Negative feedback is a mechanism commonly employed in biological processes as a means of maintaining homeostasis. We have investigated the roles of suppressor of cytokine signaling (SOCS) proteins in regulating the kinetics of negative feedback in response to cytokine signaling. In mouse livers and bone marrow-derived macrophages, both interferon-γ (IFNγ) and interleukin-6 (IL-6) rapidly induced the tyrosine phosphorylation of signal transducer and activator of transcription-1 (STAT1) and STAT3. STAT3 tyrosine phosphorylation was bi-phasic in response to continuous IL-6 signaling. In macrophages lacking Socs3, however, continuous IL-6 signaling induced uniformly high levels of STAT3 tyrosine phosphorylation, and early IL-6-inducible genes were inappropriately expressed at intermediate time points. SOCS3 therefore imposes bi-phasic kinetics upon IL-6 signaling. Compared with Socs3 mRNA, Socs1 mRNA was induced relatively slowly, and SOCS1 simply attenuated the duration of IFNγ signaling. Surprisingly, heighten Socs1 mRNA expression but minimal STAT1 tyrosine phosphorylation was observed after prolonged stimulation with IFNγ, indicating that STAT1 may not play a large role in inducing Socs1 mRNA during steady-state IFNγ signaling. We also demonstrate that both Socs1 and Socs3 can desensitize primary bone marrow-derived macrophages to IFNγ and IL-6 signaling, respectively. Consistent with the kinetics with which Socs1 and Socs3 mRNAs were induced, SOCS3 desensitized cells to IL-6 rapidly, whereas SOCS1-mediated desensitization to IFNγ occurred at later time points. The kinetics with which SOCS proteins are induced by cytokines may therefore be a parameter that is “hard-wired” into specific cytokine signaling pathways as a means of tailoring the kinetics with which cells become desensitized.

Interleukin-6 (IL-6) and interferon-γ (IFNγ) are cytokines with key roles in regulating the immune response. Signaling by IL-6 and IFNγ begins at the surface of the cell where the cytokines associate with their respective receptor complexes (1). The IFNγ-receptor complex consists of the ligand-binding IFNγ-receptor α subunit and the signal-transducing IFNγ-receptor β subunit, whereas the IL-6-receptor complex consists of the ligand-binding IL-6-receptor α subunit and the signal-transducing glycoprotein-130 subunit (1). Associated with the receptor subunits are the Janus kinases (JAKs), which become activated upon receptor dimerization, and phosphorylate signal transducers andactivators of transcription (STAT) transcription factors (2, 3). Both IFNγ and IL-6 can activate JAK1 (4–6) and JAK2 (7–10), and subsequently STAT1 (11, 12) and STAT3 (6, 13, 14). The phosphorylated STATs (P-STATs) then dimerize and are transported to the nucleus, where they bind to promoter sequences, and activate the transcription of a suite of genes, among which are those encoding the suppressor of cytokine-signaling-1 (SOCS1) and SOCS3 proteins (15, 16). STAT3 activates the transcription of Socs3 mRNA by associating with the promoter region of the Socs3 gene (17). The regulation of Socs3 mRNA by STAT1 is poorly understood, and it remains to be clarified whether STAT1 directly activates the transcription of Socs1. Interferon regulatory factor-1, which is induced by STAT1, does, however, activate the transcription of Socs1 (18).

SOCS1 and SOCS3 proteins can both inhibit JAK phosphorylation of STAT, thus creating a negative feedback loop that attenuates cytokine signal transduction, although the mechanisms by which they act appear to differ. Whereas SOCS1 functions by binding directly to JAK proteins, SOCS3 inhibits signaling by binding to phosphorylated tyrosine sites on the cytoplasmic domain of the receptor (19, 20).

Although the signaling pathways activated by IFNγ and IL-6 share common features, their biological outcomes are quite different. IFNγ acts on cells to induce inflammatory and anti-viral responses, whereas IL-6 acts on cells to regulate the production of acute phase proteins (primarily in hepatocytes) as well as the growth and differentiation of a variety of cell types. Classically, this paradox has been explained by an understanding that IFNγ primarily signals via STAT1, whereas IL-6 primarily signals via STAT3 (21). Physiologically, SOCS1 is the main inducible inhibitor of IFNγ signaling and in neonatal mice prevents the establishment of a lethal inflammatory disease characterized by heighten levels of circulating IFNγ, fatty degeneration of the liver, and necrosis of the liver and other organs (22). Mice lacking Socs3 die during embryogenesis (23); however, the generation of mice conditionally deficient for Socs3 revealed SOCS3 to be a potent physiological suppressor of IL-6 but not IFNγ signaling (24–26). Surprisingly, IL-6 signaling in the absence of SOCS3 resulted in the prolonged phosphorylation of STAT1 and a qualitative shift in the transcriptional response of IL-6 toward that typical of IFNγ (16, 24, 25). This indicates that signaling by IFNγ and IL-6 is fundamentally similar, and that the activity of SOCS3 is required to sculpt the cellular response to IL-6 and thereby discriminate it from that of IFNγ.

