nature immunology

Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity

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Suppressor of cytokine signaling 1 (SOCS1) is a critical regulator of cytokine signaling and immune responses. SOCS1-deficient mice develop severe inflammatory disease, but are very resistant to viral infections. Using neutralizing antibody to type I interferon (IFN-α and IFN-β) and mice deficient in interferon-γ or type I interferon receptor components (IFNAR1 or IFNAR2), we demonstrate here that SOCS1 deficiency amplified type I interferon antiviral and proinflammatory actions independently of interferon-γ. The mechanism of the suppression of type I interferon responses by SOCS1 was distinct from that of other cytokines. SOCS1 associated with and regulated IFNAR1- but not IFNAR2-specific signals, abrogating tyrosine phosphorylation of transcription factor STAT1 and reducing the duration of antiviral gene expression. Thus, SOCS1 is an important *in vivo* inhibitor of type I interferon signaling and contributes to balancing its beneficial antiviral versus detrimental proinflammatory effects on innate immunity.

Appropriate regulation of cytokine signaling is critical not only in maintaining homeostasis but also in the response to viral and bacterial infections and injury. Conversely, inappropriate or unrestrained cytokine action can result in unwanted toxicity, leading to neurological damage, growth suppression, cancer, inappropriate activation of the innate and acquired immune systems and ultimately chronic inflammatory disease, autoimmunity and sometimes death[1]. The interferons are cytokines that were discovered by virtue of their antiviral actions and were subsequently demonstrated to regulate cell proliferation and differentiation and to activate most effector cells of the immune system[2,3]. Interferons are classified into two types based on sequence homology, the agents that induce their production, their cellular origin and their use of distinct receptor systems. Type I interferons include the various IFN-α subtypes, IFN-β, IFN-ϖ and limitin, which all act through the receptor components designated IFN-α receptor 1 (IFNAR1) and IFNAR2 (refs. 3–6). Type I interferons are produced by a variety of cell types, including leukocytes, fibroblasts and plasmacytoid dendritic cells, in response to virus and other infectious agents[7]. In contrast, the single type II interferon, IFN-γ, acts through receptor components IFN-γ receptor 1 and IFN-γ receptor 2 and is produced mainly by T cells, natural killer cells and natural killer T cells in response to cytokines such as interleukin 12 (IL-12)[8,9]. Both classes of interferon activate two Janus kinases (Jaks) that are preassociated with a particular receptor chain: TYK2 and Jak1 for type I interferons, and Jak1 and Jak2 for type II interferons. In turn, this leads to activation of complexes of signal transducer and activator of transcription (STAT) factors: interferon-stimulated gene factor 3 (STAT1, STAT2 and interferon-regulatory factor 9) for type I interferons, and γ-activated factor (STAT1 homodimers) mainly for IFN-γ[10,11]. The interferons have properties such as potent antiviral activity and activation of immune responses that are clearly beneficial to the host. However, they also have undesirable side effects demonstrated by their dose-limiting toxicity in clinical trials in humans and neonatal toxicity in mice[1,12]. Therefore, balancing the potentially beneficial and detrimental effects of interferons is likely to be crucial to mounting a successful immune response to disease.

In addition to the cytoplasmic factors that drive signal transduction, there are negative regulators that inhibit or attenuate signaling. These include phosphatases, protein inhibitors of activated STAT proteins, and suppressor of cytokine signaling (SOCS) proteins[13–17]. The SOCS family of proteins includes eight members, designated SOCS1–SOCS7 and CIS (cytokine-inducible Src homology 2–containing protein), which inhibit signaling by binding to key phosphotyrosine residues in Jaks or cytokine receptor cytoplasmic domains and targeting them for proteasomal degradation[16,18–25].

The expression of *Socs1* and *Socs3* but neither *Socs2* nor *Cish* is inducible by IFN-α, IFN-β and IFN-γ; however, their expression is also induced by other cytokines, including growth hormone, IL-6,

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Received 2 August; accepted 4 October; published online 27 November 2005; doi:10.1038/ni1287

IL-3, IL-13, granulocyte-macrophage colony-stimulating factor, leukemia inhibitory factor, erythropoietin, IL-12 and leptin. Likewise, when constitutively overexpressed, SOCS1 and SOCS3 inhibit signaling in response to cytokines such as interferons and other cytokines acting through the Jak-STAT pathway[15,17,18,26–28]. The *in vivo* function of SOCS1 has been explored through the generation of mice with null alleles of *Socs1*. SOCS1-deficient mice die during the neonatal period with severe hepatotoxicity, multiorgan inflammation and aberrant hematopoiesis[29,30]. This phenotype requires IFN-γ, as the generation of mice deficient in both SOCS1 and IFN-γ or the injection of $Socs1^{-/-}$ mice with a neutralizing antibody to IFN-γ 'rescues' the lethal neonatal $Socs1^{-/-}$ phenotype[31]. Subsequent studies have established that mice lacking SOCS1 show both increased production of and heightened sensitivity to IFN-γ, which contribute to the perinatal lethality[32,33]. Those studies have been instrumental in demonstrating the dire consequences of 'unchecked' IFN-γ signaling. However, the lethal IFN-γ-mediated phenotype of SOCS1-deficient mice may be masking the *in vivo* importance of SOCS1 in regulating the responses to other cytokines, particularly the type I interferons.

