Interferon-γ inhibits interferon-α signalling in hepatic cells: evidence for the involvement of STAT1 induction and hyperexpression of STAT1 in chronic hepatitis C

Svetlana RADAEVA*, Barbara JARUGA*, Won-Ho KIM*, Theo HELLER†, T. Jake LIANG† and Bin GAO*[1]

*Section on Liver Biology, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 12420 Parklawn Drive, MSC 8115, Bethesda, MD 20892, U.S.A., and †Liver Diseases Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

IFN-γ (interferon-γ) modulates IFN-α therapy in chronic hepatitis C infection; however, the underlying mechanism remains unclear. Here we demonstrate that long-term (3–6 days) but not short-term (up to 1 day) IFN-γ treatment of human hepatoma Hep3B cells attenuates IFN-α activation of STAT1 (signal transducers and activators of transcription factor 1), STAT2 and STAT3, but enhances IFN-γ and interleukin 6 activation of STATs. Prolonged exposure to IFN-γ also significantly induces STAT1 protein expression without affecting STAT2, STAT3 and ERK (extracellular-signal-regulated kinase) 1/2 protein expression. To determine the role of STAT1 protein overexpression in regulation of IFN-α signalling, Hep3B cells were stably transfected with wild-type STAT1. Overexpression of STAT1 via stable transfection enhances IFN-γ activation of STAT1, but surprisingly attenuates IFN-α activation of STAT1, STAT2 and STAT3 without affecting Janus kinase activation. This STAT1-mediated inhibition does not require STAT1 tyrosine phosphorylation because overexpression of dominant-negative STAT1 with a mutation on tyrosine residue 701 also blocks IFN-α activation of STAT1, STAT2 and STAT3. Moreover, overexpression of STAT1 blocks IFN-α-activated STAT2 translocation from IFN-α receptor 2 to IFN-α receptor 1, a critical step in IFN-α signalling activation. Finally, significantly higher levels of STAT1 protein expression, which is probably induced by IFN-γ, are detected in the majority of hepatitis C virus-infected livers compared with healthy controls. In conclusion, long-term IFN-γ treatment inhibits IFN-α-activated signals most probably, at least in part, through the induction of STAT1 protein expression, which could partly contribute to IFN-α treatment failure in hepatitis C patients.

Key words: Hep3B, hepatitis, interferon (IFN), liver, signal transducers and activators of transcription factor (STAT).

INTRODUCTION

Chronic hepatitis C infection is a leading cause of liver disease worldwide. Currently, the standard treatment for hepatitis C infection is IFN-α (interferon-α) plus ribavirin, but unfortunately only a minority of patients are able to respond to such therapy [1–3]. Although the mechanism underlying resistance to IFN-α therapy is not well understood, both viral and host factors have been shown to modulate cellular response to IFN-α stimulation [4–6]. In particular, several lines of evidence indicate that an intrahepatic increase in mRNA expression of Th1-like cytokines, IL-2 (interleukin 2) and IFN-γ, in patients with chronic hepatitis C infection is strongly correlated with the severity of liver injury and is implicated in resistance to IFN-α therapy [7,8]. Additionally, treatment with ribavirin markedly reduces serum levels of IFN-γ, which may be associated with increased IFN-α response in chronic hepatitis C infection [9]. In contrast, other studies suggest that priming with IFN-γ prior to initiation of IFN-α treatment has a beneficial effect in a very small percentage of chronic hepatitis C-infected patients through changing the balance of cytokines in favour of a Th1-type response in the host [10,11]. The reasons for these opposing reports are not clear.