In addition to their role as inhibitors of cytokine signaling, a role for
SOCS proteins in the desensitization of cytokine signaling was recently postulated (27). Desensitization, as opposed to inhibition, refers to the process by which an initial signaling event causes the cell to become refractory to subsequent signals. In the case of IFNγ signaling, the T-cell protein-tyrosine phosphatase has been shown to potently desensitize cells to repeated stimulation (28). Although SOCS1 and SOCS3 are attractive candidates as mediators of desensitization to IFNγ and IL-6, respectively, detection of this activity has so far proved elusive (27).

In this study, we have explored the relationship between the kinetics of STAT tyrosine phosphorylation and the kinetics of Socs mRNA expression for both IFNγ and IL-6 signaling. We demonstrate marked differences in the kinetics with which SOCS1 and SOCS3 inhibit IFNγ and IL-6 signaling, respectively, and, furthermore, we show that both SOCS1 and SOCS3 can desensitize cells to IFNγ and IL-6 signaling, respectively.

**MATERIALS AND METHODS**

*Mouse Injections—*C57BL/6 mice housed at The Walter and Eliza Hall Institute animal facility received an intraperitoneal injection at 6–8 weeks of age with 100 μl of either saline, recombinant murine IL-6 (500 μg/kg, a gift from Dr. Richard Simpson, Ludwig Institute for Cancer Research, Melbourne, Australia), or IFNγ (250 μg/kg weight, Peprotech, Rocky Hill, NJ). Mice were asphyxiated in CO₂, and livers were dissected and frozen in liquid nitrogen at various time points following injection. Experiments were performed with the approval of the Melbourne Health Research Directorate Animal Ethics Committee.

*Culture and Stimulation of C57BL/6, Socs3−/−, Ifnγ−/−, and Socs1−/− Ifnγ−/− Macrophages—*Socs3-deficient macrophages (Socs3−/−) were derived as previously described (24, 29) from the bone marrow of mice in which one allele of Socs3 was deleted, and one allele of Socs3 was conditionally deleted from hematopoietic progenitors (29) or macrophages (24). Ifnγ−/− and Socs1−/− Ifnγ−/− macrophages were derived from the bone marrow of Ifnγ−/− and Socs1−/− Ifnγ−/− mice (22). C57BL/6 (wild-type), Socs3−/−, Ifnγ−/−, and Socs1−/− Ifnγ−/− bone marrow cells were cultured in 8-cm tissue culture-treated plates (10 × 10⁶/dish) overnight at 37 °C, 10% CO₂ in 9 ml of Dulbecco’s modified Eagle’s medium (Invitrogen). After incubation at 37 °C for 10 min, cells were resuspended in Dulbecco’s modified Eagle’s medium/10% fetal calf serum/20% LCM. Cells, of which more than 95% were macrophages (as determined by morphology), were then plated into 12-well tissue culture-treated plates (10⁶/dish) overnight at 37 °C, 10% CO₂ in 9 ml of Dulbecco’s modified Eagle’s medium/10% fetal calf serum/20% LCM at 37 °C. For mRNA stability experiments, inhibition of transcription was achieved by adding 2 μg/ml actinomycin-D (Sigma-Aldrich) in 5 μl of dimethyl sul fate (Sigma-Aldrich) to cells plated in 250 μl of media.

*Immunoprecipitation and Western Blotting—*Frozen livers were lysed in radioimmune precipitation assay buffer (30), and lysates were subjected to immunoprecipitation and then Western blotted for STAT proteins (30) and SOCS proteins (24). Immunoprecipitations were performed with antibodies to STAT1 (BD Transduction Laboratories, Lexington, KY), STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and SOCS1 or SOCS3 (generated at the Walter and Eliza Hall Institute). Western blotting was carried out with antibodies specific for either tyrosine 701-phosphorylated STAT1 (P-STAT1, Cell Signaling, Beverly, MA), STAT1, tyrosine 705-phosphorylated STAT3 (P-STAT3, Cell Signaling), STAT3, SOCS1, or SOCS3.