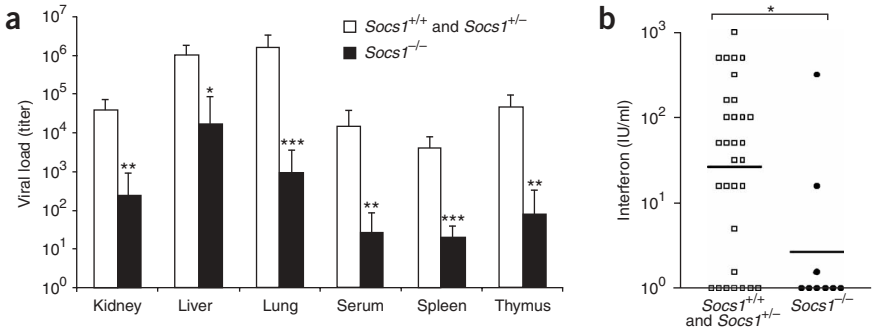

Figure 1 Viral replication and interferon secretion from SFV-infected neonatal mice with targeted mutation of *Socs1*. Neonatal mice from $Socs1^{+/-}$ matings were injected with SFV at 10× $TCID_{50}$ between 5 and 10 d of age. Neonates were monitored for 48 h, at which time they were killed and organs and serum were collected. (**a**) Viral titers, measured by CPE assay and expressed as mean log titer ± s.e.m. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, $Socs1^{-/-}$ neonatal mice ($n = 9$) versus their wild-type and heterozygous littermates ($n = 40$; Mann-Whitney rank sum tests). (**b**) Serum interferon, measured by CPE-reduction assay. Each point represents an individual mouse; horizontal lines indicate mean concentrations. *, $P < 0.05$, $Socs1^{-/-}$ neonatal mice ($n = 9$) versus their wild-type and heterozygous littermates ($n = 31$; Mann-Whitney rank sum test).

In this study, we demonstrate that mice with a targeted mutation in *Socs1* had heightened antiviral responses, resulting in decreased viral load and increased survival after infection, traits that are indicative of amplified protective effects of interferons. Using $Socs1^{-/-}$ neonatal mice, neutralizing antibody to type I interferon and crosses to $Ifng^{-/-}$ mice or $Ifnar1^{-/-}$ mice, we show that these beneficial effects were a consequence of amplification of type I interferon antiviral actions independently of IFN-γ. The $Socs1^{-/-}Ifnar1^{-/-}$ mice also survived beyond weaning and did not demonstrate the dire inflammation associated with SOCS1 deficiency. Thus, SOCS1 also acts to suppress the proinflammatory effects of type I interferons. Unlike the production of IFN-γ, the production of type I interferons was not suppressed by SOCS1; however, cells lacking SOCS1 showed a prolonged STAT1 phosphorylation and antiviral interferon-stimulated gene stimulation in response to type I interferon. Comparison of the result of crosses of $Socs1^{-/-}$ mice with $Ifnar2^{-/-}$ or $Ifnar1^{-/-}$ mice demonstrated a unique specificity of SOCS1 action on signaling through only the IFNAR1 chain of the receptor. Consistent with that observation, we demonstrate here by coimmunoprecipitation that SOCS1 associated with IFNAR1. Thus, SOCS1 uses a unique and very specific mechanism to regulate type I interferon signaling via IFNAR1 and contributes *in vivo* to balancing its various effects on innate immunity. Therefore, SOCS1 modulation may be a useful adjunct to selective antiviral therapy.

RESULTS

Amplified antiviral responses in $Socs1^{-/-}$ neonatal mice

$Socs1^{-/-}$ neonatal mice survive substantially longer than their wild-type and heterozygous littermates after infection with Semliki Forest virus (SFV); however the mechanisms of enhanced viral resistance have remain unexplored[31]. To address that issue, we measured viral loads in the serum, spleens, kidneys, livers, thymuses and lungs of $Socs1^{-/-}$ and wild-type neonatal mice 48 h after infection with SFV. Viral titers in the serum and in organs including the lung, thymus, spleen, kidney and liver in the $Socs1^{-/-}$ mice were 0.1–1% those in wild-type and heterozygous littermates (**Fig. 1a**). The reduced viral load in $Socs1^{-/-}$ organs suggested that the improved survival of the SFV infected $Socs1^{-/-}$ mice resulted from an increased ability to resolve the viral infection.

SOCS1 does not affect IFN-α or IFN-β production

The reduced viral load in $Socs1^{-/-}$ mice could have been due to either increased production of interferons after viral infection or increased antiviral response of cells to similar amounts of interferon. In untreated healthy mice, both type I and II interferon is generally undetectable in serum. However, after viral challenge, expression of type I interferons is induced rapidly, resulting in readily detectable amounts of these cytokines[34]. We measured interferon antiviral activity by cytopathic effect (CPE)–reduction bioassay of serum samples from SFV-infected neonatal mice. After viral infection, interferon was detected in serum from 77% (24 of 31) wild-type and $Socs1^{+/-}$ mice, with concentrations ranging from about 10 to 1,000 IU/ml with a mean of 30 IU/ml. In contrast, interferon activity was detected in the serum of only 33% (three of nine) $Socs1^{-/-}$ mice, with a mean concentration of 3 IU/ml, ranging from 0 to 300 IU/ml (**Fig. 1b**). Thus, the serum interferon concentrations in $Socs1^{-/-}$ mice after viral infection were significantly lower than those in wild-type mice ($P<0.05$). Therefore, the increased resistance of $Socs1^{-/-}$ mice to SFV challenge probably resulted from an increased sensitivity to interferons.

