

Expression of suppressors of cytokine signaling during liver regeneration

Jean S. Campbell,¹ Lisa Prichard,¹ Fred Schaper,² Jochen Schmitz,²
Alyssa Stephenson-Famy,¹ Maryland E. Rosenfeld,¹ Gretchen M. Argast,¹
Peter C. Heinrich,² and Nelson Fausto¹

¹University of Washington, Department of Pathology, Seattle, Washington, USA

²Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany

Address correspondence to: Nelson Fausto, Department of Pathology, University of Washington, K-078 Health Sciences Building, Box 357705, Seattle, Washington 98195, USA.

Phone: (206) 616-4550; Fax: (206) 616-1943; E-mail: nfausto@u.washington.edu.

Received for publication November 29, 2000, and accepted in revised form April 2, 2001.

The cytokines TNF and IL-6 play a critical role early in liver regeneration following partial hepatectomy (PH). Since IL-6 activates signal transducers and activators of transcription (STATs), we examined whether the suppressors of cytokine signaling (SOCS) may be involved in terminating IL-6 signaling. We show here that SOCS-3 mRNA is induced 40-fold 2 hours after surgery. SOCS-2 and CIS mRNA are only weakly induced, and SOCS-1 is not detectable. SOCS-3 induction after PH is transient and correlates with a decrease in STAT-3 DNA binding and a loss of tyrosine 705 phosphorylation. This response is markedly reduced in IL-6 knockout (KO) mice. TNF injection induces SOCS-3 mRNA in wild-type mice (albeit weakly compared with the increase observed after PH) but not in TNF receptor 1 or IL-6 KO mice. In contrast, IL-6 injection induces SOCS-3 in these animals, demonstrating a requirement for IL-6 in SOCS-3 induction. IL-6 injection into wild-type mice also induces SOCS-1, -2, and CIS mRNA, in addition to SOCS-3. Together, these results suggest that SOCS-3 may be a key component in downregulating STAT-3 signaling after PH and that SOCS-3 mRNA levels in the regenerating liver are regulated by IL-6.

J. Clin. Invest. 107:1285–1292 (2001).

Introduction

Restoration of liver mass after two-thirds partial hepatectomy (PH) is controlled by the complex interplay of cytokines, growth factors, and metabolic status (reviewed in refs. 1–5). For regeneration to take place, normally quiescent hepatocytes must gain proliferative capacity and undergo a transition from G0 to G1, a process known as priming (1, 6). Two known regulators of the priming phase of liver regeneration are the cytokines TNF and IL-6 (7–9). The levels of these cytokines increase after PH, with the peak of TNF circulating levels preceding the peak levels of IL-6 (8, 10, 11). Moreover, in mice lacking either TNF receptor 1 (p55/TNF-R1) or IL-6, hepatocyte proliferation and liver mass restoration are significantly impaired after PH (8, 9). Preoperative injection of IL-6 not only rescues the defects in hepatocyte proliferation, but returns a number of early signal transduction pathways to levels seen in wild-type (WT) mice (9). These studies and others suggest that IL-6 participates in hepatocyte proliferation *in vivo* not only in PH but in injury-induced models of liver proliferation (12).

In the liver, IL-6 activates multiple signaling pathways that culminate in the induction of genes involved in growth, hepatocyte-specific metabolic functions, and the acute-phase response (13). When IL-6 binds to its receptor complex composed of a specific receptor subunit, the IL-6 receptor (IL-6R), and a gp130 homo dimer, a cascade of signaling events is initiated, most

notably the Jak/STAT pathway (14, 15). Ligand-binding activates the receptor-associated protein tyrosine kinases, Janus kinases (Jak), and specific tyrosine residues in the intracellular domains of the receptors become phosphorylated. These phospho-tyrosine residues provide docking sites for signaling molecules, including the transcription factors' signal transducers and activators of transcription (STATs). The STATs themselves are phosphorylated on specific tyrosine residues that facilitate nuclear translocation of activated STATs and the binding of STAT dimers to specific DNA residues, thus activating transcription.

It has been demonstrated previously that STAT-3 is activated after PH as the result of increased IL-6 levels (8, 9, 16), implicating both IL-6 and STAT-3 as part of the priming mechanism for hepatocyte proliferation. However, very little is known about the downregulation or inhibition of IL-6-signaling pathways. To date, there are several mechanisms known to be involved in inactivating STAT pathways: the SH2-containing protein tyrosine phosphatases, SHP-1 and -2, the protein inhibitors of activated STATs (PIAS), and the suppressors of cytokine signaling (SOCS) (reviewed in refs. 4, 17–19). The latter is a growing family of genes that are rapidly induced by cytokines and growth factors and whose protein products negatively regulate the signaling pathways responsible for SOCS induction. The prototype, SOCS-1, also known as STAT-induced

STAT-inhibitor (SSI) or Jak-binding protein (JAB) (20–22), was identified by three different laboratories. SOCS are immediate early genes since transcripts are present at very low levels and can be rapidly induced upon stimulation. Depending on the tissue or cell type, the mRNA is short lived and the half-life of the SOCS protein is between 1 to 2 hours (23). Importantly, the STAT family of transcription factors mediates the induction of SOCS genes. Thus, SOCS proteins participate in a feedback loop to inhibit STAT activity as well as cytokine signals.

Since IL-6 has been implicated in mediating hepatocyte proliferation after PH, we hypothesized that proper coordination of the priming phase of liver regeneration must include negative regulation of the IL-6-induced signaling pathways. We show here that SOCS-3 is induced in a robust and transient manner in wild-type (WT) mouse liver after PH. The timing of the activation and inactivation of STAT-3 after PH correlates with SOCS-3 induction. Experiments with knockout (KO) mice indicate that IL-6 appears to regulate SOCS-3 directly, while TNF may regulate SOCS-3 indirectly. Although IL-6 injection can induce SOCS-3, the magnitude and spectrum of SOCS mRNA being induced are quite different from that seen during liver regeneration. Taken together, these results suggest that IL-6 is involved in SOCS-3 induction and that SOCS-3 may play a role in regulating liver regeneration after PH.

Methods

Animals. The C57BL/6 (WT) and IL-6 KO male mice were obtained from The Jackson Laboratories (Bar Harbor, Maine, USA). TNF-R1 KO (p55^{-/-}) mice have been described previously (8, 24). Animals were maintained in a specific pathogen-free facility under 12-hour dark/light cycles and given standard diet and water ad libitum. Two-thirds partial PH surgeries were performed on males between 8 to 10 weeks of age as described (8), using methoxyflurane. Laparotomy was performed as the sham operation, otherwise the sham-operated mice were treated the same as PH mice. Intraperitoneal injections of mTNF (25 µg/kg) and hIL-6 (1 mg/kg) were performed on males between the ages of 9 and 12 weeks of age. These high doses of IL-6 have been used previously to rescue defects in IL-6 KO mice (8, 9). Control mice received injections of saline. All animal work was in accordance with policies at the University of Washington.