*RNA Isolation and cDNA Synthesis—*Total RNA for real-time PCR and microarray analysis was isolated from cells using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 20 μg of total RNA (for microarray analysis) or <1 μg of total RNA (for real-time PCR) in a 20-μl reaction for 1 h at 42 °C using 100 units of Superscript II (Invitrogen) and 5 μM T7 oligo(dT) primer (synthesized by GeneWorks, Thebarton, SA, Australia) as described in the GeneChip Technical Manual (Affymetrix, Santa Clara, CA). For real-time PCR, cDNA products were diluted to 80 μl in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 8.0). Double-stranded cDNA was synthesized for microarray analysis from 20 μl of single-stranded cDNA products according to Affymetrix methodology and was purified with Phase-Lock Gels (Eppendorf, Hamburg, Germany).

*Real-time Quantitative PCR—*Real-time quantitative PCR (Q-PCR) was carried out on single-stranded cDNA samples using the Quantitect SYBR Green PCR kit (Qiagen) in 10-μl reactions containing 0.5 pmol of forward and reverse primers, 5 μl of QuantiTect Master Mix, and 4 μl of cDNA. Amplification was performed according to Qiagen’s recommendations with a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences were as follows: porphobilinogen deaminase (Pbgd)-forward: CCTGTTGTTTCACTCCTGTA; Pbgd-reverse: CAACAGCATACAAGGTTT; Socs1-forward: GTGTTGTGGAGGTTGAGAT; Socs1-reverse: CCCAGACACAGCTGC; Socs3-forward: TGAGGTCTAAGACCGTCG; Socs3-reverse: CACAGTCGAACGGGGAAC. Ttp-forward: CTTCTGCAAATCTTGTCCT; Ttp-reverse: TTCTCAGGAGAGTGTGAC. Gadd45γ-forward: GCATCCTCATTTGGAATCC; Gadd45γ-reverse: CACCCAGTCGTGGAAGCTG. Expression was determined for the gene of interest relative to a standard curve created by serial dilution of the PCR product and was normalized against the expression of the housekeeping gene Pbgd. Microarray and real-time PCR analysis confirmed Pbgd to be stably expressed in livers and primary macrophages treated with IFNγ or IL-6 (data not shown). Where actinomycin-D was used to inhibit RNA transcription, and it was not appropriate to normalize to the expression level of Pbgd, gene expression was instead normalized to the number of cells plated.

*Microarray Time-course Analysis—*Biotin-labeled cRNA was synthesized from double-stranded cDNA with the BioArray HighYield RNA Transcript Labeling Kit (Enzo, New York, NY). Samples were cleaned with the RNeasy kit (Qiagen), and 15 μg of each cRNA sample was fragmented then hybridized overnight to MUG174a2v2 Murine GeneChips (Affymetrix) following the manufacturer’s instructions. GeneChips were stained with phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) and scanned with an Affymetrix GeneChip scanner. Normalization and background correction was performed using the “Affy” package (www.bioconductor.org) of the “R” programming environment. Genes with a change in expression >1.7-fold between treated and control samples in two consecutive samples were considered to be differentially expressed. This cut-off was selected based on visual inspection of normalized quantile-quantile plots of the expression differences between pairs of microarrays. Genes displaying differential expression between control samples (either non-treated or PBS-treated) were
removed. GeneCluster2 (31) was then employed to identify sets of differentially expressed genes with common expression profiles.

RESULTS

Differential Induction of STAT1 and STAT3 Tyrosine Phosphorylation by IFNγ and IL-6 in the Liver—Gene deletion studies in mice have revealed the importance of SOCS1 for regulating IFNγ and SOCS3 for regulating IL-6 signaling, however little is known of the comparative induction of SOCS proteins by IFNγ and IL-6. To investigate the in vivo activation of STAT1 and STAT3 and expression of Socs1 and Socs3 in response to these cytokines, we injected C57BL/6 mice with IFNγ or IL-6 and examined proteins in the livers of mice after various time periods. Immunoprecipitation (9) and Western blotting demonstrated that, in response to either cytokine, tyrosine phosphorylation (a readout of STAT activation) of both STAT1 and STAT3 was maximal or near-maximal by 15 min after injection (Fig. 1, A and B). STAT3 tyrosine phosphorylation declined to near basal levels after 4 h in response to either cytokine, and a much higher level was detected following injection of IL-6 compared with injection of IFNγ (Fig. 1A). In contrast, while the maximal levels of STAT1 tyrosine phosphorylation were similar following injections of both cytokines, phosphorylation declined to basal levels after 4 h in response to IFNγ but after only 1 h in response to IL-6 (Fig. 1B). These differences in the phosphorylation of STAT1 and STAT3 by IFNγ and IL-6 are consistent with the role of STAT1 as an important mediator of the response to IFNγ and the role of STAT3 as an important mediator of the response to IL-6 (21).