Viral resistance in $Socs1^{-/-}$ mice is IFN-γ independent

Because the increased viral resistance of $Socs1^{-/-}$ mice was not due to increased interferon production after viral infection, we next investigated the regulation by SOCS1 of antiviral actions of interferons. Although SOCS1 has been shown by overexpression studies *in vitro* to suppress the antiviral effects of both type I and II interferons[35], the *in vivo* effects have not been characterized. Because SOCS1 regulates other responses to IFN-γ *in vivo*, affecting molecular signaling, hematopoiesis, inflammatory and immune responses[36–38], we addressed whether the increased antiviral response in $Socs1^{-/-}$ mice could be attributed to an increased sensitivity to IFN-γ[11,39,40]. We first examined whether removal of endogenous IFN-γ affected the outcome of SFV infection. We infected $Ifng^{-/-}$ and wild-type neonatal

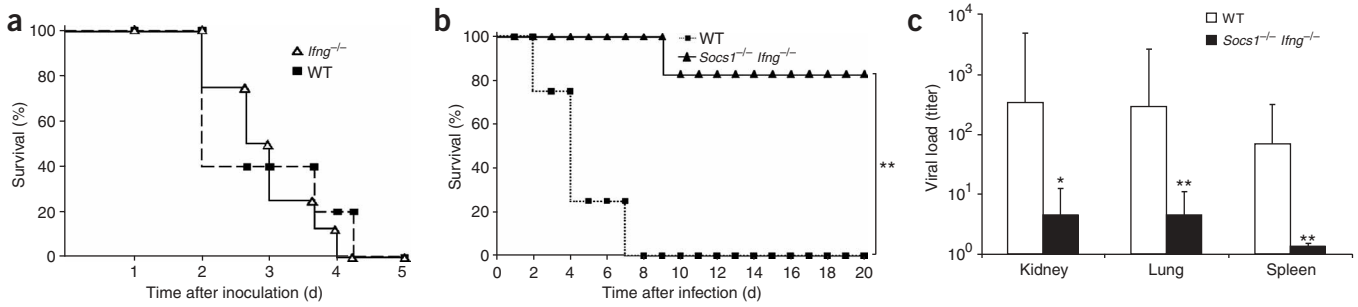

Figure 2 IFN-γ responses do not affect the 'hyper-resistance' of *Socs1*$^{-/-}$ mice to viral infection. (**a**) Resistance to infection with SFV at 10× $TCID_{50}$ in wild-type (WT; $n = 13$) and *Ifng*$^{-/-}$ ($n = 16$) neonatal mice between 4 and 11 d of age. $P > 0.05$, wild-type versus *Ifng*$^{-/-}$ (log-rank test). (**b**) *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice show more resistance in response to SFV than do wild-type neonatal mice. Survival plot reports resistance to infection with SFV at 10× $TCID_{50}$ in wild-type ($n = 4$) and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ ($n = 6$) neonatal mice between 5 and 10 d of age. **, $P < 0.01$ (log-rank test). In **a**,**b**, the health of neonatal mice was monitored and all fatalities were recorded. (**c**) Viral titers of homogenates of kidneys, lungs and spleens of in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ ($n = 6$) and wild-type ($n = 4$) neonatal mice injected with SFV at 30× $TCID_{50}$. Neonatal mice were monitored for 48 h, at which time they were killed and organs were collected. Viral titers were measured by CPE assay and are expressed as mean log titer ± s.e.m. *, $P < 0.05$, and **, $P < 0.01$, titers of wild-type mice versus *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice. (Mann-Whitney rank sum tests.) Data are representative of one experiment.

mice with SFV at 10× the half-maximal tissue culture infectious dose ($TCID_{50}$; the concentration of virus that will kill 50% of cells) and measured resistance to the virus as survival after infection. There was no significant difference in the survival of wild-type neonatal mice and *Ifng*$^{-/-}$ mice ($P > 0.05$). All mice of both genotypes were dead by 4.5 d after infection (**Fig. 2a**), indicating that IFN-γ does not have substantial involvement in the resistance of mice to SFV infection.

The survival curve of infected *Socs1*$^{-/-}$ mice closely follows that of untreated *Socs1*$^{-/-}$ mice[31], suggesting that these mice may be almost completely resistant to the SFV infection and were in fact succumbing to the inflammatory disease associated with SOCS1 deficiency. To overcome the problem of the inflammation-mediated death of *Socs1*$^{-/-}$ mice before weaning, we took advantage of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice, which fail to produce IFN-γ, are healthy and survive to adulthood[31]. *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice seemed almost completely resistant to infection with SFV, with 84% of infected neonatal mice (five of six) surviving past 22 d. In contrast, none of the infected wild-type mice survived beyond 7 d of infection (zero of four; $P < 0.01$; **Fig. 2b**). Similar to the titers of *Socs1*$^{-/-}$ mice, viral titers in the organs of SFV-infected *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice were approximately 1% those of wild-type control mice (**Fig. 2c**). We noted similar trends in experiments using a higher dose of virus (30× $TCID_{50}$; data not shown). These results suggest that the double-knockout mice were able to clear the virus from most organs more efficiently than wild-type control mice. Because these trends were similar to that demonstrated for *Socs1*$^{-/-}$ mice, the suppression of antiviral effects and enhanced resistance to infection by SOCS1 is clearly independent of IFN-γ.

