγ antibody. Only one death occurred (at day 1, of a *SOCS1*$^{+/-}$ mouse), while the remaining 28 mice remained in good health and were of normal body weight when analyzed at 3 weeks of age (Table 1). Genotyping subsequently revealed that six of these mice were homozygous *SOCS1* mutants, indicating that IFNγ was absolutely required for the premature death of *SOCS1*$^{-/-}$ mice (Figures 2A and 2B).

At 21 days, anti-IFNγ-treated *SOCS1*$^{-/-}$ mice were compared with moribund 12- to 21-day-old *SOCS1*$^{-/-}$ mice and 12- to 21-day-old *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice. Untreated *SOCS1*$^{-/-}$ mice showed a uniform series of pathological changes (Figure 3; Tables 1 and 2), including fatty degeneration and necrosis of liver cells, infiltration of the liver, lungs, pancreas, heart, and skin with macrophages, and thickening of the skin epithelium with keratinization. Untreated *SOCS1*$^{-/-}$ mice also exhibited profound alterations in hematopoiesis and lymphopoiesis with atrophy of the thymic cortex, failure of lymphoid follicle development in the spleen, and bone marrow lymphopenia (Figures 4A and 4B; Tables 1 and 2). Remarkably, most organs from anti-IFNγ antibody–treated *SOCS1*$^{-/-}$ mice were normal, the exceptions being minor cuffing of lung vessels and persistence of erythropoiesis in the spleen (Figure 3; Tables 1 and 2). Similarly, hematopoietic abnormalities, including lymphopenia, were also reduced, although not entirely eliminated (Figure 4; Tables 1 and 2). Protection from disease was specifically associated with inhibition of IFNγ because injection of *SOCS1*$^{-/-}$ mice with either anti-IL-6 or control rat immunoglobulin did not alter the kinetics of onset or typical multiorgan nature of the disease (Figure 2B).

Because treatment of *SOCS1*$^{-/-}$ mice with anti-IFNγ antibody did not entirely eliminate disease, we wished to determine whether the residual pathology was due to the involvement of other cytokines or to the inefficient

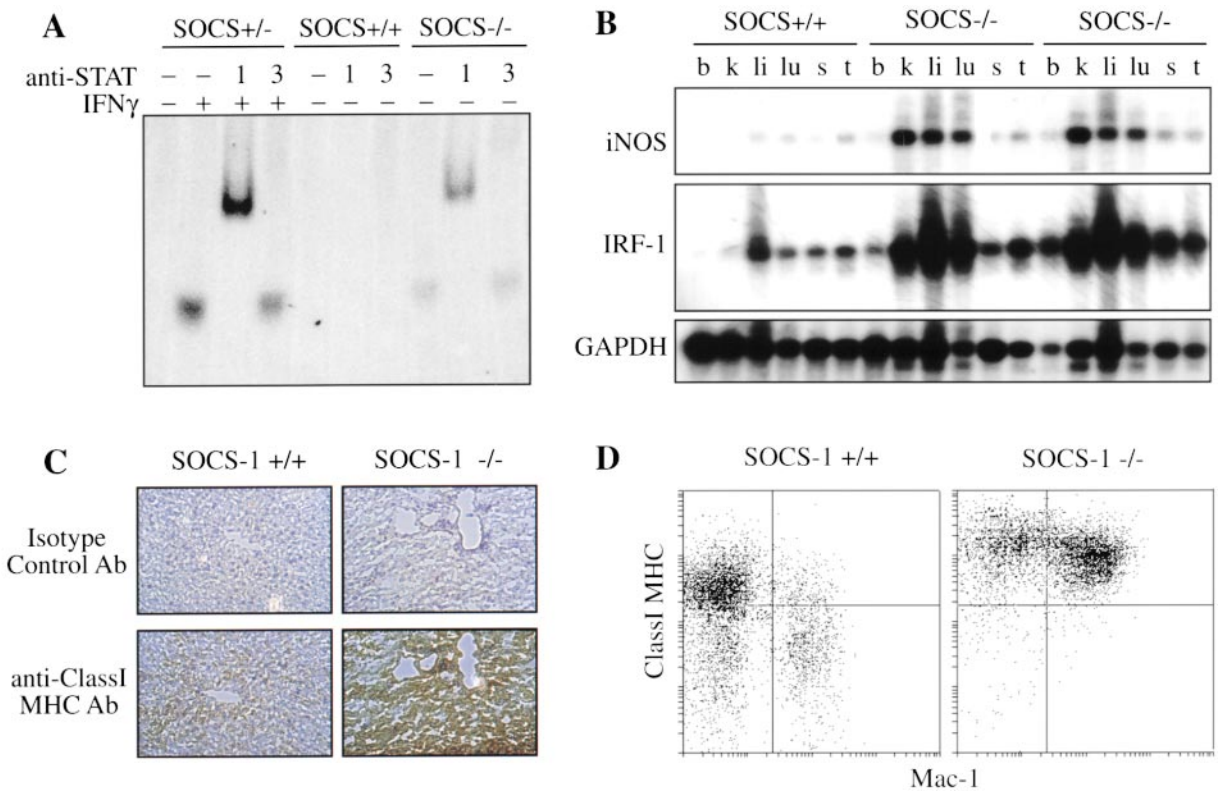

Figure 1. Experimentally Unmanipulated *SOCS1*$^{-/-}$ Mice Show Evidence of an Ongoing Response to IFNγ

(A) EMSA of extracts of livers from *SOCS1*$^{+/-}$ mice injected with 2 μg of IFNγ or untreated *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice. Prior to DNA binding, samples were treated, as indicated, with antibodies to either STAT1 or STAT3.