IFNγ induced the robust expression of SOCS1 protein, with levels reaching a maximum at 2–4 h after injection of IFNγ, at a time when STAT1 and STAT3 phosphorylation was waning (Fig. 1C; compare with Fig. 1, A and B). SOCS1 protein expression was maintained at a high level for at least 8 h, and declined to near basal levels by 16 h. In the case of SOCS3, the opposite appeared true; IL-6 induced the expression of SOCS3 protein very rapidly, with maximal levels detected 30 min to

\[^{3}\text{M. Reich, K. Ohm, M. Angelo, P. Tamayo, and J. P. Mesirov (2004) GeneCluster 2.0, available at www.broad.mit.edu/cancer/software/genecluster2/gc2.html.}\]
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1 h following injection, at a time that correlates with a decline in STAT1/STAT3 tyrosine phosphorylation (Fig. 1D; compare with Fig. 1, A and B), whereas IFNγ induced a barely detectable amount of SOCS3 (Fig. 1D).

We confirmed the pattern of SOCS1 and SOCS3 protein expression in response to IFNγ and IL-6 at the mRNA level using a Q-PCR assay (Fig. 1E). Again, IFNγ induced Socs1 to greater levels than did IL-6, whereas IL-6 induced Socs3 to greater levels than did IFNγ. IL-6 induced both Socs1 and Socs3 mRNA to a similar extent, but more SOCS3 protein than SOCS1 protein, indicating that synthesis of SOCS3 protein may be more efficient than synthesis of SOCS1 protein. The kinetics of Socs3 mRNA and SOCS3 protein expression were similar, suggesting that the SOCS3 protein is highly labile, a finding that is consistent with the reported half-life of this protein (33). Expression of SOCS1 protein, however, appeared to persist at 4 h and 8 h, despite declining levels of Socs1 mRNA, suggesting that the half-life of SOCS1 during active cytokine signaling in vivo is substantially greater than that of SOCS3.

Continuous IL-6 Signaling in Bone Marrow-derived Macrophages Produces a Bi-phasic Response—The in vivo analysis of signaling confirmed the reciprocal relationship of IFNγ with SOCS1, and IL-6 with SOCS3. However, the intrinsic variability when working with mice, and the difficulty in precisely regulating cytokine levels following injection led us to investigate the kinetics of signaling in more tractable systems: cultures of primary cells. We initially examined IL-6 signaling in Socs3-deficient primary hepatocytes, but like Socs3-deficient mouse embryonic fibroblasts (27), these cells exhibited signs of basal STAT3 activation (albeit to a lesser extent), including low level STAT3 tyrosine phosphorylation and induction of IL-6-inducible genes (data not shown). Fortunately, Socs3-deficient primary bone marrow-derived macrophages did not show signs of basal STAT3 activation (Fig. 2A, lane 10).

In bone marrow-derived macrophages stimulated with IL-6, tyrosine phosphorylation of STAT3 was observed during two distinct phases in response to IL-6 (Fig. 2, A and B). The first phase occurred between 15 and 30 min, and the second from 120 min onward. The decline in STAT3 phosphorylation from the initial peak at 15 min was rapid, and near-basal P-STAT3 levels were achieved by 60 min, suggesting that the half-life of STAT3 phosphorylation must be very short during this period. Expression of Socs3 mRNA was maximal at 30 min, 15 min after maximal STAT3 activation was observed (Fig. 3A). This was followed by a rapid reduction of Socs3 mRNA expression, with minimal expression observed between 60 to 90 min, and subsequent re-establishment of expression by 180 min.