IFNs, type I and type II, are a family of multi-functional cytokines that possess antiviral, antiproliferative and immunomodulatory activities through activation of distinct but related pathways [12–17]. These pathways involve specific type I and type II receptors, which initiate activation of the JAK (Janus kinase)-STAT (signal transducers and activators of transcription factor). IFNs directly target human hepatocytes and regulate a wide variety of genes responsible for antiviral and antitumour activities in human liver [18,19]. Upon type I IFN binding, type I IFN-receptor-associated tyrosine kinases (JAK1 and Tyk2) are activated, leading to phosphorylation of Tyr-466 of the IFNAR1 (IFN-α receptor 1), which serves as a docking site to accept STAT2 transferred from IFNAR2. The activated kinase subsequently phosphorylates STAT2 and STAT1 on Tyr-690 and Tyr-701, respectively [12–17]. Phosphorylated STAT1 and STAT2 then form heterodimers and translocate into the nucleus, where they bind p48 protein and form the ISGF3 (interferon-stimulated gene factor 3) transcription factor complex that binds ISRE (IFN-stimulated response element) to initiate transcription of a wide variety of genes, including several antiviral genes [12–17]. Phosphorylated STAT1 also forms homodimers, which translocate into the nucleus and bind GAS element to initiate transcription of a wide variety of genes. IFN-α also activates STAT3 and STAT5 in human hepatocytes, but the roles of these STATs in IFN-α-mediated activity are less clear [19]. Binding of IFN-γ to its receptors [IFNGR (IFN-γ receptor) 1 and IFNGR2] results in activation of JAK1, JAK2, STAT1 and STAT3 [12–17]. Although

Abbreviations used: IFN, interferon; IFNAR, IFN-α receptor; IFNGR, IFN-γ receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription factor; IL, interleukin; ERK, extracellular-signal-regulated kinase; ISRE, IFN-stimulated response element; RT-PCR, reverse transcriptase-PCR; SOCS, suppressors of cytokine signalling.

[1] To whom correspondence should be addressed (e-mail bgao@mail.nih.gov).

both IFN-α and IFN-γ activate related signal pathways and induce expression of a variety of antiviral proteins, IFN-α has proven to be more effective in treating chronic hepatitis C patients [1–3] than IFN-γ [20,21].

To define the effect of IFN-γ on IFN-α therapy in patients with chronic hepatitis C infection, we examined the effects of IFN-γ on IFN-α-activated signals in hepatic cells. We demonstrate that IFN-γ inhibits IFN-α-activated signals in hepatic cells, which is probably mediated via enhanced expression of STAT1. It has been previously reported that high levels of IFN-γ are associated with chronic hepatitis C patients [22–25] and we show in the present paper that levels of STAT1 are markedly elevated in the majority of chronic hepatitis C virus-infected livers. Thus high levels of IFN-γ and STAT1 could contribute to IFN-α treatment failure in patients with chronic hepatitis C infection.

MATERIALS AND METHODS

Materials

Anti-STAT1, anti-phospho-STAT1 (Tyr-701), anti-STAT3, anti-phospho-STAT3 (Tyr-705) and anti-β-actin antibodies were obtained from NEB Bio-Lab (Beverly, MA, U.S.A.). Anti-STAT2 and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Human IFN-α, IFN-β, IFN-γ, anti-JAK1, anti-Tyk2, anti-phospho-JAK1 and anti-phospho-Tyk2 were purchased from Bioscience International (Camarillo, CA, U.S.A.). Hep3B cells were obtained from ATCC (Rockville, MD, U.S.A.). Anti-IFNAR2 and anti-IFNAR1 antibodies were obtained from PBL Biomedical Laboratories (Piscataway, NJ, U.S.A.).

Stable transfection

Stable transfection of Hep3B cells with wild-type or dominant-negative STAT1 was described previously [26]. Hep3B cells stably transfected with STAT1 grow very well although more slowly than vector-transfected Hep3B cells. FACS and DNA fragmentation analyses showed Hep3B cells stably transfected with STAT1 had a similar cell-cycle pattern to that of vector-transfected cells and did not have significant apoptosis (see Figures 5C and 5D in reference [26]).

Transient transfection and luciferase assays

Transient transfections were performed using lipofectin (Gibco-BRL, Gaithersburg, MD, U.S.A.) as recommended by the manufacturer. Briefly, the cells were washed twice with Opti-MEM I reduced-serum medium (Gibco-BRL). Reporter plasmid DNA was co-transfected with a β-galactosidase vector (Promega, Madison, WI, U.S.A.) to allow for adjustments in transfection efficiencies. After transfection, the cells were continuously incubated in reduced-serum medium for 8 h then changed to normal growth medium. After an additional 8 h period, cells were harvested, lysed by freeze-thawing, and assayed for β-galactosidase and luciferase activity. The IFN-responsive reporter gene (pISG54-Luc), which was constructed by incorporating a fragment of the hamster ISG54 promoter from $+429$ to $+31$ fused to the luciferase gene, was kindly provided by Dr D. Levy (New York University School of Medicine, NY, U.S.A.). All transfections were replicated at least three times with similar results.