Type I IFN mediates SFV resistance in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice

The results presented above suggested that type I interferons mediated the heightened antiviral response noted in both the *Socs1*$^{-/-}$ and the *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice. To establish directly the involvement of type I interferons, we injected 1,000 IU of neutralizing antibody to type I IFN (which recognizes both IFN-α and IFN-β) into the peritoneal cavities of 5- to 10-day-old *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice 6 h before SFV infection. We then monitored the survival of mice for 10 d as described above. In the absence of antibody treatment, the survival of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice was 100% after 3 d, 75% after 4 d and 55% after 10 d of viral infection. Pretreatment of *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice with neutralizing antibody to type I IFN reversed the relative resistance of these mice to viral infection and resulted in increased sensitivity to SFV, with all mice dead by 4 d, similar to wild-type mice (**Fig. 3a**).

To demonstrate by an independent method that SOCS1 regulates type I interferons, we generated mice lacking both SOCS1 and IFNAR1 and assessed the survival of 5- to 10-day-old mice in response to SFV. *Ifnar1*$^{-/-}$ mice are reported to have reduced type I interferon signaling and to be highly susceptible to viral infection[41,42]. *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice infected in this experiment demonstrated 100% survival at 4 d, 90% survival at 5 d and 50% survival at 10 d after infection. When we crossed *Socs1*$^{-/-}$ mice with *Ifnar1*$^{-/-}$ mice, however, the offspring were more sensitive to infection, with 65% survival at 4 and 5 d after infection and 0% survival at 6 d after infection ($P < 0.001$; **Fig. 3b**). These data collectively suggest that SOCS1 regulation of type I but not type II interferon antiviral action has a profound effect on viral replication (by up to 1,000-fold) and survival after infection.

Mechanism of SOCS1 suppression of type I IFN signaling

To examine the mechanism of SOCS1 regulation of type I interferon responses *in vivo* independently of IFN-γ, we first examined the duration of tyrosine phosphorylation of STAT1, a primary mediator of interferon-induced antiviral activity[10]. Treatment of bone marrow macrophages (BMMs) from *Ifng*$^{-/-}$ mice with IFN-α1 resulted in a transient increase in tyrosine phosphorylation of STAT1, which began to decrease by 60 min, was barely detectable after 80 min (**Fig. 4a**) and was not detectable after 100 min (data not shown). In contrast, BMMs deficient in SOCS1 reproducibly demonstrated prolonged STAT1 tyrosine phosphorylation that was still strongly detectable 80 min (**Fig. 4a**) and 100 min (data not shown) after treatment. Notably, STAT1 serine phosphorylation was unaffected by the presence of SOCS1 (data not shown), consistent with inactivation of a tyrosine kinase associated with IFNAR, such as a Jak, by SOCS1.

To determine whether the actions of SOCS1 affected interferon-stimulated antiviral gene activity, we next monitored the activity of a type I interferon–inducible, antiviral enzyme, 2′-5′-oligoadenylate synthetase (2′-5′-OAS), which is regulated by STAT1 via IFNAR-mediated signaling[42]. This assay has the advantage of being able to accurately quantify basal as well as inducible enzyme activity. The basal 2′-5′-OAS activity in *Socs1*$^{-/-}$ cells was not significantly different from that of wild-type cells (275.2 ± 43.9 and 356.4 ± 35.27 mmol/μg protein in wild-type ($n = 3$) and *Socs1*$^{-/-}$ ($n = 5$) mouse embryo fibroblasts, respectively; $P < 0.05$). After stimulation with IFN-α4, the

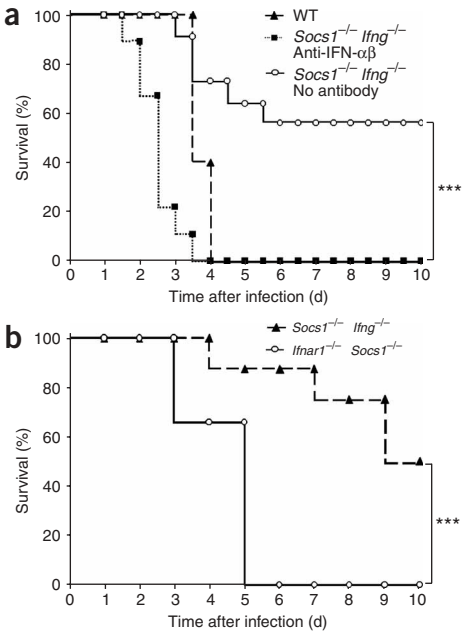

Figure 3 Type I interferon mediates resistance to SFV in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice. (**a**) Survival after SFV infection in *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice is due to the type I interferon antiviral response. *Socs1*$^{-/-}$*Ifng*$^{-/-}$ neonatal mice between 5 and 10 d of age were divided into two groups, control ($n = 11$) and test ($n = 9$). The test group was given 1,000 IU sheep anti-type I IFN (anti-IFN-α/β); the control group was injected with PBS. After 6 h, both groups were infected with SFV at 100× $TCID_{50}$ and were monitored. As a reference, wild-type neonatal mice (WT; $n = 5$) of the same age were infected with SFV at 100× $TCID_{50}$ and were monitored. ***, $P < 0.001$, *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice with anti-type I IFN versus no antibody (log-rank test). (**b**) Deletion of *Ifnar1* in *Socs1*$^{-/-}$ neonatal mice decreases their resistance to viral infection. Survival plot measures the resistance to infection with SFV at 30× $TCID_{50}$ in *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ ($n = 3$) and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ ($n = 8$) neonatal mice between 5 and 10 d of age. The health of neonatal mice was monitored and fatalities were recorded. ***, $P < 0.001$ (log-rank test).