SOCS3 Is Responsible for Bi-phasic Tyrosine Phosphorylation of STAT3 and Expression of Genes in Response to IL-6—The observation of two separate phases of signaling by IL-6 in bone marrow-derived macrophages suggested that negative feedback by SOCS3 might impose a period of oscillation upon signaling by IL-6. To test this hypothesis, levels of phosphorylated STAT3 were determined in response to IL-6 in Socs3−/− bone marrow-derived macrophages (Fig. 2, A and B). In the absence of Socs3, STAT3 phosphorylation was strongly induced after 15 min and was not substantially reduced for the remainder of the time course. SOCS3 is therefore responsible for imposing bi-phasic kinetics upon the signaling of IL-6.
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To determine how the expression levels of IL-6-responsive genes are affected by SOCS3-mediated regulation of IL-6 signaling, the responses of the IL-6-inducible genes growth arrest and DNA damage-inducible gene 45γ (Gadd45γ) (34) and tristetraprolin (Ttp) (35), were examined following stimulation with IL-6 in bone marrow-derived macrophages. In wild-type cells, the response of Gadd45γ mRNA expression to IL-6 was bi-phasic, with peaks of expression occurring at 30 and 180 min. In the absence of Socs3, however, Gadd45γ mRNA expression was sustained at a high level in response to IL-6 (Fig. 3B). Expression of Ttp mRNA in wild-type cells peaked at 30 min and was sustained at a low level for the remainder of the time course (Fig. 3C). In Socs3−/− cells, however, stimulation with IL-6 resulted in heightened levels of Ttp mRNA expression for the duration of the time course. These results suggest that loss of Socs3 results in the prolonged expression of genes that are normally induced by IL-6 at early time points and in the heightened and uniform expression of genes that are normally induced by IL-6 with bi-phasic kinetics.

Disparate Kinetics of STAT1 TyrosinePhosphorylation and Socs1 Expression in Response to Continuous IFNγ Signaling—Although the role of SOCS1 in regulating the kinetics of STAT1 tyrosine phosphorylation in response to IFNγ has been explored previously (30), it is unknown how the kinetics with which SOCS1 and SOCS3 regulate cytokine signaling compare. We therefore examined the tyrosine phosphorylation of STAT1 and expression of Socs1 mRNA in response to IFNγ in bone marrow-derived Ifnγ−/− and Ifnγ−/− Socs1−/− macrophages. Consistent with previous observations (30), STAT1 tyrosine phosphorylation in response to IFNγ was prolonged in the absence of SOCS1 (Fig. 4A). The effects of SOCS1 were not apparent until ~90 min after stimulation with IFNγ, ~60 min later than the effects of SOCS3 were first observed in response to IL-6 (compare Fig. 2, A and B, with Fig. 4, A and B). The pattern of Socs1 mRNA expression in Ifnγ−/− cells was quite different to the pattern of STAT1 phosphorylation (Fig. 4, compare B with C). Whereas STAT1 tyrosine phosphorylation occurred rapidly and declined to near-basal levels by 240 min, expression of Socs1 mRNA was induced slowly, but remained at near-maximal levels from 60 min until at least 480 min. This was surprising, given that, after 240 min, STAT1 tyrosine phosphorylation remained low for the duration of the time course. These results seem to differ from the response to injection of IFNγ that is observed in whole livers (Fig. 1, B and E), probably because the livers were taken from mice subjected to a single stimulation of IFNγ, whereas IFNγ signaling in the bone marrow-derived macrophages was continuous.

Socs1 and Socs3 mRNAs Are Degraded at Similar Rates—One possible explanation for the discrepancy between STAT1 phosphorylation and Socs1 mRNA expression after 240 min of IFNγ signaling could be that Socs1 mRNA is degraded relatively slowly. To test this, we compared the stability of Socs1 mRNA with the stability of Socs3 mRNA, which presumably has a short half-life because it exhibits rapid bi-phasic kinetics in response to IL-6. As shown in Fig. 5A, actinomycin-D efficiently suppresses synthesis of Socs3 mRNA in response to IL-6. To assess the rates at which Socs1 mRNA and Socs3 mRNA are degraded, cells were stimulated with either IFNγ (at 0 min) or IL-6 (at 30 min) and then treated with actinomycin-D (at 60 min). No difference was observed in the kinetics with which mRNAs for Socs1 and Socs3 are degraded in the absence of transcription (Fig. 5B). By 120 min (60 min after treatment with actinomycin-D), levels of both Socs1 mRNA and Socs3 mRNA were ~25% of the maximal level, indicating that the mRNAs of both Socs1 and Socs3 have a half-life of ~30 min (Fig. 5B). Because this value is relatively short, we conclude that a high rate of transcription must be required to sustain the levels of Socs1 mRNA shown in Fig. 4C at between 240 and 480 min of continuous IFNγ signaling. Because the corresponding levels of STAT1 phosphorylation were minimal (Fig. 4, A and B), it seems likely that some other IFNγ-responsive transcription factor (such as interferon regulatory factor-1) is responsible for the majority of Socs1 mRNA transcription observed at these points.