RT-PCR (reverse transcriptase-PCR)

RT-PCR was performed as described previously [19]. Primers for β-actin, IFNAR1, IFNAR2 and SOCS1 (suppressors of cytokine signalling 1) were described previously [19,27]. The primer sequences of other genes were as follows: STAT1, 5′-TCG TTT GTG GTG GAA AGA CA-3′ (forward), 5′-GGT GCC AGC ATT TTT CTG TT-3′ (reverse).

Cell extraction, SDS/PAGE, immunoprecipitation and Western blotting

Cells were lysed for 15 min at 4 °C in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 1 % Nonidet P-40 and 10 % glycerol), vortexed and centrifuged at 20 900 ***g*** at 4 °C for 10 min. The supernatants were mixed in Laemmli loading buffer, boiled for 4 min and then subjected to SDS/PAGE. After electrophoresis, proteins were transferred on to nitrocellulose membranes and blotted against primary antibodies for 16 h. Membranes were washed with 0.05 % (v/v) Tween 20 in PBS (pH 7.4) and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Biosciences, Piscataway, NJ, U.S.A.).

For the detection of STAT2 tyrosine phosphorylation, cell extracts were first immunoprecipitated with anti-phosphotyrosine antibody, and then blotted with anti-STAT2 antibody.

For immunoprecipitation assays, cell extracts (2.5 mg of total proteins) prepared in lysis buffer [20 mM Hepes, pH 7.6, 10 mM NaCl, 10 % glycerol, 0.5 % Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor mixture P8340 (Sigma-Aldrich), 10 mM sodium molybdate and 10 mM β-glycerolphosphate] were incubated with 5 μg of the specific antibody overnight at 4 °C under constant shaking. A 50 μl aliquot of Protein A–agarose beads (Invitrogen) was added and incubated for 2 h, followed by centrifuging at 735 ***g*** for 5 min. The beads were washed three times with lysis buffer. Finally, the immunoprecipitates were mixed with SDS loading buffer and analysed by SDS/PAGE (7.5 % gels; Bio-Rad) followed by Western blotting using a specific antibody.

Human liver tissues of chronic hepatitis C infection and normal controls

The LTPDS (Liver Tissue Procurement Distribution System, Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis, MN, U.S.A.) provided 24 liver specimens from patients with chronic hepatitis C virus infection. Chronic hepatitis C virus infection was diagnosed histopathologically and serologically by the LTPDS programme. These hepatitis C patients had not been treated with IFN-α therapy in the 2 years prior to this study. Liver pathology showed piecemeal necrosis, bridging necrosis and various stages of cirrhosis in these specimens. Lobules were dissected by irregular septa resulting from collapse of hepatic parenchyma and fibrosis. LTPDS also provided normal healthy liver specimens that were obtained from human donor livers not used for transplantation. Another 15 liver biopsy specimens from patients with chronic hepatitis C virus infection were also obtained from National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, U.S.A. These patients were not treated with IFN-α in the 6 months before collection. Liver pathology showed no cirrhosis and no or very early fibrosis in these specimens.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated with PBS, followed by proteinase K treatment [30 μg/ml in 100 mM Tris/HCl buffer/50 mM EDTA