Chemicals and reagents. Recombinant murine TNF and human IL-6 were purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA). STAT-1, -3, and -5 double-stranded oligonucleotides were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, California, USA). Ab's to STAT-1 (C-111), STAT-3 (C-20, H-190), and SOCS-3 (M-20) were purchased from Santa Cruz Biotechnologies Inc., while anti-phosphotyrosine 705 and anti-phosphoserine 729 STAT-3 Ab's (9131 and 9134, respectively) were purchased from New England Biolabs Inc. (Boston, Massachusetts, USA). Anti-β-actin Ab was purchased

from Sigma Chemical Co. (St. Louis, Missouri, USA). Horseradish peroxidase-conjugated (HRP-conjugated) anti-mouse, anti-rabbit, and anti-goat secondary Ab's were purchased from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA) and from Pierce Chemical Co. (Rockford, Illinois, USA), respectively.

Electrophoretic mobility-shift assay. Hepatocytes were lysed and nuclei extracted as reported previously (8). For the assay, 5 µg of nuclear protein was incubated at room temperature for 30 minutes with 0.2 ng of ³²P-end-labeled, double-stranded STAT oligonucleotide followed by electrophoresis through a 5% polyacrylamide Tris-glycine-EDTA gel. Gels were dried under a vacuum and exposed overnight to Kodak X-AR film at -80°C with an intensifying screen. For supershifts, specific Ab's for STAT-3 (C-20X) and STAT-1 (C-111) were added to the nuclear extracts for 30 minutes at room temperature before the addition of labeled probe.

Immunoblot analysis. Liver tissue (1–2 mm³) was homogenized in 1 ml of a 1% Triton X-100 lysis buffer described previously (25), supplemented with soybean trypsin inhibitor (50 µg/ml), E-64 (10 µg/ml), and microcystin (200 nM). Protein quantification was performed using Bradford reagent (Bio-Rad Laboratories Inc., Hercules, California, USA), and 50 µg of total protein lysate was subjected to SDS-PAGE and transferred to PVDF (Millipore Corp., Bedford, Massachusetts, USA). For STAT-3 immunoprecipitations, 1 µg of anti-STAT3 Ab (C-20X) was added to 300 µg of protein lysate and incubated overnight at 4°C. Protein A-Sepharose (40 µl of 1:1 [vol/vol] slurry in Triton X-100 lysis buffer) was added for 2 hours, and the bound complexes were washed two times in lysis buffer. Immunoprecipitated proteins were subjected to immunoblot analysis. Membranes were blocked in TBST (Tris-buffered saline with 0.1% Tween 20) containing 5% milk (blotting grade; Bio-Rad Laboratories Inc.) at 4°C and incubated with primary Ab's. The following dilutions were used: phosphotyrosine STAT-3 (1:2,000), phosphoserine STAT-3 (1:1,000), and SOCS-3 (1:1,000) in 0.5% milk in TBST overnight. The appropriate secondary Ab's were added for 2–3 hours in 0.5% milk in TBST and antigen-Ab complexes were detected with enhanced chemiluminescent reagents purchased from either NEN Life Science Products (Boston, Massachusetts, USA) or Pierce Chemical Co. The indicated blots were stripped as recommended by NEN Life Science Products and reprobed with STAT-3 (1:1,000; H-190) or β-actin (1:5,000; AC-15). Western blot analysis detection of CIS, SOCS-1, and SOCS-2 proteins was unsuccessful as the commercially available Ab's (Santa Cruz Biotechnologies Inc. and Zymed Inc., Camarillo, California, USA) were unable to detect these proteins in liver lysates (data not shown).

RNA preparation and Northern analysis. Total RNA was prepared from liver tissue using a guanidine thiocyanate buffer and ultracentrifugation as described previously (24). Total RNA (10 µg) was separated on a 0.8% formaldehyde agarose gel and transferred to