Uniform Regulation by SOCS1 of the Transcriptional Response to IFNγ in the Liver—Given the dramatic inflammatory response to IFNγ of mice deficient for Socs1 (22), we were interested in determining how SOCS1 regulates the response to IFNγ at a genome-wide level, and in particular, whether distinct sets of genes respond differently to the loss of SOCS1 following stimulation with IFNγ. Sets of IFNγ-inducible genes with abnormally dramatic responses to the loss of Socs1, for instance, could provide additional insight into the development of the inflammatory disease to which neonatal Socs1−/− mice succumb (22). Microarrays were used to profile the transcriptional responses of livers from Ifnγ−/− Socs1−/− and Ifnγ−/− mice subjected to various durations of stimulation with IFNγ, and the expression profiles of genes found to be differentially regulated due to loss of Socs1 were clustered into six common groups using GeneCluster2 (31). The number of cluster

FIGURE 4. Kinetics of STAT1 tyrosine phosphorylation and Socs1 mRNA expression in response to IFNγ in Ifnγ−/− and Ifnγ−/− Socs1−/− macrophages. A, Western analysis of STAT1 tyrosine phosphorylation in Ifnγ−/− and Ifnγ−/− Socs1−/− bone marrow-derived macrophages stimulated with IFNγ. B, ImageJ quantification of Western blots for P-STAT1 relative to total STAT1 (± S.E.). C, Q-PCR analysis of Socs1 mRNA expression for two separate experiments in which Ifnγ−/− and Ifnγ−/− Socs1−/− bone marrow-derived macrophages were stimulated with IFNγ.

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Three groups of genes with variable expression profiles were also identified by GeneCluster2 (data not shown). Several of the genes included in these groups were later determined by Q-PCR analysis to be regulated in the liver with a high degree of variability and not specifically in response to IFNγ (data not shown).

SOCS3 Desensitizes Bone Marrow-derived Macrophages to Repeated Stimulation with IL-6—SOCS proteins are tempting candidates as mediators of cytokine desensitization. However, previous attempts to detect SOCS3-mediated desensitization of cytokine signaling in Socs3-deficient mouse embryonic fibroblasts were hampered by the constitutive activation of STAT3 in these cells (27). The kinetics with which IL-6 and IFNγ induced the tyrosine phosphorylation of STAT proteins and expression of Socs mRNAs suggested that both SOCS1 and SOCS3 might be able to desensitize cells to repeated stimulation with cytokine. Furthermore, given the differences in the kinetic relationships between STAT activation and Socs mRNA expression, it appeared that SOCS1 may be substantially more potent at desensitizing cells to repeated IFNγ stimulation than SOCS3 would be at desensitizing cells to repeated IL-6 stimulation. To determine whether SOCS3 can desensitize IL-6 signaling, we employed the experimental approach taken by Fischer et al. (27), in which cells are subjected to a pulse of cytokine, rested for various periods of time, and then subjected to a second pulse prior to analysis.

In C57BL/6 bone marrow-derived macrophages, a 15-min pulse of IL-6 resulted in the tyrosine phosphorylation of STAT3 at 15 and 30 min (Fig. 7A). In the absence of Socs3, tyrosine phosphorylation of STAT3 in response to a 15-min pulse of IL-6 was extended until at least 90 min, with low level phosphorylation also apparent at later time points (Fig. 7A). To determine whether IL-6 could desensitize cells to subsequent IL-6 signaling, cells were stimulated again after various periods of time had elapsed. Consistent with the previous observations of Fischer et al., pre-treatment of C57BL/6 cells with IL-6 resulted in the desensitization of cells to a further pulse of IL-6 after 60, 90, 120, and 240 min (Fig. 8A). There appeared to be some alleviation of the desensitization at 90 and 120 min, although this was not consistent. A similar pattern of pronounced desensitization was not observed in cells deficient for Socs3, indicating that SOCS3 desensitizes cells to IL-6 signaling (Fig. 8A). Limited desensitization to IL-6 was, however, apparent for all re-stimulated Socs3-deficient cells, and was more pronounced at later time points, indicating that factors other than SOCS3 are required for long term desensitization to IL-6 (Fig. 8A).