(B) Northern blot analyses showing mRNA from brain (b), kidney (k), liver (li), lung (lu), spleen (s), and thymus (t) from *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice hybridized with an *iNOS* probe (upper panel), *IRF1* probe (central panel), or *GAPDH* probe (lower panel).

(C) Immunohistochemistry showing class I MHC expression in the liver. Liver sections were stained with anti-class I MHC antibody or isotype control antibody and a peroxidase-labeled secondary antibody. Sections were counterstained with Giemsa.

(D) FACS analysis of class I MHC and Mac1 expression on bone marrow cells from SOCS1$^{+/+}$ and SOCS1$^{-/-}$ mice. Cells were stained with a biotinylated anti-class I MHC antibody and streptavidin-phycoerythrin followed by an FITC-conjugated anti-Mac1 antibody and were analyzed by flow cytometry. Data shown are representative of at least three independent experiments.

neutralization of IFNγ. To distinguish between these possibilities, we interbred *SOCS1*$^{+/-}$ and *IFN*γ$^{-/-}$ mice to yield animals lacking both SOCS1 and IFNγ. As previous studies of *IFN*γ$^{-/-}$ mice have reported no postnatal death (Dalton et al., 1993), it was not surprising to observe that all *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice remained healthy irrespective of their *IFN*γ genotype (data not shown). All *SOCS1*$^{-/-}$ *IFN*γ$^{+/+}$ mice died or became moribund in the second and third weeks of life (Figure 2D), and their disease was identical to that of unmanipulated *SOCS1*$^{-/-}$ mice (Figures 3 and 4; Tables 1 and 2). Consistent with the antibody administration studies, all *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice survived normally to weaning age and appeared overtly healthy (Figure 2D). A detailed analysis of 12 *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice at 3 weeks of age revealed normal body and organ weight and no hematological abnormalities (Figure 4D and Table 1). Only 2 of 12 mice showed lymphoid cuffing of lung vessels, and 8 of 12 mice had thymi with an enlarged medulla but normal cortex (Table 2), neither of which were histological features of the typical *SOCS1*$^{-/-}$ disease. *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice, some of which are now over 6 months old, have remained uniformly healthy, and both males and females have proven fertile. Interestingly, 8 out of 12 *SOCS1*$^{-/-}$ *IFN*γ$^{+/-}$ mice became moribund between 2 and 12 weeks of age (Figure 2D), while the remaining 4 mice were alive after 15 weeks and were fertile and healthy.

Together, these data from IFNγ antibody treatment and intercrosses with *IFN*γ$^{-/-}$ mice indicate that the full spectrum of disease that results in the postnatal death of *SOCS1*$^{-/-}$ mice is dependent on the action of IFNγ.

SOCS1 Deficiency Results in IFNγ Hyperresponsiveness

The disease in *SOCS1*$^{-/-}$ mice could reflect either an intrinsic cellular hypersensitivity to IFNγ and/or the production of elevated amounts of the cytokine. In this regard, *SOCS1*$^{-/-}$ granulocyte-macrophage progenitor cells have been shown to be hypersusceptible to inhibition by IFNγ (Metcalf et al., 1999). To further investigate these possibilities, the capacity of macrophages grown from bone marrow of wild-type or *SOCS1*$^{-/-}$ mice to kill the intracellular parasite *Leishmania major* following IFNγ stimulation was determined. Macrophages were infected 6 hr after stimulation with various doses of IFNγ

Table 1. Influence of IFNγ on the Development of Hematopoietic Changes in *SOCS1*$^{-/-}$ Mice

SOCS1 genotype	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$
Interferon γ genotype	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{-/-}$	*IFN*γ$^{-/-}$
Treatment	Untreated	Untreated	Anti-IFNγ Ab	Anti-IFNγ Ab	Untreated	Untreated
Number of mice analyzed	10	11	15	4	11	12
Age (days)	12–21	12–21	21	21	21	21
Total peripheral blood white cells/μl	3610 ± 1780	2160 ± 1190	4820 ± 2620	4660 ± 2620	3330 ± 1890	4780 ± 1430
Peripheral blood cells/μl						
Neutrophils	260 ± 100	1130 ± 710	560 ± 300	1890 ± 520	310 ± 150	460 ± 230
Lymphocytes	2850 ± 1580	620 ± 380	3580 ± 1680	1940 ± 1480	2640 ± 1620	3770 ± 1270
Monocytes	370 ± 210	360 ± 220	560 ± 290	440 ± 370	290 ± 220	390 ± 150
Eosinophils	140 ± 100	50 ± 80	70 ± 90	390 ± 320	90 ± 110	160 ± 120
Platelets × 10^{-3}	978 ± 138	630 ± 296	694 ± 195	862 ± 238	748 ± 160	770 ± 180
Hematocrit (%)	33 ± 2	30 ± 3	39 ± 3	36 ± 4	39 ± 3	39 ± 4
Total cells per femur × 10^{-6}	17.5 ± 4.1	5.5 ± 2.1	28.7 ± 9.5	21.6 ± 1.9	21.1 ± 3.0	23.7 ± 3.1
Morphology of bone marrow cells (%)						
Blasts	5 ± 2	6 ± 2	4 ± 2	4 ± 2	4 ± 1	4 ± 1
Myeloblasts/myelocytes	3 ± 2	10 ± 3	5 ± 2	7 ± 1	4 ± 3	3 ± 2
Metamyelocytes/neutrophils	15 ± 5	30 ± 12	17 ± 4	20 ± 7	14 ± 4	15 ± 5
Lymphocytes	38 ± 8	6 ± 4	37 ± 16	30 ± 11	38 ± 6	38 ± 5
Monocytes	8 ± 3	10 ± 4	4 ± 2	4 ± 2	6 ± 2	5 ± 2
Eosinophils	3 ± 2	4 ± 4	3 ± 3	12 ± 3	2 ± 2	4 ± 2
Nucleated erythroid cells	28 ± 6	34 ± 15	30 ± 11	24 ± 15	31 ± 6	31 ± 6
Spleen weight (mg)	41 ± 10	21 ± 8	57 ± 9	90 ± 32	67 ± 16	67 ± 26
Morphology of spleen cells (%)						
Blasts	3 ± 2	3 ± 2	4 ± 2	6 ± 1	2 ± 2	3 ± 2
Myeloblasts/myelocytes	0.4 ± 0.7	3 ± 4	0.2 ± 0.5	0.6 ± 1.1	0.2 ± 0.4	0.4 ± 0.7
Metamyelocytes/neutrophils	2 ± 2	6 ± 4	4 ± 2	2 ± 3	1 ± 1	1 ± 1
Lymphocytes	53 ± 12	8 ± 5	84 ± 4	53 ± 5	47 ± 17	51 ± 18
Monocytes	2 ± 2	6 ± 4	2 ± 1	1 ± 1	1 ± 2	2 ± 2
Eosinophils	2 ± 1	2 ± 3	0.6 ± 0.9	2 ± 1	0.5 ± 0.5	2 ± 1
Nucleated erythroid cells	38 ± 13	72 ± 18	5 ± 3	35 ± 5	48 ± 17	41 ± 19
Thymus weight (mg)	69 ± 11	8 ± 4	71 ± 14	52 ± 12	82 ± 16	83 ± 14
Body weight (g)	8.9 ± 0.7	4.2 ± 0.8	10.2 ± 1.0	9.3 ± 1.7	10.7 ± 0.9	10.6 ± 1.2