activity in wild-type mouse embryo fibroblasts peaked after 72 h and thereafter decreased steadily to approximately 40% of the maximum value by 144 h (**Fig. 4b**). In contrast, although 2′-5′-OAS activity reached a similar peak in *Socs1*$^{-/-}$ mouse embryo fibroblasts after 48 h, the activity was maintained at this peak for the entire time course ($P < 0.05$; **Fig. 4b**) without the decrease noted in wild-type cells. These results show that although SOCS1 had no apparent effect on the amplitude of the IFN-α induction of this interferon-stimulated gene, it was crucial in its temporal regulation, acting via IFNAR on receptor-proximal signaling events that regulate the duration of subsequent interferon-stimulated gene activation.

Because type I interferons transduce signals through the IFNAR1 and IFNAR2 components each with specific interaction domains for signal-transducing proteins, we compared and characterized mice deficient in SOCS1 and either IFNAR1 or IFNAR2 to categorize the SOCS1 effects on IFNAR signaling. The survival of uninfected *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice was different from that of *Socs1*$^{-/-}$ mice, with no deaths at 3 weeks of age, when all *Socs1*$^{-/-}$ mice had died (**Fig. 5a**). Histological examination of organs from the *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice showed that these mice did not have the familiar 'inflammatory syndrome' of the *Socs1*$^{-/-}$ mice (data not shown), demonstrating the important contribution to this phenotype of signaling through IFNAR1. In contrast, *Ifnar2*$^{-/-}$*Socs1*$^{-/-}$ mice were all dead by 3 weeks of age, similar to the original *Socs1*$^{-/-}$ mice (**Fig. 5a**). Because this phenotype was unexpectedly different from the survival pattern of *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice, it demonstrates that signals transduced through the IFNAR2 receptor component are not influenced by SOCS1. These results represent an important advance in elucidating the mechanism of SOCS1 action *in vivo*. In regulating the type I interferon system, SOCS1 interacts with only one receptor chain, IFNAR1 and/or its associated signal-transducing proteins.

Because the result reported above constituted compelling genetic evidence for an association of SOCS1 with IFNAR1, we next sought to demonstrate this association directly. First, we coimmunoprecipitated IFNAR1 and Flag-tagged SOCS1 overexpressed in 293T cells (**Fig. 5b**). Notably, we then demonstrated association of endogenous SOCS1 with IFNAR1 by coimmunoprecipitation from IFN-α1-treated BMM lysates using a monoclonal antibody to immunoprecipitate IFNAR1 and a monoclonal antibody to SOCS1 to detect this protein by immunoblot (**Fig. 5c**). Thus, our results demonstrate that SOCS1 interacts endogenously with IFNAR1 after stimulation and that this interaction is independent of Jak1.

DISCUSSION

In this study we have demonstrated that SOCS1 is crucial in attenuating type I interferon signaling *in vivo* and hence in limiting the host response to viral infection. Mice and cells lacking SOCS1 responded to type I interferons for longer to limit viral replication and to clear virus more efficiently and, in doing so, allowed survival of an otherwise lethal infection. Unlike the situation with IFN-γ, in which SOCS1 deficiency led to both increased production and

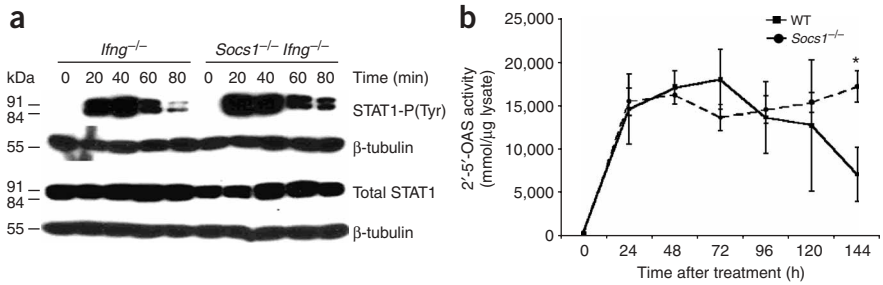

Figure 4 Mechanism of SOCS1 suppression of type I interferon antiviral signaling. (**a**) Duration of STAT1 tyrosine phosphorylation in BMMs, analyzed by immunoblot of cell lysates. BMMs from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice were 'pulse-treated' for 20 min with 1,000 IU/ml of IFN-α1, then cell lysates were prepared at 20-minute intervals up to 80 min after treatment. Immunoblots used an antibody specific for tyrosine-phosphorylated STAT1 (STAT1-P(Tyr)) or for STAT1; β-tubulin, loading control. (**b**) Duration of 2′-5′-OAS stimulation in response to IFN-α4 is prolonged in SOCS1-deficient mice. Wild-type (WT; $n = 3$) and *Socs1*$^{-/-}$ ($n = 5$) mouse embryo fibroblast cell lines at day 13 were individually 'pulse-treated' for 24 h with 1,000 IU/ml of IFN-α4. Cells were collected every 24 h until 144 h after initial treatment and were lysed at a density of 1×10^4 cells/μl of lysis buffer; lysates were analyzed by enzyme assay measuring 2′-5′-OAS activity in a radioactive 'readout'. *, $P < 0.05$ (Mann-Whitney rank sum tests).