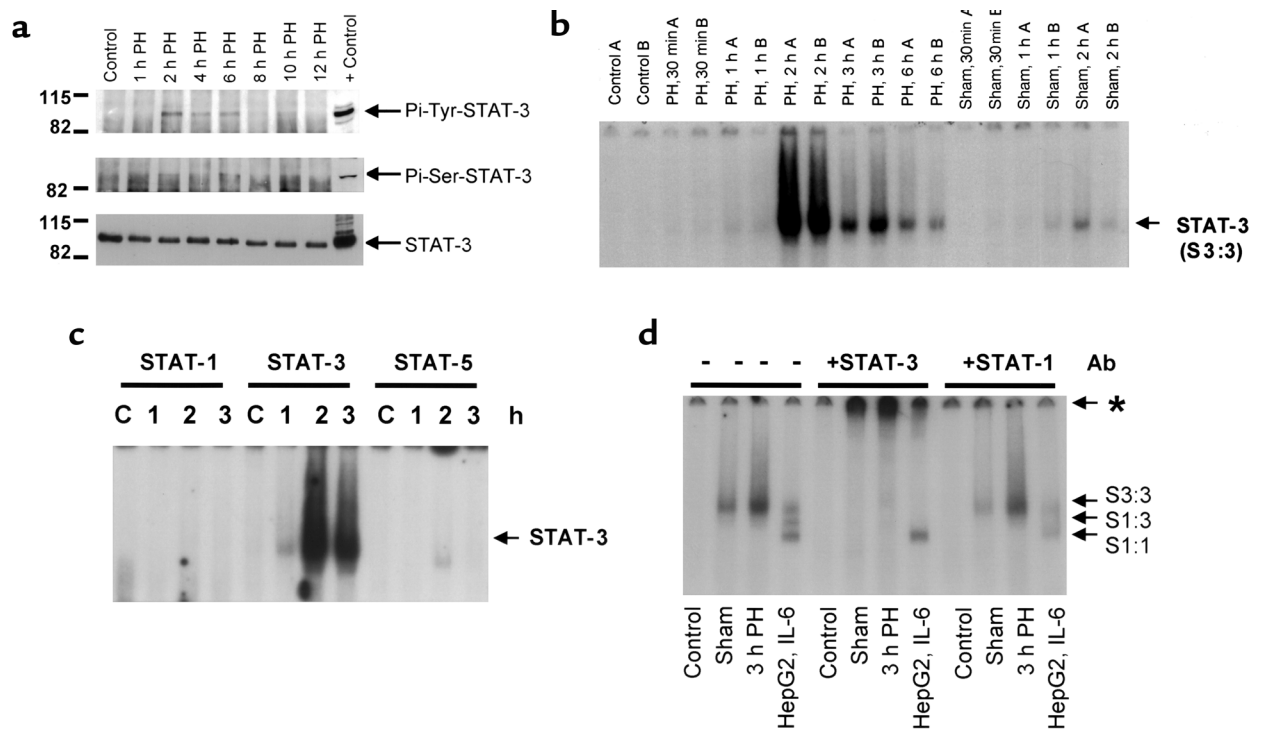


Figure 1

Transient activation of STAT-3 after PH in WT mice. Nuclear extracts and whole-cell lysates were prepared from the remnant liver at the indicated times after PH. (a) STAT-3 was immunoprecipitated from whole-cell lysates and analyzed for phosphotyrosine 705 and phosphoserine 727 content using immunoblot analysis. The top panel is phosphotyrosine 705-STAT-3, the middle panel is phosphoserine 727-STAT-3, and the bottom panel is immunoprecipitated STAT-3. As a positive control, STAT-3 was immunoprecipitated from whole-cell lysates from IL-6-stimulated AML12 hepatocytes (10 ng/ml, 15 minutes) and is shown on the right. Molecular-weight markers are shown on the left in kilodaltons. (b) DNA-binding ability of nuclear STAT-3 was analyzed by electrophoretic mobility-shift assay (EMSA) as described in Methods. Data from duplicate mice are shown. (c) DNA-binding ability of nuclear STAT-3 was compared with nuclear STAT-1 and STAT-5 DNA-binding activity. Control (C), 1-, 2-, and 3-hour time points were analyzed by EMSA as described in Methods. (d) Supershift analysis of STAT-3 and STAT-1 DNA-binding activity. Nuclear extracts from HepG2 cells stimulated with IL-6 (10 ng/ml) for 15 minutes are shown as an example of STAT-3 homodimers (S3:3), STAT-1:STAT-3 heterodimers (S1:3), and STAT-1 homodimers (S1:1). Ab-DNA-protein complexes are indicated by an asterisk. Control (C), 2-hour sham-operated, and 3-hour PH surgical time points were analyzed.

GeneScreen Plus (NEN Life Science Products). SOCS-1, -2, -3, and CIS cDNA probes were generated from *Xba*I digests of SOCS expression plasmids provided by Doug Hilton and Tracy Willson (26). Probes were labeled with α^{32} -ATP using a random prime kit (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Murine cyclophilin riboprobe and the RNA Millennium markers were purchased from Ambion Inc. (Austin, Texas, USA). Quantification of SOCS and cyclophilin mRNA levels was carried out using a Storm phosphorimager (Molecular Dynamics, Sunnyvale, California, USA). SOCS mRNA levels were normalized to cyclophilin levels at each time point. Fold induction is the relative induction compared with control mice (i.e., nonanesthetized, nonsurgery mice). Statistical analysis of the data was performed using GraphPad Prism (GraphPad Software for Science Inc., San Diego, California, USA). Data was analyzed by one-way ANOVA followed by the Newman-Keuls test.

Tissue culture. HepG2 and RAW 267.4 cultures were maintained according to the manufacturer's recommendation (American Type Culture Collection, Man-

assas, Virginia, USA). AML12 hepatocytes, a well-differentiated, nontransformed murine hepatocyte cell line, were cultured as described previously (25). To generate positive controls, cultures were stimulated with IL-6 (10 ng/ml) for the indicated times, and whole-cell lysates were prepared as described above.

Results

PH activates STAT-3 in a transient manner. The time course of activation of STAT transcription factors was examined after PH to corroborate and extend previous studies (8, 9, 16) and to determine the time frame of STAT-3 inactivation. Since activation of STAT-3 requires phosphorylation on tyrosine 705 to facilitate DNA binding (14, 15), this parameter was examined. At the indicated time points, whole-cell lysates were prepared from hepatic tissue, STAT-3 was immunoprecipitated, and the phosphorylation status was determined by phospho-specific Ab's. STAT-3 tyrosine 705 phosphorylation is first detected by 2 hours after surgery and decreases to background levels by 8 hours (Figure 1a, top). Phosphorylation of serine 727 of STAT-3 can

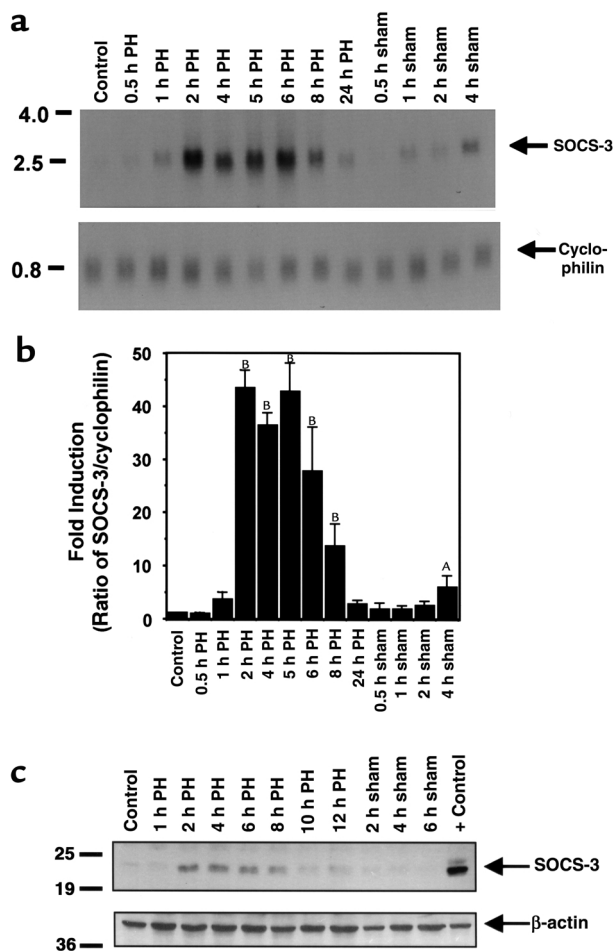


Figure 2 Induction of SOCS-3 mRNA and protein after PH in WT mice. Total RNA was prepared from the remnant liver at the indicated times after PH as described in Methods. (a) Total RNA was probed for SOCS-3 and cyclophilin mRNA levels as described in Methods. Molecular-weight markers are shown on the left in kilobases. (b) Induction of SOCS-3 mRNA was quantified by phosphorimager analysis relative to cyclophilin levels. The error bars represent SEM of the data from three independent experiments. ^A $P < 0.01$, ^B $P < 0.001$ vs. control values. (c) Whole-cell lysates were prepared from the remnant liver at the indicated times after PH. SOCS-3 protein was detected by immunoblot analysis using SDS-PAGE (top panel). As a positive control, cell lysate from IL-6-stimulated RAW 264.7 cells is shown on the right and molecular-weight markers are shown on the left in kilodaltons. To ensure equal loading, the blot was stripped and reprobbed for β -actin (bottom panel).