Data represent mean values ± standard deviations. The data should be read by pairwise comparison of column 1 versus column 2, column 3 versus column 4, and column 5 versus column 6. Note that the mice in columns 1 through 4 were generated by crossing *SOCS1*$^{-/+}$ mice on an otherwise wild-type background and are therefore all IFNγ$^{+/+}$, whereas the mice in columns 5 and 6 were generated by crossing *SOCS1*$^{-/+}$ *IFN*γ$^{-/+}$ mice or *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice, and their identity was established by genotyping.

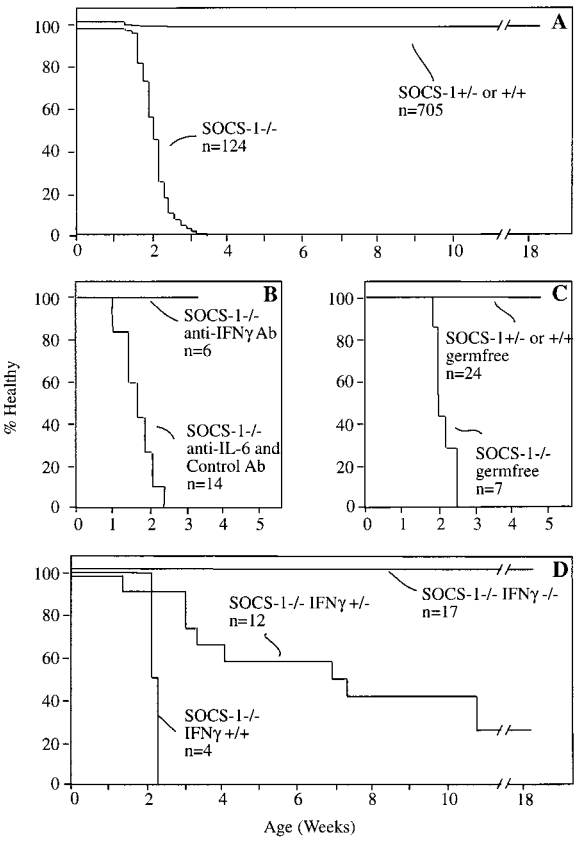

Figure 2. Development of Disease in *SOCS1*$^{-/-}$ Mice Is Dependent on IFNγ and Occurs in a Germ-Free Environment

(A) Rapid onset of morbidity of untreated *SOCS1*$^{-/-}$ but not *SOCS1*$^{+/+}$ or *SOCS1*$^{+/-}$ mice.

(B) Twice weekly injection of neonatal *SOCS1*$^{-/-}$ mice with anti-IFNγ but not anti-IL-6 or control antibody prevents morbidity.

(C) Rapid onset of morbidity in *SOCS1*$^{-/-}$ but not *SOCS1*$^{+/+}$ or *SOCS1*$^{+/-}$ mice born and reared in a germ-free environment.

(D) Prevention of morbidity in *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice and amelioration of morbidity in *SOCS1*$^{-/-}$ *IFNγ*$^{+/-}$ mice in comparison with *SOCS1*$^{-/-}$ *IFNγ*$^{+/+}$ mice. Seventeen *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice have been examined to date, with twelve animals being sacrificed for detailed histological and hematological analysis at 3 weeks of age and the remaining five being left for long-term observation.

in the presence of 100 ng/ml lipopolysaccharide. After 2 hr at all IFNγ concentrations, 30% to 50% of macrophages from both *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice were infected, and this percentage persisted for up to 48 hr in macrophages of both genotypes in the absence of cytokine (Figure 5A). The ability of IFNγ activation to enhance macrophage killing of *L. major* was demonstrable, because only 1% to 3% of cells from both *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice were still infected with live parasites 48 hr after treatment with 10 U/ml of IFNγ (Figure 5A). Strikingly, although ≤0.1 U/ml IFNγ had little or no effect on killing by *SOCS1*$^{+/+}$ macrophages, cells from *SOCS1*$^{-/-}$ mice maintained efficient killing when stimulated with as little as 0.01 U/ml IFNγ (Figure 5A), thus exhibiting at least 100-fold increased sensitivity to IFNγ. The hypersensitivity of macrophages from *SOCS1*$^{-/-}$ mice to IFNγ was confirmed by the demonstration of an increased capacity to produce nitric oxide, the major effector involved in the intracellular killing of *L. major* (data not shown).