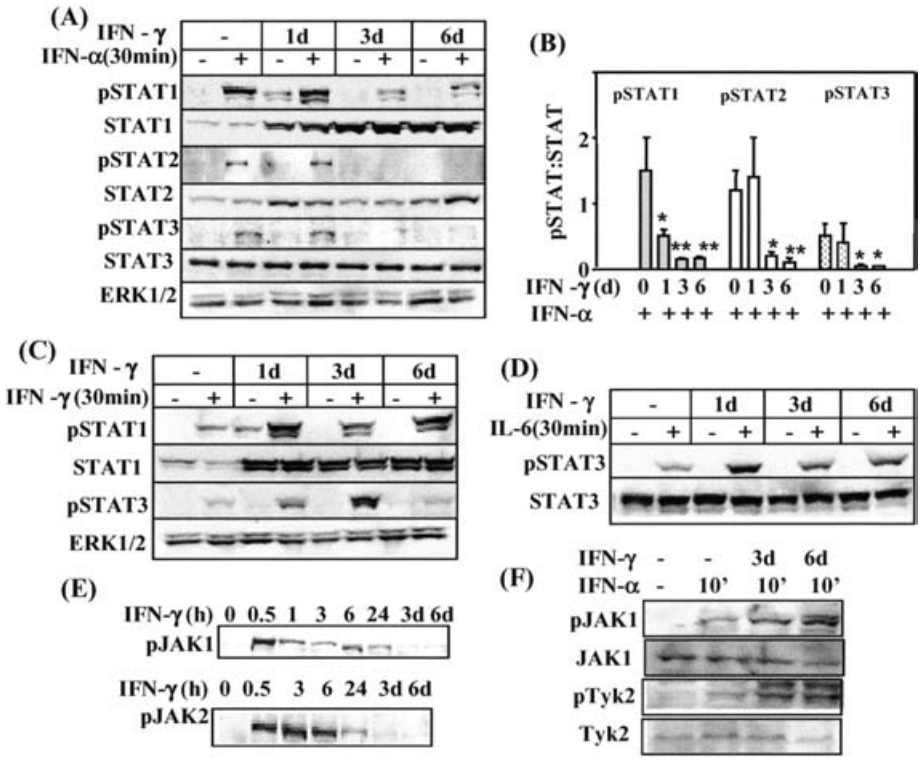

Figure 1 Long-term exposure to IFN-γ induces STAT1 protein expression, attenuates IFN-α activation of STAT1, STAT2 and STAT3, but enhances IFN-γ and IL-6 activation of STATs

Hep3B cells were incubated with IFN-γ (10 i.u./ml) for 1, 3 and 6 days, followed by stimulation with IFN-α (500 i.u./ml) for 30 min (**A**) or IFN-γ (10 i.u./ml) for 30 min (**C**) or IL-6 (10 ng/ml) for 30 min (**D**). Cellular protein extracts were then prepared and immunoblotted with various antibodies as indicated. (**A**) Representative of three independent experiments. Densitometric readings and statistic analyses of these three independent experiments are shown in (**B**). Bars represent means $\pm$ S.E.M; $^{*}P < 0.01$, $^{**}P < 0.001$ in comparison with corresponding control groups that were not treated with IFN-γ. (**C**, **D**) Representative of two independent experiments. (**E**) Hep3B cells were treated with IFN-γ (10 i.u./ml) for various times. Cellular extracts were then immunoblotted with anti-phospho-JAK1 and anti-phospho-JAK2 antibodies. (**F**) Hep3B cells were treated with IFN-γ (10 i.u./ml) for 3 and 6 days, followed by treatment with IFN-α (500 i.u./ml) for 10 min. Cellular extracts were then immunoblotted with various antibodies as indicated. Phosphorylated STAT2 (pSTAT2) in (**A**) was detected as described in the Materials and methods section. p represents the tyrosine-phosphorylated form.

(pH 8.0), 30 min at 37 °C]. Next, sections were incubated in 0.3 % H_2O_2 in methanol to block endogenous peroxidase activity. Non-specific binding sites were blocked by 20 min incubation in normal blocking serum. Sections were incubated with 1:50 diluted primary antibodies overnight at 4 °C. Biotinylated secondary antibodies and ABC Reagent were applied according to the manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.). Colour development was induced using 3,3′-diaminobenzidine as a substrate during a 5–10 min incubation period. Using this substrate, specific staining could be visualized by light microscopy.

Statistical analysis

For comparing values obtained in three or more groups, one-factor ANOVA was used, followed by Tukey's *post hoc* test, and P values less than 0.05 were taken to imply statistical significance.