be weakly detected (Figure 1a, middle), but the signal is variable. Importantly, tyrosine 705 phosphorylation of STAT-3 correlates with its ability to bind DNA (Figure 1b). As has been demonstrated previously (8, 9, 16), robust STAT-3 DNA-binding activity is detected by 2 hours and rapidly disappears between 3 to 6 hours. Sham-operated mice have a detectable, but much weaker STAT-3 DNA-binding activity (Figure 1b). Use of STAT-1 and STAT-5 oligonucleotide probes demonstrates that STAT-3 is the major STAT family member activated after PH (Figure 1c). Supershift analysis con-

firms that the STAT-3 DNA-binding activity being measured is due to a STAT-3 homodimer, not a STAT-1:STAT-3 heterodimer (Figure 1d). No STAT-1 homodimer activity was seen in these experiments. Thus, STAT-3 tyrosine 705 phosphorylation and DNA-binding activity are transiently activated after surgery.

PH induces SOCS-3 in a robust and transient manner. STAT-3 activity can be downregulated by a number of mechanisms (14), but none of these pathways have been examined in the context of liver regeneration. We examined the induction of a family of immediate early genes that are upregulated by STAT proteins and are potent inhibitors of the Jak/STAT pathway (20–22). SOCS-1, -2, -3, and CIS mRNA levels were examined using Northern analysis after PH in livers of WT mice. SOCS-3 mRNA is rapidly induced in the mouse liver after PH compared with sham-operated animals (Figure 2, a and b). A 40- to 50-fold increase of SOCS-3 mRNA is seen at 2 hours and is sustained for 4 hours when the levels start declining at 8 hours after PH and return to baseline levels by 24 hours (Figure 2b). A weak induction of SOCS-3 is detectable in sham-operated mice (Figure 2). This is perhaps due to the weak, but detectable, STAT-3 activation also observed in these mice (Figure 1b). While SOCS-1 mRNA was not induced by PH, CIS and SOCS-2 mRNA was detectable, albeit much weaker than SOCS-3 (data not shown). PH induced CIS and SOCS-2 levels only between fourfold and tenfold compared with controls, and in some experiments no induction was detected. Thus, PH appears to selectively upregulate SOCS-3 and not other members of the SOCS family.

To examine SOCS-3 protein induction, whole-cell lysates were prepared from hepatic tissue at the indicated time points after surgery. Increased SOCS-3 protein levels were first detected by 2 hours with sustained expression until 8 and 10 hours (Figure 2c, top). The blots were stripped and reprobbed with β -actin to ensure equal loading (Figure 2c, bottom). Detection of SOCS-3 protein is consistent with the induction of SOCS-3 mRNA (Figure 2a). Taken together, these results indicate that SOCS-3 is the major SOCS family member induced after PH, and the induction of SOCS-3 mRNA and protein levels is transient. Importantly, the induction of SOCS-3 correlates with the activation and inactivation of STAT-3, suggesting that in liver regeneration SOCS-3 participates in a negative-feedback loop to decrease STAT-3 activity.

Blunted induction of SOCS-3 in IL-6 KO mice after PH. We next examined the role of IL-6 in the regulation of SOCS-3 induction since IL-6 serum levels are elevated after PH and this cytokine stimulates STAT-3 activity (8, 9). IL-6 KO mice were subjected to PH, and total RNA was prepared from liver tissue obtained at the indicated time points. Although SOCS-3 message levels were induced compared with either control or sham-operated mice, SOCS-3 mRNA induction in the IL-6 KO liver was severely reduced after PH compared with WT tissue (Figure 3, a and b). None of the other SOCS messages were detectable (data not shown). Moreover, SOCS-3

protein was detected in WT by immunoblot analysis, but not in IL-6 KO liver (Figure 3c). Densitometry analysis showed that at 2 hours after PH there was approximately a threefold induction of SOCS-3 in livers of WT mice compared with IL-6 KO mice (data not shown). These results suggest that IL-6 is a major regulator of SOCS-3 induction after PH.

Defective induction of STAT-3 in IL-6 KO mice after PH. The blunted induction of SOCS-3 in IL-6 KO mice is likely due to a deficit of STAT-3 DNA-binding observed previously in these mice (9). We examined both the DNA-binding ability of STAT-3 and its tyrosine 705 phosphorylation status after surgery. STAT-3 was immunoprecipitated from whole-cell lysates prepared from remnant liver after PH, and the tyrosine 705 phosphorylation status was determined using Western blot analysis. In IL-6 KO mice, STAT-3 tyrosine phosphorylation was detectable by 2 hours after surgery, although it was weaker than the levels seen in WT animals (Figure 4a, top). However, the ability of STAT-3 to bind DNA was impaired (Figure 4b). Similar to Cressman et al. (9), our results show that STAT-3 DNA-binding activity is greatly impaired after PH in IL-6 KO mice liver. Specific STAT-3 binding was analyzed by supershift analysis with a specific STAT-3 Ab, and competition with a 200-fold excess of unlabeled probe confirmed that only STAT-3 bound to the SIE probe (Figure 4b). The low level of tyrosine 705 phosphorylation of STAT-3 in the KO mice after PH correlates with weak STAT-3 DNA binding (Figure 4b) and weak induction of SOCS-3 (Figure 3a). Sham-operated mice also have a detectable, but much weaker STAT-3 tyrosine 705 phosphorylation and DNA-binding capacity (Figure 4a and Figure 1b).