The hyperresponsiveness of cells from *SOCS1*$^{-/-}$ mice to IFNγ in vitro was paralleled by an increased resistance of *SOCS1*$^{-/-}$ mice to viral infection in vivo. When 5-day-old mice were infected with Semliki forest virus, all *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice died within 2 to 3 days. However, *SOCS1*$^{-/-}$ mice largely survived this early postinfection period and only became ill 3 to 21 days after viral challenge (Figure 5B). This suggests an almost complete resistance to infection, because the survival pattern of infected and uninfected *SOCS1*$^{-/-}$ mice was similar.

IFNγ levels in the serum and in media conditioned by organs taken from *SOCS1*$^{-/-}$ or control littermate mice were investigated using ELISA and/or antiviral assays. At a limit of detection of 3 U/ml, we were unable to detect IFNγ in serum of mice of either genotype, and media conditioned by organs from *SOCS1*$^{-/-}$ mice did not reproducibly contain more IFNγ than those from their wild-type littermates.

Neonatal Death of *SOCS1*$^{-/-}$ Mice Occurs under Germ-Free Conditions

Because IFNγ was clearly central to the development of disease in *SOCS1*$^{-/-}$ mice, we were interested to determine whether subclinical infection in conventional mice might induce increased production of IFNγ and precipitate the early neonatal death of *SOCS1*$^{-/-}$ mice. Three litters of pups produced from the mating of *SOCS1*$^{+/-}$ mice were delivered by cesarean section into a strictly germ-free environment. As expected, *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ pups were among the progeny, and these were weaned normally and as adults exhibited enlarged ceca characteristic of mice in a germ-free environment. In contrast, germ-free *SOCS1*$^{-/-}$ pups died during the second and third weeks of life, the same age at which conventional *SOCS1*$^{-/-}$ mice die (Figure 2C). Analyses of seven of these moribund germ-free *SOCS1*$^{-/-}$ mice revealed that their organ pathology and peripheral blood abnormalities were identical to *SOCS1*$^{-/-}$ mice raised under conventional conditions.

Discussion

SOCS1 Is a Critical Regulator of IFNγ-Mediated Signal Transduction

SOCS1 is indispensable for survival beyond the postnatal period. Mice lacking this regulator die before weaning from a complex disease characterized by fatty degeneration of the liver, macrophage infiltration of several organs, and severe lymphopenia (Starr et al., 1998). We demonstrate here that this fatal syndrome is associated with the specific activation of STAT1 and elevated expression of IRF1, iNOS, and class I MHC, strongly implying that IFNγ signaling pathways are aberrantly active in vivo in the absence of SOCS1. Remarkably, all organ pathology was eliminated or markedly reduced, and death was prevented by treatment of neonatal *SOCS1*$^{-/-}$ mice with a neutralizing anti-IFNγ antibody. The amelioration of disease was specific to the inhibition of IFNγ because treatment of *SOCS1*$^{-/-}$ mice with neutralizing anti-IL-6 antibodies or control immunoglobulin had no

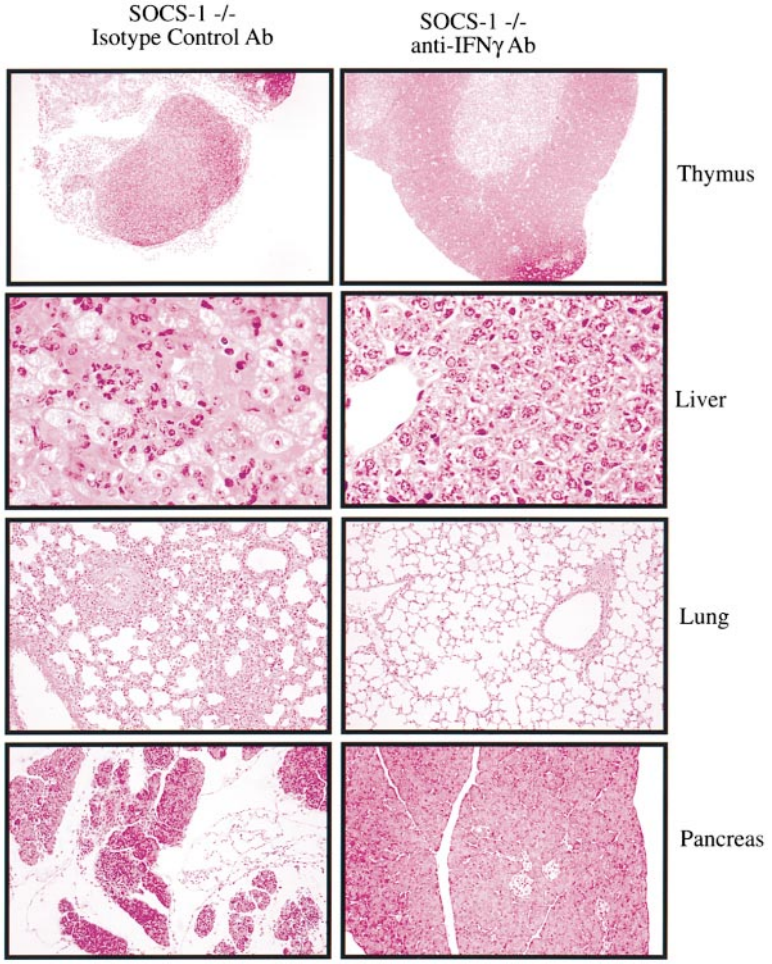

Figure 3. Prevention of Tissue Pathology in *SOCS1*$^{-/-}$ Mice by Treatment with Anti-IFNγ Antibody