FIGURE 5. Analysis of Socs1 and Socs3 mRNA stability. A, 2.5 × 10^5 C57BL/6 bone marrow-derived macrophages were either left untreated, or treated with actinomycin-D (A-D) for 30 min. Cells were then either left unstimulated, or stimulated with IL-6 for a further 30 min. Expression levels were determined by Q-PCR for Pbgd mRNA (open boxes) and Socs3 mRNA (shaded boxes). B, 2.5 × 10^5 C57BL/6 bone marrow-derived macrophages were stimulated with IFNγ (for 60 min) or IL-6 (for 30 min) prior to the addition of actinomycin-D (at 60 min). Levels of Socs1 or Socs3 mRNA were determined by Q-PCR for cells stimulated with IFNγ or IL-6, respectively.

FIGURE 6. Cluster analysis of the role of SOCS1 in regulating IFNγ-inducible gene expression. Ifnγ−/− and Ifnγ−/− Socs1−/− mice were injected with IFNγ or PBS (to provide controls), and livers were taken at various times afterward for transcriptional profiling by microarray. Data have been deposited in the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo/), and have the GEO Series accession id GSE4232. Genes that were differentially expressed in the livers of IFNγ-injected Ifnγ−/− Socs1−/− mice relative to the livers of IFNγ-injected Ifnγ−/− mice (at the same time point after injection) were clustered into sets of commonly differentially expressed genes using GeneCluster2. While only a single liver was used for each time point, differential expression was defined as relative induction or suppression of expression over at least two consecutive time points. Average expression profiles of clusters of genes are displayed as heat plots, where red represents high relative expression, and blue represents low relative expression. Heat plots labeled “γ−−0 h” depict the response of livers from IFNγ-injected Ifnγ−/− mice, relative to the liver of a non-injected control. Heat plots labeled “S1γ−−γ−” depict the response of livers from IFNγ-injected Ifnγ−/− Socs1−/−, relative to the livers of IFNγ-injected Ifnγ−/− mice.
SOCS1 Desensitizes Bone Marrow-derived Macrophages to Repeated Stimulation with IFNγ—Having found that the induction of SOCS3 by IL-6 could desensitize cells to subsequent signaling by IL-6, we wondered whether the induction of SOCS1 by IFNγ could desensitize cells to subsequent signaling by IFNγ. In response to a 15-min pulse of IFNγ, STAT1 tyrosine phosphorylation was observed until 60 min in Ifnγ−/− cells, and until 360 min in Socs1−/− Ifnγ−/− cells (Fig. 7B). Desensitization of Ifnγ−/− macrophages (as measured by STAT1 tyrosine phosphorylation) following 15 min of IFNγ signaling was observed after 240 min and until 360 min (Fig. 8B). In Socs1−/− Ifnγ−/− macrophages, desensitization to IFNγ occurred after 240 min, probably a result of the activity of the phosphatase SHP-2 (36). Thus, SOCS1 clearly desensitizes cells to re-stimulation with IFNγ at 120 min; however, it is difficult to clarify the specific role of SOCS1 in desensitizing cells to IFNγ at later times. Consistent with the relative kinetics of SOCS1 and SOCS3-mediated inhibition of cytokine signaling (as determined above), desensitization of cytokine signaling was mediated much more rapidly by SOCS3 than by SOCS1 (compare Fig. 8A with 7B).

DISCUSSION

Despite the physiologically distinct functions of IFNγ and IL-6, the signaling mechanisms by which they elicit their cellular responses are similar, and in the case of IL-6 are distinguished at least in part through the activity of SOCS3 (16, 24, 25). To further clarify the importance of SOCS proteins in regulating signaling by IFNγ and IL-6, we compared the roles of SOCS1 and SOCS3 in regulating the kinetics of cytokine
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signal transduction. Consistent with the known physiological activities of SOCS proteins, Socs1 mRNA and SOCS1 protein were induced to a greater extent by IFNγ than by IL-6, whereas Socs3 mRNA and SOCS3 protein were induced more by IL-6 than by IFNγ. Both IFNγ and IL-6 rapidly induced the activation of STATs, and in the absence of Socs1 and Socs3, respectively, continuous cytokine stimulation resulted in the sustained activation of STATs.