TNF injection induces SOCS-3 in WT, but not in TNF-R1 and IL-6 KO mice. Current models of liver regeneration suggest that IL-6 serum levels are regulated by a prior release of TNF (1). We examined if TNF injection could induce SOCS-3 induction and whether the induction was dependent on the TNF receptor, TNF-R1, or the cytokine, IL-6. WT, TNF-R1 KO and IL-6 KO mice were injected with TNF and liver tissue obtained at the indicated time points. Total RNA was probed for SOCS-1, -2, -3, and CIS mRNA. Figure 5 shows that TNF injection into WT mice induces SOCS-3 in a time-dependent manner compared with control and saline injection. SOCS-3 message is first detected at 1 hour and disappears rapidly (Figure 5a). TNF injection also transiently induces CIS mRNA (Figure 5b) and SOCS-2 and SOCS-1 mRNAs (data not shown). These results suggest that while TNF can induce SOCS-3, this induction is much weaker and more transient compared with the induction following PH.

When TNF-R1 KO or IL-6 KO mice were injected with TNF, we found that TNF did not induce SOCS-3 in the liver of either mouse strain (Figure 5b). In contrast to SOCS-3, CIS is induced in the liver of IL-6 KO mice but not in TNF-R1 KO mice. These results suggest that SOCS-3 induction is dependent on IL-6 and, perhaps, TNF-signaling pathways. In contrast, induction of CIS after TNF injection seems to require only TNF-R1 and not IL-6. It is

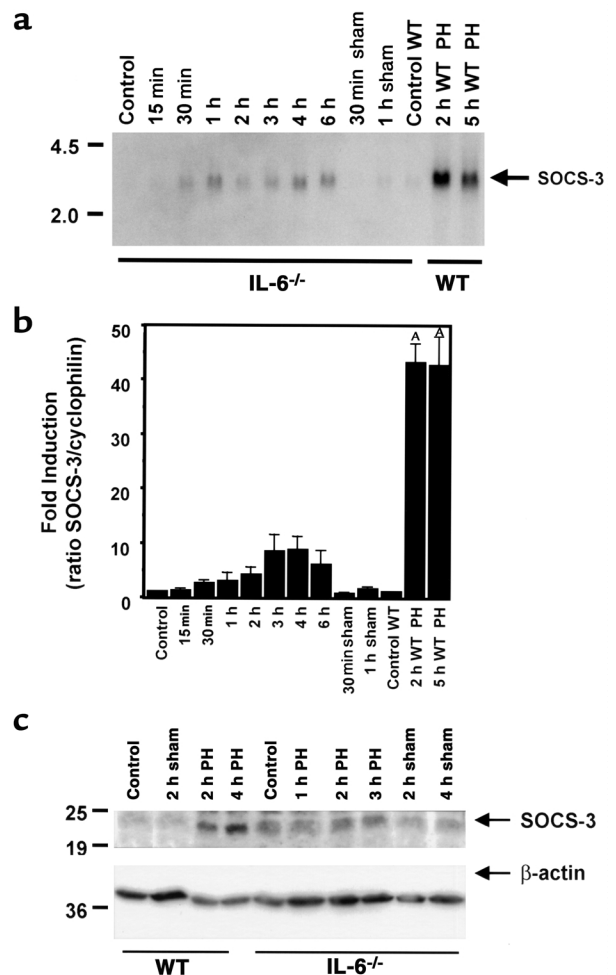


Figure 3 Blunted induction of SOCS-3 mRNA and protein in IL-6 KO mice after PH. Total RNA was prepared from the remnant liver at the indicated times after PH as described in Methods. (a) Total RNA was probed for SOCS-3 and cyclophilin message levels as described in Methods. Molecular-weight markers are shown on the left in kilobases. (b) Induction of SOCS-3 mRNA was quantified by phosphor imager analysis as described in Figure 2. The error bars represent SEM of the data from three independent experiments. ^AP < 0.001 vs. IL-6 KO, 3-hour PH values. (c) Whole-cell lysates were prepared from the remnant liver at the indicated times after PH. SOCS-3 protein was detected by immunoblot analysis using SDS-PAGE (top panel). The blot was stripped and reprobed for β -actin (bottom panel). Molecular-weight markers are shown on the left in kilodaltons.

important to note that PH induces SOCS-3 to much higher levels (>40-fold) than TNF injection (<15-fold) (compare Figure 5b to Figure 2b). Additionally, we find that the magnitude of induction and spectrum of SOCS molecules that are induced is markedly different after TNF injection when compared with that seen after PH. After TNF injection, we see that SOCS-3 induction is about twofold greater than CIS induction. In contrast, after PH, SOCS-3 induction is approximately sixfold greater than CIS. Moreover, SOCS-1 and -2 mRNA levels are detectable after TNF injection. These data suggest that TNF is not the sole mediator of SOCS-3 induction after PH.

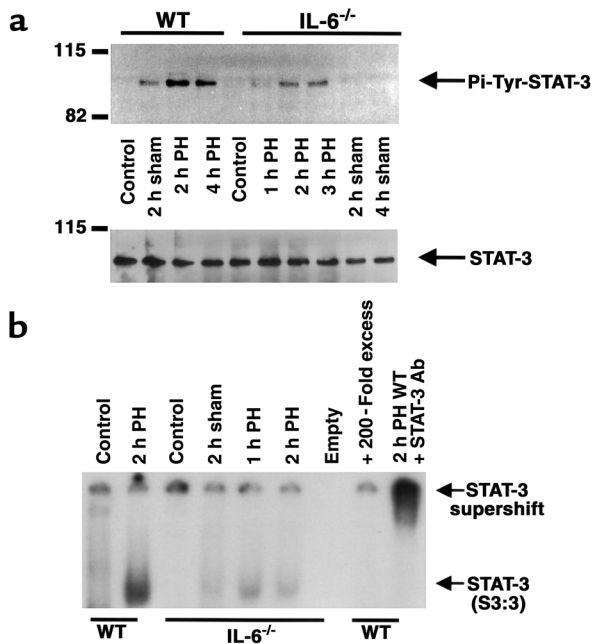


Figure 4 Defective activation of STAT-3 after PH in IL-6 KO mice. Nuclear extracts and whole-cell lysates were prepared from the remnant liver of WT and IL-6 KO mice at the indicated times after PH. (a) STAT-3 was immunoprecipitated from whole-cell lysates and analyzed for phosphotyrosine 705 content by immunoblot analysis (top panel). The bottom panel shows the total immunoprecipitated STAT-3. Molecular-weight markers are shown on the left in kilodaltons. (b) DNA-binding ability of nuclear STAT-3 was analyzed by EMSA as described in Methods. The 2-hour PH time point from WT mouse liver was used both for supershift analysis with a specific STAT-3 Ab and for competition with a 200-fold excess of unlabeled probe.