Neonatal *SOCS1*$^{-/-}$ mice were treated twice weekly with either anti-IFNγ antibody or control antibody. Anti-IFNγ antibody–treated mice were healthy at 21 days of age and were sacrificed. Control antibody–treated mice developed disease between 9 and 16 days of age and were sacrificed when moribund. The normal histological appearance of organs from anti-IFNγ antibody-treated *SOCS1*$^{-/-}$ mice (right-hand panels) is contrasted with the atrophy of the cortex of the thymus, the fatty degeneration, hematopoietic infiltration and necrosis of the liver, macrophage accumulation around the bronchi and in the alveolar walls in the lung, and macrophage infiltration and acinar tissue destruction of the pancreas observed in *SOCS1*$^{-/-}$ mice treated with control antibody (left-hand panels).

protective effect. We were also unable to find any evidence of dysregulated activation of STAT3 in *SOCS1*$^{-/-}$ mice, the STAT protein used preferentially by inflammatory cytokines such as IL-6 and LIF (Ihle et al., 1994). In addition, the full spectrum of disease development was completely prevented in mice genetically deficient in *IFN*γ as well as *SOCS1*, and a delayed disease developed in *SOCS1*$^{-/-}$ mice that had only a single functional copy of the *IFN*γ gene. The absence of *SOCS1* correlated with marked in vitro hypersensitivity to IFNγ in macrophages infected with the intracellular parasite *L. major*. Hypersensitivity to IFNγ was also observed when nitric oxide (NO) production was measured following stimulation of macrophages with LPS and IFNγ. Likewise, hematopoietic cells from *SOCS1*$^{-/-}$ mice have also been found to be more susceptible to inhibition of proliferation by IFNγ but normally responsive to most hematopoietic cytokines that also act through the JAK/STAT pathway (Metcalf et al., 1999). Together these data clearly establish that SOCS1 is an essential physiological regulator of cellular sensitivity to IFNγ signaling.

Our results do not exclude the possibility that an elevated production of IFNγ in *SOCS1*$^{-/-}$ mice might contribute to disease development. However, within the sensitivity of the assays used, as in normal animals, we were unable to detect IFNγ in the circulation of *SOCS1*$^{-/-}$ mice. Media conditioned by organs of *SOCS1*$^{-/-}$ or normal mice also displayed no consistent differences in IFNγ concentrations. Finally, while *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice have remained disease free, some *SOCS1*$^{-/-}$ *IFN*γ$^{+/-}$ mice do die with a more slowly developing disease. The delayed disease in mice containing only a single functional *IFN*γ allele suggests that IFNγ concentrations may be limiting in these mice and therefore unlikely to be grossly elevated in *SOCS1*$^{-/-}$ mice. IFNγ is thought to be usually produced by T cells and NK cells in response to infection. Since *SOCS1*$^{-/-}$ mice succumb to disease in strictly germ-free conditions, the IFNγ required for disease development in *SOCS1*$^{-/-}$ mice might either represent basal production or be produced aberrantly. While our studies have clearly demonstrated an indispensable role for IFNγ in the multiorgan pathology of *SOCS1*$^{-/-}$ mice, the source of this cytokine and the identity of potential downstream effectors of IFNγ action remain to be examined.

Specificity of SOCS1 Action In Vivo

Previous in vitro data suggested that SOCS1 can directly interact with all four members of the JAK family, resulting in the inhibition of their catalytic activity (Endo et al., 1997; Naka et al., 1997). Consistent with this range of action, overexpression of SOCS1 in cell lines inhibited signal transduction from a large number of cytokines,

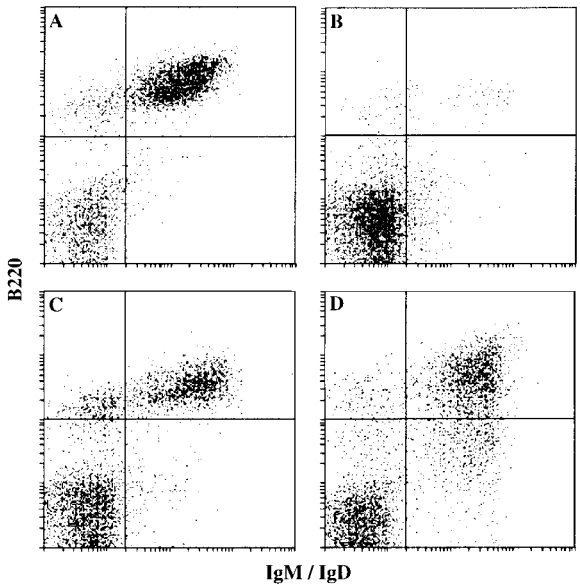

Figure 4. Prevention of B Lymphopenia in *SOCS1*$^{-/-}$ Mice by Treatment with Anti-IFNγ Antibody or Generation of *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ Mice

Spleen cells were collected from (A) wild-type mice, (B) *SOCS1*$^{-/-}$ mice treated with a control antibody, (C) *SOCS1*$^{-/-}$ mice treated with anti-IFNγ antibody, and (D) *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice. Cells were stained with anti-B220 antibody and a mixture of anti-IgM and anti-IgD antibodies and analyzed by FACS.

including IFNα, IFNβ, IFNγ, IL-6, LIF, OSM, thrombopoietin, growth hormone, and stem cell factor (SCF). Moreover, each of these cytokines is able to rapidly induce the synthesis of *SOCS1* mRNA (Endo et al., 1997; Starr et al., 1997; Adams et al., 1998; Auernhammer et al., 1998; Bjorbaek et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999; De Sepulveda et al., 1999).