RESULTS

Prolonged exposure to IFN-γ induces expression of STAT1 protein, suppresses IFN-α activation of STAT1, STAT2 and STAT3 in hepatic cells, but enhances IFN-γ and IL-6 activation of STATs

The effect of IFN-γ on the IFN-α signalling pathway was examined using Western blot analysis. As shown in Figure 1(A), short-term treatment with IFN-γ (up 1 day) significantly induced STAT1 protein expression and slightly enhanced IFN-α-activated STAT1 tyrosine phosporylation, but did not affect STAT2 and STAT3 protein expression. In contrast, prolonged treatment with IFN-γ (3 and 6 days) markedly inhibited IFN-α-induced STAT1, STAT2 and STAT3 tyrosine phosphorylation. Additionally, such long-term treatment with IFN-γ (3 and 6 days) induced significantly higher levels of STAT1 protein expression compared with control and 1-day treatment but did not affect STAT2 and STAT3 protein expression. Densitometric analyses showed that the ratio of IFN-α-activated pSTAT1 to STAT1, pSTAT2 to STAT2, pSTAT3 to STAT3 was significantly reduced in Hep3B cells treated with IFN-γ for 3 and 6 days (Figure 1B). Finally, IFN-γ treatment did not affect expression of mitogen-activated protein kinase ERK (extracellular-signal-regulated kinase) 1/2 (Figure 1A).

We have previously shown that treatment with a low dose of IFN-γ for 1 day did not cause changes in cell cycle of Hep3B cells [26], treatment with such a low dose of IFN-γ (10 i.u./ml) for 3 and 6 days slightly induced cell-cycle arrest but did not induce apoptosis in Hep3B cells (results not shown). To confirm the integrity of intracellular signalling compartments and cellular responsiveness to cytokine stimulation in IFN-γ-treated cells, IFN-γ and IL-6 signalling were examined in these cells. As shown in Figures 1(C) and 1(D), pretreatment with IFN-γ did not inhibit, but rather enhanced IFN-γ activation of STAT1 and STAT3, and IL-6 activation of STAT3 tyrosine phosphorylation.

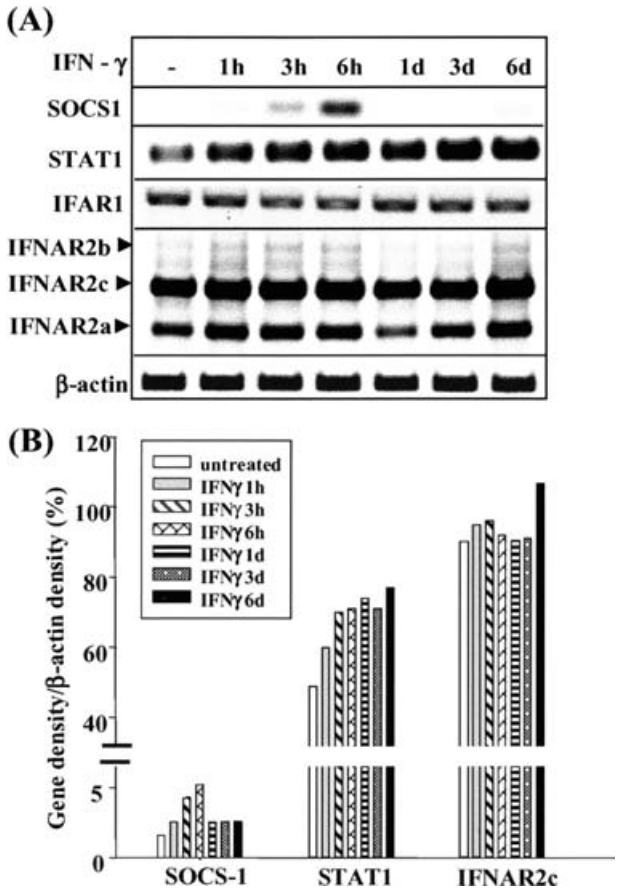

Figure 2 Effects of IFN-γ on expression of SOCS1, STAT1 and IFNAR2 mRNA

(**A**) Hep3B cells were incubated with IFN-γ (10 ng/ml) for various times. Total RNA was then purified and subjected to RT-PCR using primers as indicated. (**B**) RT-PCR band densities from (**A**) were quantified by PhosphorImager analysis. Photographs in (**A**) are representative of three independent experiments with similar results.