IL-6 injection induces SOCS-3 mRNA in TNF-R1- and IL-6 KO mice. We next examined whether IL-6 injection could directly induce SOCS-3 and bypass the lack of SOCS-3 mRNA induction in TNF-R1- and IL-6 KO mice after TNF injection. WT, TNF-R1-, and IL-6 KO mice were injected with IL-6 or TNF, and liver tissue was obtained 1 or 1.5 hours after injection, respectively. Total RNA was probed for SOCS-1, -2, -3, and CIS. Figure 6 shows that IL-6 injection induces SOCS-3 mRNA in all three genetic strains, indicating that IL-6 can bypass the defects in TNF-R1- and IL-6 KO mice. IL-6 injection induces SOCS-3 message levels 40- to 60-fold (Figure 6, a and b). The magnitude of SOCS-3 induction after IL-6 injection is now comparable with that seen after PH. However, IL-6 injection also induces SOCS-1, -2, and CIS mRNA expression, a pattern not seen after PH. SOCS-3 protein can be detected after IL-6 injection, but not after TNF injection (Figure 6c). In summary, IL-6 injection bypasses the lack of TNF and IL-6 signaling and induces all SOCS molecules examined in murine liver.

Discussion

A complex growth process such as liver regeneration requires the precise orchestration of a multitude of

hormonal, metabolic, cytokine, and growth-factor signals to ensure the precise timing and coordination of DNA replication. Priming and progression of hepatocyte proliferation after PH requires the actions of TNF and IL-6 and growth factors such as TGF- α and HGF (1, 3, 5, 27). However, the mechanisms that downregulate or “turn off” these signaling pathways is unclear despite their critical role in controlling growth. The studies presented here show that SOCS-3 transcripts and protein are induced during the priming phase of liver regeneration and that this induction is greatly diminished in IL-6 KO mice.

TNF levels increase after PH, activating the transcription factor, NF- κ B, which in turn upregulates IL-6 mRNA, resulting in elevated serum levels of IL-6. IL-6 stimulates hepatic STAT-3 by inducing phosphorylation at tyrosine 705, which allows nuclear translocation and DNA binding. STAT-3 mediates transcription of a variety of genes including genes involved in proliferation such as cyclins,

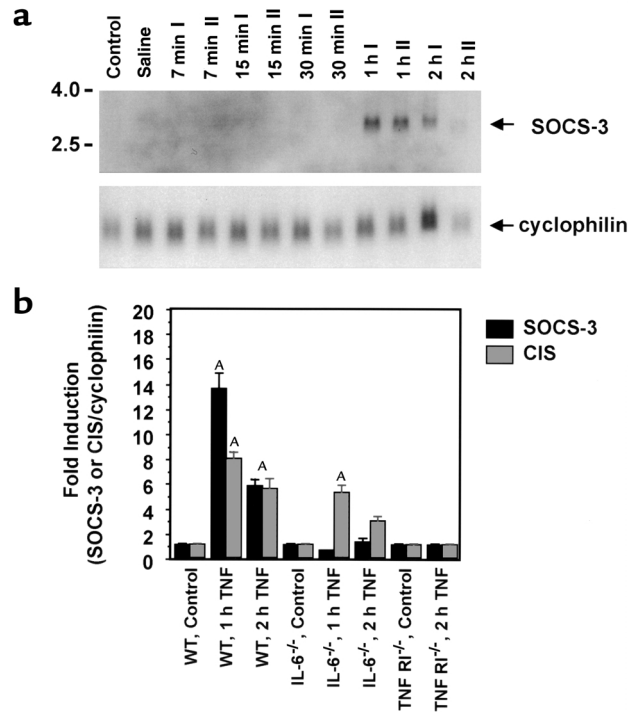
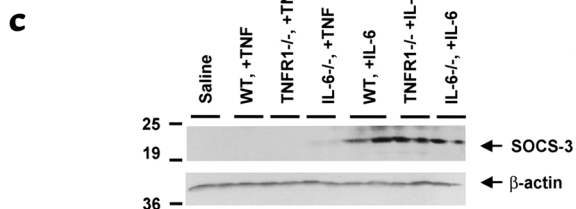
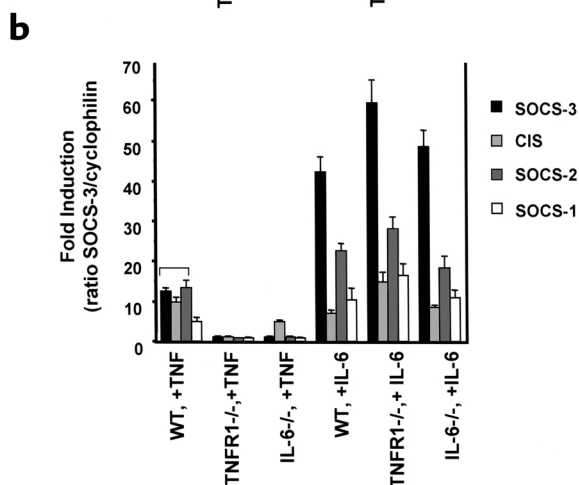
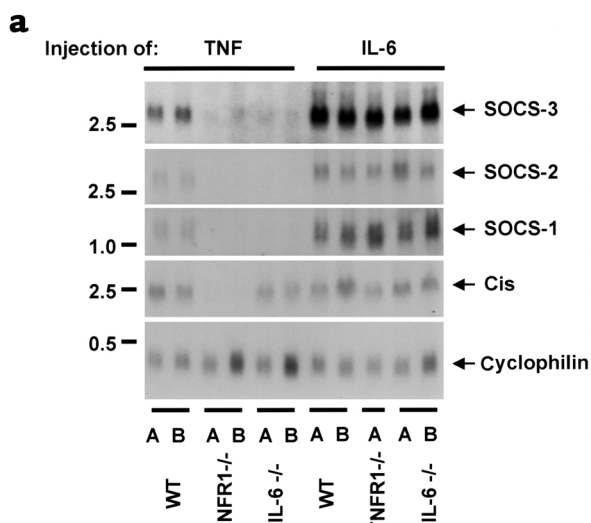


Figure 5 TNF injection induces SOCS-3 and CIS mRNA in a time-dependent manner in WT, but not in TNF-R1 KO and IL-6 KO mouse liver. Total RNA was prepared from tissue obtained from the median liver lobe at the indicated times after TNF injection (25 μ g/kg, intraperitoneally). (a) Total RNA from WT mice was probed for SOCS-3, CIS, and cyclophilin message levels as described in Methods. Molecular-weight markers are shown on the left in kilobases. I and II represent RNA prepared from duplicate mice. (b) Induction of SOCS-3 and CIS mRNA levels was quantified by phosphorimager analysis as described in Figure 2. Quantification of SOCS-3 and CIS level is shown in black and gray bars, respectively, for WT, TNF-R1-, and IL-6 KO mice. Similar time-course experiments were done on TNF-R1- and IL-6 KO mice, and total RNA was prepared and probed as described in a for WT mice. The error bars represent SEM of the data from three independent experiments. $^{\wedge}P < 0.001$ vs. control values.