Given the promiscuity of SOCS1 action in vitro, disease in mice lacking this protein might potentially have been due to deregulation of signals from a multitude of cytokines. Remarkably, however, the full spectrum of the complex disease in *SOCS1*$^{-/-}$ mice, including liver degeneration, hematopoietic infiltration of multiple tissues, and profound loss of T and B lymphocytes, can be attributed to a failure to regulate signal transduction by a single cytokine, IFNγ. This implies that in contrast to its actions in vitro, under physiological conditions SOCS1 has no indispensable role in the regulation of signal transduction from the majority of cytokines that utilize the JAK-STAT pathway.

As the in vitro studies all involved overexpression of SOCS1, it is possible that these approaches overestimated the breadth of SOCS action, simply because the level of *SOCS* gene expression achieved experimentally is likely to be far higher than expression controlled by an endogenous promoter. Moreover, ectopic expression may also be temporally inappropriate because physiological transcription of *SOCS* genes appears to be dependent on exposure to cytokine, allowing SOCS proteins to act in vivo only after signaling has commenced. However, although it is clear that a lack of SOCS1 causes a lethal hyperresponsiveness to IFNγ, abnormal responses to other cytokines may not be manifested so dramatically. Our studies to date suggest that responses to most hematopoietic cytokines are not altered in *SOCS1*$^{-/-}$ mice (Metcalf et al., 1999). However, the postnatal lethality in mice lacking this protein has prevented analysis of signaling by these or other cytokines in the adult mouse. Clearly, the availability of healthy *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice will now allow the full spectrum of in vivo SOCS1 activity to be definitively resolved.

Binding of IFNγ to its cell surface receptor triggers the activation of JAK1 and JAK2. While in vitro studies have shown SOCS1 is able to inhibit the function of both these kinases, the biochemical specificity of SOCS1 action in vivo remains to be determined. The inhibition of JAK activity by SOCS1 may regulate IFNγ signaling in several ways, for example, by determining the threshold concentration of IFNγ capable of triggering a biological response or by regulating the magnitude or length of a response to a particular concentration of IFNγ. Distinguishing between these possibilities will require a careful comparison of the capacity of different doses of IFNγ to induce JAK1 and JAK2 activation, IFNγ receptor phosphorylation, STAT1 activation, and expression of STAT1-regulated genes in wild-type and SOCS1-deficient cells.

As SOCS1 is a member of a larger family of related proteins, it is also feasible that a regulatory role for this protein in other cytokine signaling pathways is not evident in *SOCS1*$^{-/-}$ mice due to the compensatory actions of other SOCS proteins. In vitro studies have clearly shown that SOCS1 and SOCS3 have overlapping activities, and both molecules are induced by and inhibit the actions of a similar spectrum of cytokines when overexpressed (Adams et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999). However, for regulation of IFNγ signaling, SOCS1 appears to be considerably more active than SOCS3 (Sakamoto et al., 1998; Song and Shuai, 1998). Consistent with these in vitro studies, the phenotype of *SOCS1*$^{-/-}$ mice reveals key roles for SOCS1 in regulating IFNγ responses that cannot be compensated by SOCS3 in vivo. Whether SOCS3 also acts to regulate a unique set of cytokine signal transduction pathways and whether there are other pathways regulated by both SOCS1 and SOCS3 under physiological conditions remains to be clarified.

SOCS1 Balances the Beneficial and Potentially Deleterious Actions of IFNγ

The potentially devastating actions of IFNγ in vivo have been well established in studies in which IFNγ levels have been experimentally elevated (Gresser et al., 1981, 1987; Gresser, 1982), and these pathologies were faithfully reproduced in mice lacking SOCS1. In addition, inappropriate IFNγ production and action is implicated in the pathogenesis of disease following certain infections. In a mouse transgenic model of hepatitis B, the destruction of liver cells and resultant morbidity appears to be caused by excess endogenous IFNγ (Ando et al., 1993). In other models of infection, however, the actions of IFNγ are beneficial (Huang et al., 1993; Newport et al., 1996; Lu et al., 1998), and this action is also amplified in mice lacking SOCS1. SOCS1-deficient macrophages

Table 2. Influence of IFNγ on the Development of Histological Changes in Organs of *SOCS1*$^{-/-}$ Mice