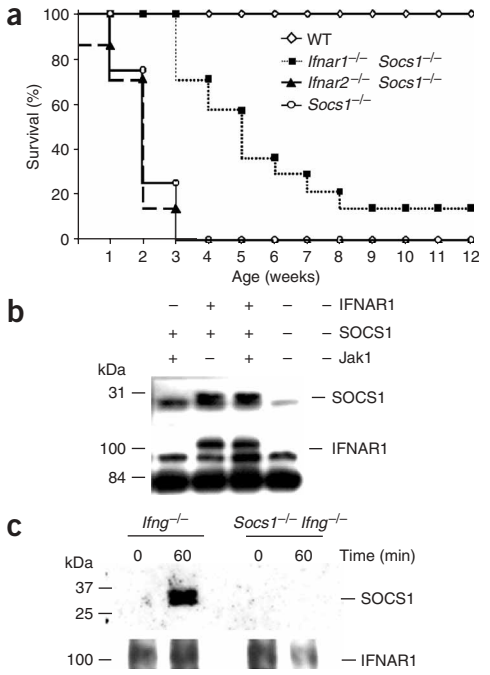

Figure 5 IFNAR1 and SOCS1 show a direct interaction. (**a**) Survival of uninfected *Socs1*$^{-/-}$ mice ($n = 8$), *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice ($n = 14$) and *Ifnar2*$^{-/-}$*Socs1*$^{-/-}$ mice ($n = 7$) versus wild-type control mice ($n = 6$). (**b**) Immunoblot of cells overexpressing IFNAR1 and SOCS1. Cells (293T) were infected with various constructs, pEF-BOS-Flag-Jak1, pEF-BOS-Flag-SOCS1 and pEF-BOS-IFNAR1; cell lysates were immunoprecipitated with monoclonal antibody to IFNAR1 followed by immunoblot with biotinylated anti-SOCS1 and horseradish peroxidase–conjugated anti-Flag. (**c**) Immunoblot of BMM lysates from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice treated with 1,000 IU/ml of IFN-α1, showing an interaction between IFNAR1 and SOCS1. Lysates were immunopreciptated with monoclonal antibody to IFNAR1 followed by immunoblot with biotinylated monoclonal antibody to SOCS1. Data are representative of three experiments.

heightened sensitivity, only the latter was affected with respect to type I interferons. The difference in the effect of SOCS1 on the production of the two types of interferon could have been due to differences in the mechanism of their induction. One prominent means of induction of IFN-γ is by IL-12, a cytokine known to be regulated by SOCS1 (ref. 43). Conversely, type I interferons are activated directly by viral activation of interferon-regulatory factors[4,44], a pathway that is not known to be sensitive to SOCS1.

We used several independent methods to demonstrate that SOCS1 regulation of type I interferon activity was responsible for the resistance to viral infection independently of IFN-γ. Those methods included the use of *Socs1*$^{-/-}$ mice in combination with *Ifng*$^{-/-}$ mice, *Ifnar1*$^{-/-}$ mice and injection of neutralizing antibody to type I IFN. Those results have provided an independent demonstration that SOCS1 inhibition of type I interferon responses *in vivo* modulated resistance or sensitivity to viral infection.

Notably, in the absence of exogenous viral infection, these *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice survived beyond 3 weeks of age and did not die from the inflammation that characterized the *Socs1*$^{-/-}$ mice, which all died before 3 weeks of age[29]. That result demonstrates the proinflammatory potency of type I interferons, which is increasingly being recognized as an important mediator of inflammation and a product of selective Toll-like receptor activation[42,44,45]. Furthermore, that pathway may contribute to the function of SOCS1 in regulating Toll-like receptor signaling[45]. Type I interferons contribute to the IFN-γ response, possibly by recruitment of IFNAR1 into the IFN-γ receptor signaling complex[46]. It is therefore possible that the abrogation of inflammation in *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ mice reflected a (partial) reduction in IFN-γ signaling, albeit through type I interferon production and action via IFNAR1. We consider this possibility unlikely, because deficiency in either IFNAR chain reduced IFN-γ signaling (P.J.H. *et. al.*, unpublished observations) but only one affects survival from the inflammatory response (discussed below).

To determine the selectivity of SOCS1 regulation of interferon signaling, we also crossed the *Socs1*$^{-/-}$ mice with *Ifnar2*$^{-/-}$ mice that we have generated and characterized (P.J.H. *et al.*, unpublished data). This cross did not 'rescue' the inflammatory phenotype of the *Socs1*$^{-/-}$ mice, as *Ifnar2*$^{-/-}$*Socs1*$^{-/-}$ mice all died before 3 weeks of age, like the *Socs1*$^{-/-}$ mice. That result demonstrates that the mechanism whereby SOCS1 regulates type I interferon responses *in vivo* is via action selectively on IFNAR1 or its associated signaling molecules such as the TYK2 kinase[11] and does not affect IFNAR2-mediated signaling. Notably, we demonstrated the mechanism of selective action of SOCS1 on IFNAR1 by coimmunoprecipitation of endogenous IFNAR1 with SOCS1 from IFN-α1-treated BMMs and of IFNAR1 and SOCS1 proteins overexpressed in 293T cells.