Figure 6

IL-6 injection induces SOCS-3 mRNA and protein in TNF-R1⁻ and IL-6 KO mice. (a) Total RNA was prepared from tissue obtained from the median liver lobe after intraperitoneal TNF (25 μg/kg; 1.5 h) or hIL-6 injection (1,000 μg/kg; 1 hour) in WT, TNF-R1 KO, and IL-6 KO mice. Total RNA was probed for SOCS-1, -2, -3, CIS, and cyclophilin mRNA levels as described in Methods. Molecular-weight markers are shown on the left in kilobases. I and II represent RNA prepared from duplicate mice. (b) Induction of SOCS-3, -1, -2, and CIS mRNA levels was quantified by phosphorimager analysis relative to cyclophilin levels. Fold induction is compared with TNF-R1 KO mice injected with TNF. The error bars represent SEM of the data from three to five mice per strain and cytokine. **P* < 0.01, ***P* < 0.001 vs. TNF-R1 KO mice injected with TNF. Quantification of SOCS-3, -1, -2, and CIS levels is shown in filled, open, red, and blue bars, respectively. (c) Whole-cell lysates were prepared from the median liver lobe, and SOCS-3 protein was detected by immunoblot analysis using SDS-PAGE (top panel). The blot was stripped and reprobed for β-actin (bottom panel). Molecular-weight markers are shown on the left in kilodaltons.



cdc25A, and c-myc (reviewed in refs. 28 and 29). STAT-3 also induces SOCS-3 mRNA leading to an increase in SOCS-3 protein levels, which downregulate IL-6-stimulated STAT-3. Both STAT-3 activity and SOCS-3 protein are no longer detectable by 8 hours after PH. Thus in liver regeneration, SOCS-3 participates in a negative-feedback loop turning off IL-6-mediated STAT-3 activation. Inhibition of STAT-3 signaling may ensure the termination of the priming phase of liver regeneration.

SOCS-3 appears to be the major isoform regulating cytokine signaling during liver regeneration. Compared with SOCS-3, which is induced 40- to 60-fold after PH, CIS and SOCS-2 were only weakly induced. Since SOCS-3 induction is severely blunted in IL-6 KO liver after PH, the induction of SOCS-3 after PH is, for the most part, dependent on IL-6. Interestingly, when IL-6 is injected into mice, there are striking differences in the SOCS profiles compared with induction after PH. SOCS-3 mRNA is induced by 1 hour and rapidly declines by 2 hours after IL-6 injection (data not shown). After PH, SOCS-3 mRNA is much more sustained because mRNA is detected between 2 and 8 hours after PH. In addition to SOCS-3, injection of IL-6 induces CIS, SOCS-1, and SOCS-2 mRNA (Figure 6) in the liver, an observation reported by other laboratories (20). In contrast, CIS, SOCS-1, and SOCS-2 are not strongly induced after PH. Besides IL-6, there must be other factors, cytokines, or growth factors, that directly or indirectly influence SOCS-3 induction after PH. Moreover, the lack of induction of the other SOCS isoforms after PH suggests that a single injection of IL-6 does not faithfully reproduce the signaling parameters of PH.

The SOCS-3 induction is not completely abolished in IL-6 KO mice after PH, perhaps uncovering the actions of other cytokines. Clues to the identity of these cytokines or factors maybe revealed in the limited pattern of STAT family members that can bind DNA after PH. Only STAT-3 homodimers are seen after PH (Figure 2c and ref. 16). Moreover, both hepatocytes in vivo and in culture respond to IL-6 treatment in a similar manner;

that is, only STAT-3 homodimers (SIF A) are found (unpublished observations and ref. 30). One notable exception is the human hepatoma cell line, HepG2 (Figure 1c), where SIF A, B (STAT-3:STAT-1), and C (STAT-1:STAT-1) DNA complexes are all detected after IL-6 treatment (30). Oncostatin M (OSM), leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1), members of the IL-6 cytokine family that can induce STAT-3 DNA binding, could be the cytokines that cooperate with IL-6 during regeneration. These cytokines have been implicated in liver functions such as development, hepatocyte differentiation, or inflammation (31–33), but have yet to be examined in liver regeneration.

SOCS-3 has not been shown to have catalytic phosphatase activity, yet by 8 hours after PH, STAT-3 is no

longer tyrosine phosphorylated (Figure 1a). Thus two mechanisms, SOCS-3 and a tyrosine phosphatase, may coexist to ensure complete inhibition of cytokine signals mediated by STATs. In the first mechanism, nascent SOCS-3 blocks the activation of STAT-3 by binding to the receptor complex via gp130 at tyrosine 759 (34, 35) and/or by binding and inhibiting Jak2 activity (36). Secondly, existing, activated pools of STAT-3 (i.e., tyrosine phosphorylated) may be inactivated by dephosphorylation through the actions of a tyrosine phosphatase. Possible tyrosine phosphatases include an unidentified nuclear tyrosine phosphatase activity that has been reported to inactivate IFN- γ -stimulated STAT-1 (37) and the SH2-containing phosphatases, SHP-1 and SHP-2, which regulate cytokine signals in other tissues (38). However, the roles of any of these candidate tyrosine phosphatases in regeneration remain to be examined. Other mechanisms such as PIAS (39) that downregulate cytokine signaling in other cells may also play a role in inhibiting early cytokine signaling during PH.

The priming phase of hepatocyte replication probably ends at 5–6 hours after PH, perhaps even earlier. Many cytokine signals are downregulated by the end of this time period. SOCS-3 appears to play an important role in inhibiting the IL-6–signaling pathway. While SOCS-3 is induced after PH and the timing of its expression correlates very well with the inactivation of STAT-3 DNA binding, more precise analysis of the role of SOCS-3 awaits the establishment of SOCS-3–transgenic or KO mice. These expression systems require conditional expression of SOCS-3 within the parenchymal cells of the liver since constitutive expression or ablation results in embryonic death (40). Nevertheless, this analysis of SOCS-3 contributes to our understanding of the termination of signaling pathways involved in liver regeneration.