SOCS1 genotype	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$
Interferon γ genotype	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{+/+}$	*IFN*γ$^{-/-}$	*IFN*γ$^{-/-}$
Treatment	Untreated	Untreated	Anti-IFNγ Ab	Anti-IFNγ Ab	Untreated	Untreated
Age (days)	12–21	12–21	21	21	21	21
Liver						
Fatty degeneration	0/21	12/18	0/22	0/6	0/8	0/12
Necrosis	0/21	6/18	0/22	0/6	0/8	0/12
Excess hematopoiesis	0/21	18/18	0/22	0/6	0/8	0/12
Lung						
Cuffing by mϕ and neut	1/21	17/18	0/22	2/6	0/8	2/12
Heart						
Infiltration by mϕ and neut	0/21	15/17	0/19	1/6	0/8	0/12
Kidney						
Glomerular immaturity	2/21	16/18	1/18	0/6	1/8	1/12
Tubular immaturity	6/21	11/18	0/18	0/6	0/8	0/12
Spleen						
Lymphoid follicles	20/21	1/19	21/21	5/5	8/8	12/12
Excess erythropoiesis	1/21	15/17	0/21	4/5	3/8	9/12
Thymus						
Cortical atrophy	0/21	15/16	0/22	1/5	0/8	0/12
Medullary enlargement	1/21	1/16	0/22	1/5	1/8	8/12
Pancreas						
Acinar atrophy	2/19	16/18	0/22	0/5	0/8	0/12
Infiltration by mϕ	0/19	13/18	0/22	0/5	0/8	0/12
Skin						
Mϕ infiltration	0/20	14/17	0/10	1/4	0/8	0/12
Epidermal thickening	0/20	15/17	0/10	0/4	0/8	0/12
Keratinization	0/20	14/17	0/10	0/4	0/8	0/12
Eosinophil infiltration	0/20	6/17	0/10	0/4	0/8	0/12
Marrow						
Monos > granulocytes	16/20	2/18	16/19	3/5	8/8	12/12
Granulocytes > monos	4/20	16/18	3/19	2/5	0/8	0/12

The ratios indicate the number of organs showing a particular lesion versus the total number of organs examined. The data should be read by pairwise comparison of column 1 versus column 2, column 3 versus column 4, and column 5 versus column 6. Note that the mice in columns 1 through 4 were generated by crossing *SOCS1*$^{-/+}$ mice on an otherwise wild-type background and are therefore all *IFN*γ$^{+/+}$, whereas the mice in columns 5 and 6 were generated by crossing *SOCS1*$^{-/+}$ *IFN*γ$^{-/+}$ mice or *SOCS1*$^{-/-}$ *IFN*γ$^{-/-}$ mice, and their identity was confirmed by genotyping. Not all organs were sectioned from all animals. Neut, neutrophil; monos, mononuclear cells.

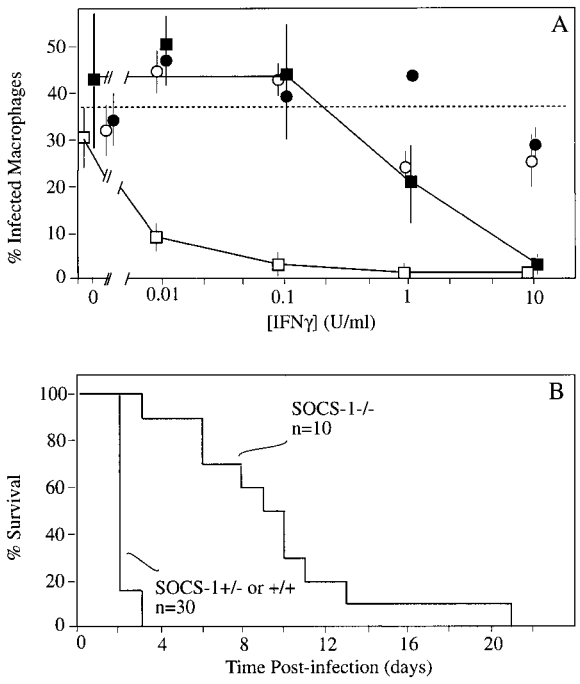

Figure 5. Absence of SOCS1 Renders Cells Hyperresponsive to IFNγ and Mice Hyperresistant to Semliki Forest Virus Infection

(A) Macrophages from the bone marrow of *SOCS1*$^{+/+}$ (filled symbols) and *SOCS1*$^{-/-}$ mice (open symbols) were stimulated with 100 ng/ml LPS and the indicated concentration of IFNγ and infected with *L. major*. After 2 hr (circles) and 48 hr (squares), the percentage of macrophages containing parasites was determined.

(B) Mice generated by crossing *SOCS1*$^{+/-}$ mice were infected with Semliki forest virus at 5 days of age and monitored.

stimulated with IFNγ killed intracellular parasites considerably more efficiently than their wild-type counterparts, and *SOCS1*$^{-/-}$ mice were more resistant than normal mice to Semliki forest virus infection. Clearly, the actions of IFNγ in vivo require exquisite control. The data presented here establish that SOCS1 is central to the balance between the beneficial and potentially harmful effects of IFNγ. While the behavior of neonatal mice may not necessarily predict the occurrence of similar responses to overstimulation by IFNγ in humans, these observations nevertheless raise the prospect that small molecule mimetics or antagonists of SOCS1 might prove clinically valuable in enhancing the actions of IFNγ in combating viral and parasitic infection or in dampening its pathological side effects in other disease states.

Experimental Procedures

Generation and Maintenance of Mice and Injection of Antibodies, Cytokine, and Virus

SOCS1$^{-/-}$ mice were generated as described previously (Starr et al., 1998) and maintained on a mixed 129/Sv and C57BL/6 genetic background. *IFN*γ$^{-/-}$ mice on an inbred C57BL/6 background (C57BL/6-Ifng^{tm1Ts}) were obtained from the Jackson Laboratories via Monash University (Dalton et al., 1993). Mice were routinely housed in clean but not specific pathogen-free conditions (SPF) at the Walter and Eliza Hall Institute of Medical Research. To raise mice under germ-free conditions, pups were delivered by cesarean section and placed with BALB/c foster mothers that had been maintained in germ-free microisolators for several generations. The sterility of this environment was monitored closely and tested continually for the absence of bacterial organisms and a variety of viral pathogens.