Next, we examined the effects of IFN-γ on IFN-α activation of JAK1 and Tyk2. As shown in Figures 1(E) and 1(F), IFN-γ treatment induced a sustained activation of JAK1 and JAK2 up to 1 day, and returned to basal levels at 3 and 6 days. Prolonged treatment with IFN-γ for 3 and 6 days neither inhibited IFN-α-activation of JAK1 and Tyk2 nor affected the expression of JAK1 and Tyk2.

IFN-γ treatment induces expression of SOCS1 and STAT1 mRNA without affecting IFNAR1 and IFNAR2c mRNA expression

Next, we studied the effect of IFN-γ on expression of STAT1, SOCS1 and IFNAR2 that were involved in the regulation of IFN-α-stimulated gene expression. As shown in Figure 2, treatment with IFN-γ markedly induced expression of SOCS1 mRNA and STAT1 mRNA. Unlike induction of STAT1 mRNA that lasted for 6 days, SOCS1 mRNA was significantly induced after 3 h, reached maximum expression at 6 h, and returned to undetectable basal levels on day 1. RT-PCR in Figure 2 showed that multiple forms of IFNAR2 were detected, which is consistent with our previous study [19], and quantitative analysis revealed that expression of IFNAR2c, the functional form, remained unchanged after IFN-γ treatment (Figure 2B). Expression of IFNAR1 also remained unchanged after IFN-γ treatment (Figure 2A).

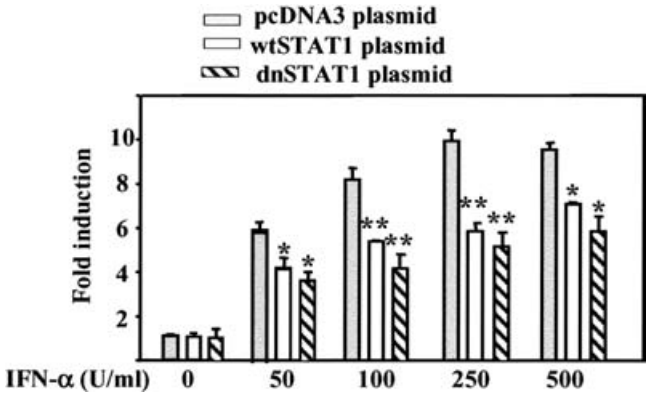

Figure 3 Transient overexpression of STAT1 attenuates IFN-α activation of ISRE-luciferase reporter activity

Hep3B cells in a six-well plate were transiently cotransfected with an IFN-responsive reporter pISG54-luciferase plasmid (0.5 μg/well), a β-galactosidase DNA plasmid (0.5 μg/well), and with control pcDNA3 plasmid (1.5 μg/well) or pEF-BOS expression vector containing either full-length STAT1 cDNA (wtSTAT1; 1.5 μg/well) or mutated STAT1 cDNA (dnSTAT1; 1.5 μg/well), followed by stimulation with IFN-α (500 units/ml). After 8 h, luciferase and β-galactosidase activities were measured. All values of luciferase activity in cell extracts were normalized to the β-galactosidase activity in the same extracts and finally expressed as counts of luciferase activity/β-galactosidase activity. The fold induction was calculated in IFN-α-treated samples compared with untreated samples. The values are shown as means $\pm$ S.E.M. from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with corresponding group transfected with pcDNA3.