Acknowledgments

The authors wish to thank Doug Hilton and Tracy Willson for providing the SOCS expression plasmids. The technical assistance of Seth L. Mercer is gratefully acknowledged. This work was supported by the NIH grants CA-23226 and CA-74131 (N. Fausto), M.E. Rosenfeld was supported by a postdoctoral training grant F32-DK09920-01, and G.M. Argast was supported by training grant T32-ES07032-23. F. Schaper and P.C. Heinrich were supported by grants from the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the Fonds der Chemischen Industrie (Frankfurt, Germany).

1. Fausto, N. 2000. Liver regeneration. *J. Hepatol.* **32**:19–31.
2. Fausto, N., Laird, A.D., and Webber, E.M. 1995. Liver regeneration. II. Role of growth factors and cytokines in hepatic regeneration. *FASEB J.* **9**:1527–1536.
3. Michalopoulos, G.K., and DeFrances, M.C. 1997. Liver regeneration. *Science.* **276**:60–66.
4. Holecek, M. 1999. Nutritional modulation of liver regeneration by carbohydrates, lipids, and amino acids: a review. *Nutrition.* **15**:784–788.
5. Taub, R., Greenbaum, L.E., and Peng, Y. 1999. Transcriptional regulatory signals define cytokine-dependent and -independent pathways in liver regeneration. *Semin. Liver Dis.* **19**:117–127.
6. Steer, C.J. 1995. Liver regeneration. *FASEB J.* **9**:1396–1400.
7. Plumpe, J., Streetz, K., Manns, M.P., and Trautwein, C. 1999. Tumour necrosis factor alpha: mediator of apoptosis and cell proliferation of

- hepatocytes. *Ital. J. Gastroenterol. Hepatol.* **31**:235–243.
8. Yamada, Y., Kirillova, I., Peschon, J.J., and Fausto, N. 1997. Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc. Natl. Acad. Sci. USA.* **94**:1441–1446.
9. Cressman, D.E., et al. 1996. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science.* **274**:1379–1383.
10. Trautwein, C., Rakemann, T., Niehof, M., Rose-John, S., and Manns, M.P. 1996. Acute-phase response factor, increased binding, and target gene transcription during liver regeneration. *Gastroenterology.* **110**:1854–1862.
11. Akerman, P., et al. 1992. Antibodies to tumor necrosis factor-alpha inhibit liver regeneration after partial hepatectomy. *Am. J. Physiol.* **263**:G579–G585.
12. Streetz, K.L., Luedde, T., Manns, M.P., and Trautwein, C. 2000. Interleukin 6 and liver regeneration. *Gut.* **47**:309–312.
13. Heinrich, P.C., Castell, J.V., and Andus, T. 1990. Interleukin-6 and the acute phase response. *Biochem. J.* **265**:621–636.
14. Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**:297–314.
15. Imada, K., and Leonard, W.J. 2000. The Jak-STAT pathway. *Mol. Immunol.* **37**:1–11.
16. Cressman, D.E., Diamond, R.H., and Taub, R. 1995. Rapid activation of the Stat3 transcription complex in liver regeneration. *Hepatology.* **21**:1443–1449.
17. Kovanen, P.E., and Leonard, W.J. 1999. Inhibitors keep cytokines in check. *Curr. Biol.* **9**:R899–R902.
18. Starr, R., and Hilton, D.J. 1999. Negative regulation of the JAK/STAT pathway. *Bioessays.* **21**:47–52.
19. Yasukawa, H., Sasaki, A., and Yoshimura, A. 2000. Negative regulation of cytokine signaling pathways. *Annu. Rev. Immunol.* **18**:143–164.
20. Starr, R., et al. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature.* **387**:917–921.
21. Endo, T.A., et al. 1997. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature.* **387**:921–924.
22. Naka, T., et al. 1997. Structure and function of a new STAT-induced STAT inhibitor. *Nature.* **387**:924–929.
23. Siewert, E., Muller-Esterl, W., Starr, R., Heinrich, P.C., and Schaper, F. 1999. Different protein turnover of interleukin-6-type cytokine signalling components. *Eur. J. Biochem.* **265**:251–257.
24. Yamada, Y., Webber, E.M., Kirillova, I., Peschon, J.J., and Fausto, N. 1998. Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. *Hepatology.* **28**:959–970.
25. Pierce, R.H., et al. 2000. Disruption of redox homeostasis in tumor necrosis factor-induced apoptosis in a murine hepatocyte cell line. *Am. J. Pathol.* **157**:221–236.
26. Nicholson, S.E., et al. 1999. Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO J.* **18**:375–385.
27. Brenner, D.A. 1998. Signal transduction during liver regeneration. *J. Gastroenterol. Hepatol.* **13**(Suppl.):S93–S95.
28. Hirano, T., Ishihara, K., and Hibi, M. 2000. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene.* **19**:2548–2556.
29. Fukada, T., et al. 1998. STAT3 orchestrates contradictory signals in cytokine-induced G1 to S cell-cycle transition. *EMBO J.* **17**:6670–6677.
30. Bode, J.G., et al. 1999. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett.* **463**:365–370.
31. Miyajima, A., et al. 2000. Role of Oncostatin M in hematopoiesis and liver development. *Cytokine Growth Factor Rev.* **11**:177–183.
32. Peters, M., Roeb, E., Pennica, D., Meyer zum Buschenfelde, K.H., and Rose-John, S. 1995. A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1). *FEBS Lett.* **372**:177–180.
33. Omori, N., et al. 1996. Expression of leukemia inhibitory factor and its receptor during liver regeneration in the adult rat. *Lab. Invest.* **75**:15–24.
34. Schmitz, J., Weissenbach, M., Haan, S., Heinrich, P.C., and Schaper, F. 2000. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. *J. Biol. Chem.* **275**:12848–12856.
35. Nicholson, S.E., et al. 2000. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc. Natl. Acad. Sci. USA.* **97**:6493–6498.
36. Krebs, D.L., and Hilton, D.J. 2000. SOCS: physiological suppressors of cytokine signaling. *J. Cell Sci.* **113**:2813–2819.
37. Haspel, R.L., and Darnell, J.E., Jr. 1999. A nuclear protein tyrosine phosphatase is required for the inactivation of Stat1. *Proc. Natl. Acad. Sci. USA.* **96**:10188–10193.
38. Feng, G.S. 1999. Shp-2 tyrosine phosphatase: signaling one cell or many. *Exp. Cell Res.* **253**:47–54.
39. Shuai, K. 2000. Modulation of STAT signaling by STAT-interacting proteins. *Oncogene.* **19**:2638–2644.
40. Marine, J.C., et al. 1999. SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell.* **98**:617–627.