In C57BL/6 bone marrow-derived macrophages, regulation of continuous IL-6–signaling by SOCS3 was bi-phasic, with the first peak of IL-6 signaling appearing rapidly and transiently. Bi-phasic STAT3 activation has been reported in response to a variety of stimuli, including granulocyte-colony stimulating factor (37), leukemia inhibitory factor (38), IL-6 (27, 38), and lipopolysaccharide (39). Interestingly, each of these stimuli activates the glycoprotein-130/STAT3 or the granulocyte-colony stimulating factor-receptor/STAT3 signaling pathways, which are known to be inhibited by SOCS3 in vivo (24–26, 29, 40, 41). To ascertain whether negative feedback by SOCS3 is necessary for the bi-phasic response to IL-6, we examined IL-6 signaling in Socs3-deficient macrophages, and found the response to IL-6 of these cells to be maximal and constant. Therefore, SOCS3 is required for suppression of the early phase of IL-6 signaling. It follows that the second phase of IL-6 signaling seen in wild-type cells is probably due to the degradation of the SOCS3 protein synthesized during the first phase of signaling, and the subsequent re-establishment of STAT3 activation (a model for this is shown in Fig. 9A).

As was the case for STAT3 in response to IL-6, tyrosine phosphorylation of STAT1 was detected in Ifnγ−/− macrophages immediately after stimulation with IFNγ. Yet despite the rapid phosphorylation of STAT1, the induction of Socs1 mRNA was slow. In contrast to the bi-phasic manner in which IL-6 signaling was regulated by SOCS3, IFNγ signaling was simply attenuated in duration by SOCS1, a finding consistent with previous observations (30). Using microarray expression profiling of an IFNγ time course, all genes regulated by SOCS1 were regulated with uniform kinetics, suggesting that separate sets of genes are not differentially regulated by SOCS1.

The steady-state outcomes of signaling by IFNγ and IL-6 were remarkably dissimilar. After prolonged IL-6 signaling, medium levels of STAT3 activity, Socs3 mRNA expression, and presumably SOCS3 protein expression were observed. Surprisingly, prolonged IFNγ signaling resulted in high levels of Socs1 mRNA expression, and low levels of STAT1 tyrosine phosphorylation. We calculated the half-life of Socs1 mRNA to be ~30 min, which is clearly too short to account for the substantial duration over which heightened Socs1 mRNA expression but minimal STAT1 activation was observed (from 240 to 480 min). This suggests that an IFNγ-inducible transcription factor other than STAT1 may be responsible for sustaining Socs1 mRNA expression after prolonged IFNγ signaling. One likely candidate is interferon regulatory factor-1, because it is induced by STAT1 (32, 42, 43) and can associate with the Socs1 promoter to activate the transcription of Socs1 mRNA (18). A model for the proposed relationship between STAT1 activation, Socs1 mRNA expression, and SOCS1 protein expression, is given in Fig. 9B.

We demonstrated that both SOCS3 and SOCS1 desensitized bone marrow-derived macrophages to signaling by IL-6 and IFNγ, respectively, with the activity of SOCS1 occurring later than the activity of SOCS3. This is consistent with the finding that IL-6 induces the expression of Socs3 mRNA earlier than IFNγ induces the expression of Socs1 mRNA. As such, the kinetics with which cytokines induce SOCS proteins appears to be a parameter that is tailored to different cytokines to ensure that cells are desensitized to the cytokine with appropriate kinetics. Why cells need to be desensitized to IL-6 earlier than they are to IFNγ is not currently apparent, although it is clear that this mechanism also allows cells to become re-sensitized to IL-6 more quickly than to IFNγ. It could be speculated that IFNγ signaling is more dangerous to the health of the cell and the organism than is IL-6 signaling, so the desensitization of cells to IFNγ may be more critical. Consistent with this idea, desensitization in macrophages lacking Socs genes was more pronounced for IFNγ signaling than for IL-6 signaling. This result also highlighted the important role that factors other than SOCS1, such as T-cell protein-tyrosine phosphatase for instance (28), also have in desensitizing cells to IFNγ.

In conclusion, both SOCS1 and SOCS3 are able to desensitize cells to signaling by IFNγ and IL-6, respectively. The kinetics with which SOCS proteins are induced by cytokine is a critical factor in determining how the cell responds. IFNγ appears to induce SOCS1 protein expression relatively slowly, leading to slow inhibition and desensitization of signaling. In contrast, IL-6 appears to induce SOCS3 protein expression rapidly, resulting in bi-phasic IL-6 signaling, and relatively rapid desensitization. It should be interesting to see how the kinetics with which SOCS proteins are induced determines the kinetics of desensitization for cytokines other than IFNγ and IL-6.

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