Transient overexpression of STAT1 inhibits IFN-α-induced ISRE-luciferase reporter activity

The above data showed that pre-treatment with IFN-γ for 3 and 6 days inhibited IFN-α activation of STAT1; however, SOCS expression was not detected at these time points, suggesting that SOCS is not involved in the inhibition of STAT1 activation. Since levels of STAT1 protein were markedly elevated in IFN-γ-treated cells, it was hypothesized that STAT1 overexpression affected the IFN-α signalling pathway. To test this hypothesis, the effects of transient STAT1 overexpression on IFN-α-induced ISRE-luciferase reporter activity in hepatic cells were examined. Hep3B cells were transiently co-transfected with pISG54-luciferase, the IFN-responsive reporter plasmid, and control pcDNA3 plasmid or the pEF-BOS expression vector containing either full-length STAT1 cDNA (wtSTAT1) or mutated STAT1 (dnSTAT1), and then stimulated with IFN-α. As shown in Figure 3, as expected, co-transfection of a mutated form of STAT1 (dnSTAT1) inhibited IFN-α-induced ISG54-luciferase reporter activity, but surprisingly, transient overexpression of STAT1 also resulted in a significant suppression of IFN-α-induced ISG54-luciferase reporter activity.

Stable overexpression of wild-type STAT1 inhibits IFN-α activation of STAT1, STAT2 and STAT3, but potentiates IFN-γ activation of STAT1 without affecting JAK tyrosine phosphorylation

To further test the inhibitory role of STAT1 overexpression in IFN-α signalling, Hep3B cells were stably transfected with wild-type STAT1 (wtSTAT1). Three positive clones (STAT1-1, STAT1-2 and STAT1-3) were selected, and overexpression of STAT1 in these clones was confirmed by Western blotting (Figure 4). Next, IFN-α-mediated activation of STATs was examined in these clones. As shown in Figure 4(B), although STAT1 was over-expressed in these clones, IFN-α-induced STAT1 tyrosine phosphorylation was markedly suppressed. Interestingly, IFN-α-induced STAT2 and STAT3 tyrosine phosphorylation were also