Mice were genotyped by Southern blot analysis of genomic DNA obtained from tail tips, as described (Starr et al., 1998). For analysis of the *SOCS1* gene, genomic DNA was digested with EcoRI and filters were probed with a 1.5 kilobase pairs (kbp) EcoRI/HindIII fragment of the mouse *SOCS1* gene, which hybridizes to a 5.3 kbp band for the wild-type allele and an 8.0 kbp fragment for the null allele. For analysis of the *IFN*γ gene, DNA was cut with BamHI and filters were probed with a 450 bp PstI fragment of the mouse *IFN*γ cDNA that detects a fragment of 11.0 kbp for the wild-type allele and 13.0 kbp for the null allele.

Neonatal mice were injected intraperitoneally twice weekly for up to 3 weeks with 16 mg/kg of neutralizing rat anti-IFNγ antibody (R4-6A2; American Type Culture Collection, Manassas, VA), 200 mg/kg of an ammonium sulfate–precipitated neutralizing polyclonal rabbit anti-IL-6 antisera, as described (Liu et al., 1995), 16 mg/kg control purified rat IgG antibody (Sigma Chemical Co., St. Louis, MO), or 200 mg/kg ammonium sulfate–precipitated preimmune rabbit sera and were sacrificed when moribund or at the end of the experiment. The efficacy of anti-IL-6 treatment was verified at the end of the experiment by testing serum from injected mice for its capacity to inhibit IL-6-induced differentiation of M1 cells. In a titration experiment, a 1 in 100 dilution of serum from mice injected with the anti-IL-6 but not control antibody was found to inhibit 60 ng/ml of IL-6.

In some experiments, 5-day-old mice were injected intraperitoneally with 50 μl of Semliki forest virus containing 100 times the virus dose that kills 50% of fibroblasts in vitro. The mice were monitored daily and sacrificed when moribund.

IFNγ Assays

IFNγ in serum was assayed using cytopathic effect reduction with mouse L929 cells as targets for Semliki forest virus challenge as described (Hertzog et al., 1991). A standard cytokine sandwich ELISA was also used to quantitate IFNγ in mouse serum and organ-conditioned media. IFNγ-specific antibodies (R4-6A2 as the capture antibody, XMG1.2 for detection) were obtained from Pharmingen (San Diego, CA), and the assay was performed essentially as described by the manufacturer.

Hematological Analysis, Histology, and Immunohistochemistry

Hematological analyses were performed as described (Starr et al., 1998; Metcalf et al., 1999). For histological examination, tissues were fixed in 10% (v/v) formalin in phosphate-buffered saline (PBS) and sections were prepared and stained by standard techniques (Starr et al., 1998). Tissue preparation for immunohistochemistry was carried out as described (Thomas et al., 1998), and sections were stained with a rat anti-mouse class I MHC antibody, 34-12S, and a peroxidase-labeled secondary antibody. Sections were counterstained with Giemsa (Ozato et al., 1982).

Culture of Macrophages and Leishmanicidal Assays

To obtain bone marrow–derived macrophages, femoral bone marrow cells collected in PBS were dispersed, centrifuged, and resuspended in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 ng/ml murine macrophage colony-stimulating factor (M-CSF; Cetus Corporation, Emeryville, CA) and 10% (v/v) fetal bovine serum (FBS). After 3 days of culture in a humidified incubator at 37°C in 5% (v/v) CO_2 in air, medium and nonadherent cells containing an expanded precursor population were resuspended at 10^4 cells/ml and cultured for an additional 5 days in the presence of M-CSF, after which a relatively pure and homogeneous population of adherent mature bone marrow macrophages were present.

The cloned line of *L. major* LRC-L137 V 121 has been described in detail (Handman, 1983). Promastigotes were grown in M199 medium with 10% FBS and were in the stationary phase of growth. Infection of macrophages was carried out using a modification of a method described previously (Proudfoot et al., 1995). Briefly, 5×10^4 bone marrow–derived macrophages were transferred onto glass coverslips in 24-well trays, allowed to adhere for 24 hr, and stimulated with 100 ng/ml LPS and various concentrations of IFNγ. After 6 hr of cytokine stimulation, cells were infected at a ratio of five parasites

per cell for 2 hr at 37°C. The free parasites were removed by vigorous washing before incubation for a further 24 or 48 hr, after which cells were stained with Giemsa. The percentage of macrophages infected with parasites was determined at each time point, with at least 400 cells per sample counted.

Northern Blots

Following sacrifice, organs were removed from neonatal wild-type and *SOCS1*$^{-/-}$ mice. PolyA^{+} mRNA was purified, and Northern blot hybridization was performed essentially as described (Alexander et al., 1995). Probes used were as follows: a 1.1 kbp PstI fragment of the chicken *glyceraldehyde 6-phosphate dehydrogenase* (*GAPDH*) cDNA, a 2 kbp EcoRI fragment of the mouse *IRF1* gene, and a 1.8 kbp NcoI fragment of the mouse *iNOS* cDNA.

Flow Cytometry

Single-cell suspensions of femoral bone marrow, thymocytes, and splenocytes were prepared and erythrocytes were lysed by incubation in 156 mM ammonium chloride (pH 7.3) at 37°C for 3 min. The cells were stained with a biotinylated or FITC-conjugated rat monoclonal antibody specific for the cell surface markers of interest (Class I MHC, CD4, CD8, surface IgM, surface IgD, B220, or Mac1), followed where necessary by streptavidin-phycoerythrin and analyzed by flow cytometry as described (Strasser et al., 1991).

Electrophoretic Mobility Shift Assays

Nuclei were extracted from the livers of unmanipulated *SOCS1*$^{-/-}$ mice and control littermates or from mice 15 min after intraperitoneal administration of 2 μg IFNγ (Ruff-Jamison et al., 1993). Electrophoretic mobility shift assays (EMSA) were performed on 1–2 μg of nuclear protein using the m67 oligonucleotide probe as described (Novak et al., 1995).

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