We have further characterized the molecular mechanisms responsible for the amplified interferon responses noted in the *Socs1*$^{-/-}$ neonatal mice as resulting from a sustained response to type I interferons. Tyrosine phosphorylation of STAT1, probably by a Jak family member such as TYK2, was prolonged, whereas serine phosphorylation was unaffected. Also, the interferon-inducible 2′-5′-OAS antiviral enzyme activity in cells isolated from *Socs1*$^{-/-}$ mice produced a signal that increased to the same intensity as in wild-type mice but was sustained for a longer period.

Despite the impression that SOCS1 has a relatively specific function *in vivo* in suppressing IFN-γ-mediated neonatal death, it in fact has multiple functions *in vivo*. When examined more closely (in the case of IL-12, IL-2, IL-4, IL-7 and IL-15), SOCS1 has more subtle but nevertheless important functions in regulating other aspects of the immune response[43,47,48]. The loss of IFN-γ regulation has an early and profound effect on the survival of young animals, and we have now demonstrated that type I interferon signals contribute to that phenotype. We have further demonstrated here that after challenge with virus, an essential function of SOCS1 on regulating type I interferon responses *in vivo* is uncovered. That result may extend beyond the antiviral actions of these interferons to bacterial infections, as reports have indicated that SOCS1-deficient mice are hyporesponsive to challenge with lipopolysaccharide. Notably, type I interferon signals are important in Toll-like receptor 4–mediated lipopolysaccharide responses[42,44,45]. Because type I interferons regulate both innate immunity (dendritic cells, macrophages and Toll-like receptor–mediated responses) and acquired immunity (memory T cells and CD8$^+$ T cells) and are relatively more specific in action than are other cytokines, regulation of SOCS1 could be considered as an adjunct to type I interferons in the clinical context and could provide a therapeutic target in type I interferon–sensitive diseases.

METHODS

Mice. *Socs1*$^{-/-}$ and *Socs1*$^{+/-}$ mice and their equivalent wild-type mice were generated and maintained on a mixed 129/Sv and C57BL/6 background[29] and were housed in clean but not specific pathogen–free conditions before viral infection experiments. These mice were genotyped by Southern blot analysis of *Eco*RI-digested genomic DNA and hybridization with a 1.5-kilobase (1.5-kb)

*Eco*RI–*Hin*dIII fragment of mouse *Socs1* (ref. 29). This fragment hybridized to a 5.3-kb band for the wild-type allele and an 8.0-kb band for the knockout allele. *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice, *Ifng*$^{-/-}$ mice and their equivalent C57BL/6 wild-type mice were maintained as separate colonies[31]. *Ifnar1*$^{-/-}$, *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ and wild-type control mice were generated by intercrossing of *Ifnar1*$^{+/-}$*Socs1*$^{+/-}$ mice housed in conventional conditions and genotyped by Southern blot analysis of *Eco*RI-digested genomic DNA. Hybridization with a 1.7-kb *Bam*HI–*Sac*I fragment of mouse *Ifnar1* (ref. 42) identified a 3.8-kb band for the wild-type allele and a 4.8-kb band for the knockout allele. The *Socs1* genotype of these mice was determined as described above. *Ifnar2*$^{-/-}$ mice were generated by targeting of exon 4 of *Ifnar2*, resulting in a null mutation whereby both the transmembrane and soluble isoforms of IFNAR2 are not expressed (P.J.H. *et al.*, data not shown). *Ifnar2*$^{-/-}$, *Ifnar2*$^{-/-}$*Socs1*$^{-/-}$ and wild-type control mice were generated by intercrossing of *Ifnar2*$^{+/-}$*Socs1*$^{+/-}$ mice housed in conventional conditions and genotyped by PCR. All mice were age- and sex-matched for experiments. Animal experimentations were in compliance with guidelines set by the institutional ethics committee (Monash Medical Centre AEC-A, Monash University, Clayton, Australia).

Viral infection of neonatal mice. Pups between 5 and 10 d of age were injected intraperitoneally with 50 µl SFV at 10×, 30× and 100× $TCID_{50}$, determined by CPE bioassay of mouse L929 cell cultures[44]. One of the experiments using *Socs1*$^{-/-}$*Ifng*$^{-/-}$ and equivalent control mice involved preinjection of a neutralizing antibody (1,000 IU) specific to type I interferons (sheep antibody to mouse IFN-α and IFN-β; Research Diagnostics) 6 h before viral infection. The neutralization activity of this antibody against virus-induced type I interferons was verified in the CPE-reduction bioassay described below. Experiments with *Socs1*$^{-/-}$ and *Ifnar1*$^{-/-}$*Socs1*$^{-/-}$ used littermate controls and were conducted by researchers 'blinded' to experimental conditions, with genotypes being determined after completion of the experiments. Experiments with *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice and their equivalent controls used offspring from separate colonies as described above. Mice were monitored at 3- to 6-hour intervals and resistance was recorded.

In a separate series of experiments, mice were killed 48 h after infection and blood, spleens, kidneys, livers, thymuses, lungs and brains were collected. The blood was left to coagulate, then was centrifuged, and serum was collected and was stored at 4 °C before assay. The organs were 'snap-frozen' in liquid nitrogen and were stored at –80 °C.

Virus titers. Each frozen organ was weighed and then was transferred to 1 ml of cold PBS on ice. Samples were sonicated for three 5-second pulses on ice with an Ultrasonics Homogenizer set at an amplitude of 40, ensuring the sample remained cold. Organ homogenates were centrifuged at 9,000*g* for 30 min at 4 °C. Supernatants were serially 'titrated' in semi-log steps in duplicate across monolayers of L929 cells plated in 96-well microtiter plates at a density of 1.3×10^5 cells/ml in RPMI medium supplemented with 3% (vol/vol) FBS and 0.5% (vol/vol) penicillin and streptomycin. Virus was 'titrated' onto plates as a positive control and, as a negative control, cells were left in culture for the duration of the assay without additions. Plates were incubated for 3 d at 37 °C in 5% CO_2. After that time, plates were viewed under a microscope and each well was assigned a score for CPE according to the following scale: 4, approximately 100% death; 3, 75% death; 2, 50% death; 1, 25% death; 0, 0% death. Viral titers were then determined as the dilution at which 50% death occurred and are expressed as $-\log_{10}$ titers or as 'fold dilution'. Comparisons of viral titers for each sample group were then assessed by statistical analysis by the Mann-Whitney rank sum test.

Serum interferon assays. Serum samples from neonatal mice were diluted in semi-$\log_{10}$ steps in duplicate into 96-well flat-bottom tissue culture plates containing L929 cell monolayers, as described above. Media was removed 24 h later and SFV was added at a concentration of 10× $TCID_{50}$ in fresh RPMI medium. Plates were subsequently incubated for 3 d at 37 °C in 5% CO_2, after which plates were assigned scores for CPE. This assay measures the activity of all type I and type II interferons. The interferon activity in 'IU/ml' was calculated by comparison of the titer of the sample with a laboratory IFN-α standard calibrated to the National Institutes of Health reference standard Ga02-901-511.

STAT1 phosphorylation. BMMs were isolated from mice and cultured as described[49]. Cells were plated at a density of 1×10^7 cells/plate and were cultured for a further 24 h without colony-stimulating factor 1. Cells were treated with 1,000 IU/ml of IFN-α1 for a 20-minute pulse, then were collected at 20, 40, 60 and 80 min after initial treatment. Cells were collected into boiling lysis buffer (66 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM sodium vanadate and 2% SDS, plus one protease inhibitor 'tablet' (Roche) per 10 ml buffer, with resulting lysate concentrations determined by the Lowry assay. Protein (50 µg) was separated by 8% SDS-PAGE, followed by immunoblot for phosphorylated STAT1 (Tyr; Cell Signaling), total STAT1 (Cell Signaling) and β-tubulin (Chemicon).

2′-5′-OAS assays. Wild-type, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ mouse primary embryo fibroblasts were generated as described[50]. Cells were treated with 1,000 IU/ml of mouse IFN-α4 (obtained from D. Gewert, BioLauncher, Cambridge) for a 24-hour pulse and were washed, then were incubated for a further 48, 72, 96, 120 or 144 h. Cell lysates were prepared and 2′-5′-OAS activity was determined as described[42]. Protein concentration was determined by the Bradford assay. Enzyme activity was calculated as millimoles phosphate incorporated per microgram protein at each time point[42].

Transient transfection. The 293T cells were maintained in DMEM containing 10% FBS. Cells expressing the PEF-BOS-Flag-Jak1+pEF-BOS-Flag-SOCS1, pEF-BOS-IFNAR1+pEF-BOS-Flag-SOCS1 and pEF-BOS-FLAG-Jak1+pEF-BOS-IFNAR1+pEF-BOS-Flag-SOCS1 constructs were generated using the fuGENE6 transfection reagent (Roche) according to the manufacturer's instructions and were collected 48 h later.

Immunoprecipitation and immunoblot analysis. BMMs were isolated from mice and cultured as described[49]. Cells were plated at a density of 1×10^7 cells/plate and were cultured for a further 24 h with the addition of colony-stimulating factor 1. Cells were pretreated for 40 min with 10 µM MG132 (Calbiochem), then were treated for a further 60 min with 1,000 IU/ml of IFN-α1. Cells were collected in ice-cold lysis buffer (0.5% Nonidet-P40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM sodium vanadate, 1 mM PMSF and 1 mM NaF, plus one protease inhibitor 'tablet' (Roche) per 25 ml buffer). The resulting lysates were precleared by the addition of a 50% protein A–Sepharose slurry, then were immunoprecipitated with a monoclonal antibody to mouse IFN-αR1 (ref. 51). Protein A beads were incubated with the immunoprecipitating lysates for 2 h, after which they were washed and were boiled in reducing or nonreducing sample buffer. Proteins were separated by 8% SDS-PAGE, followed by immunoblot for IFNAR1, or were separated by 15% SDS-PAGE, followed by immunoblot with monoclonal antibody to SOCS1 (obtained from J. Zhang, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

Transfected cells were collected in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM sodium vanadate, 10 mM NaF and 1 mM PMSF plus one protease inhibitor 'tablet' (Roche) per 50 ml buffer). The resulting lysates were immunoprecipitated with monoclonal antibody to IFNAR1. Protein A beads were incubated with the immunoprecipitating lysates for 2 h, after which they were washed and boiled in 2× reducing sample buffer. Proteins were separated by 10–20% SDS-PAGE (precast gels; Novex), followed by immunoblot for Flag (rat monoclonal antibody to Flag (9H1; ref. 52) is an 'in-house' antibody of the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) or IFNAR1.

ACKNOWLEDGMENTS

Supported by the National Health and Medical Research Council of Australia.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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