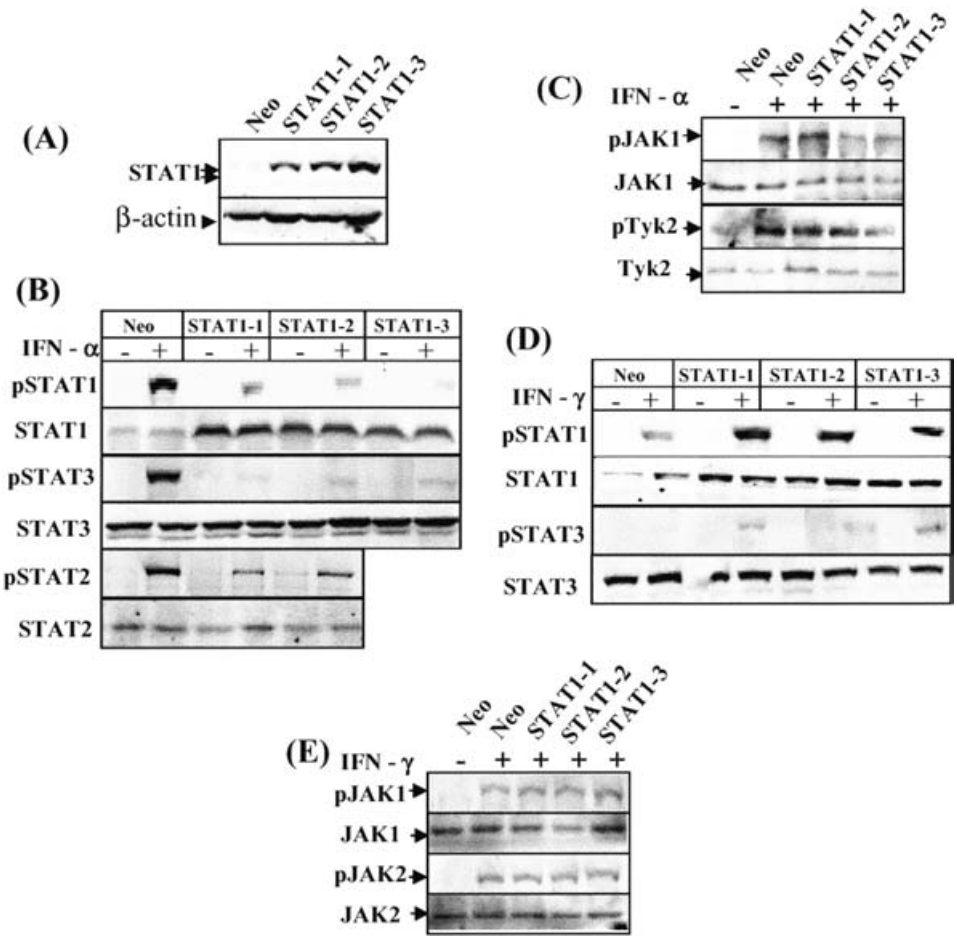

Figure 4 Overexpression of wild-type STAT1 by stable transfection attenuates IFN-α-activated signals, but potentiates IFN-γ-activated STAT1

Hep3B cells were stably transfected with empty vector or wild-type STAT1 cDNA. Three positive clones were selected and named STAT1-1, STAT1-2 and STAT1-3. Cells stably transfected with empty vectors are labelled Neo. (**A**) Total cell extracts were prepared from Neo or STAT1 clones and immunoblotted with anti-STAT1 and anti-β-actin antibodies. (**B**, **C**) Neo- and STAT1-transfected cells were treated with IFN-α (500 units/ml) for 30 min (**B**) or 10 min (**C**), followed by Western blot analysis using antibodies as indicated. Determination of STAT2 phosphorylation was described in the Materials and methods section. (**D**, **E**) Neo- and STAT1-transfected cells were treated with IFN-γ (1 ng/ml) for 30 min (**D**) or 10 min (**E**), followed by Western blot analysis using antibodies as indicated. p represents the tyrosine-phosphorylated form.

markedly attenuated (Figure 4B), whereas expression of STAT2 and STAT3 proteins was not significantly altered, and IFN-α-induced activation of Tyk2 and JAK1 kinases, the upstream of STATs, was similar in these clones compared with Neo clones (Figure 4C). In contrast, overexpression of STAT1 significantly enhanced IFN-γ activation of STAT1 tyrosine phosphorylation and slightly enhanced IFN-γ activation of STAT3 without affecting activation of JAK1 and JAK2 (Figures 4D and 4E). Similarly, IL-6 activation of STAT3 signals was not inhibited, but rather slightly enhanced in these clones compared with that in Neo clones (results not shown).

Stable overexpression of dominant-negative STAT1 inhibits IFN-α activation of STAT1, STAT2 and STAT3 without affecting JAK activation

To define whether STAT1 hyperexpression-mediated inhibition of IFN-α signalling required STAT1 tyrosine phosphorylation on wild-type STAT1, Hep3B cells were stably transfected with dominant-negative STAT1 (dnSTAT1) that had a mutation of Tyr-701. Three positive clones were selected and confirmed by Western blotting (Figure 5A). As expected, overexpression of dominant-negative STAT1 blocked IFN-α-mediated activation of STAT1 in all clones (Figure 5B). Interestingly, IFN-α-induced STAT2 and STAT3 tyrosine phosphorylation were also markedly suppressed in these clones (Figure 5B), whereas STAT2 and STAT3 protein expression remained unchanged. Moreover, IFN-α-induced activation of JAK1 and Tyk2, upstream of STATs, was similar in these three clones compared with vector-transfected Neo clones (Figure 5C). Finally, Figures 5(D) and 5(E) show that IFN-γ-mediated activation of STAT1 was significantly inhibited in these dnSTAT1-transfected clones, whereas IFN-γ activation of STAT3, JAK1 and JAK2 remained unchanged.

Transient overexpression of wild-type or dominant-negative STAT1 attenuates IFN-α activation of STAT1

STAT1-mediated suppression of IFN-α signalling in stably transfected clones may have occurred during the process of clonal selection. To distinguish between changes that occurred within days from those occurring more slowly, we transiently transfected STAT1 and STAT3 into cells. Since transfection efficiency in Hep3B cells is low, 2fTGH cells, in which high transfection efficiencies have been reported, were used [28]. As shown in Figure 6(A), transient transfection of STAT1 and STAT3 enhanced protein levels of STAT1 and STAT3 in 2fTGH cells respectively.

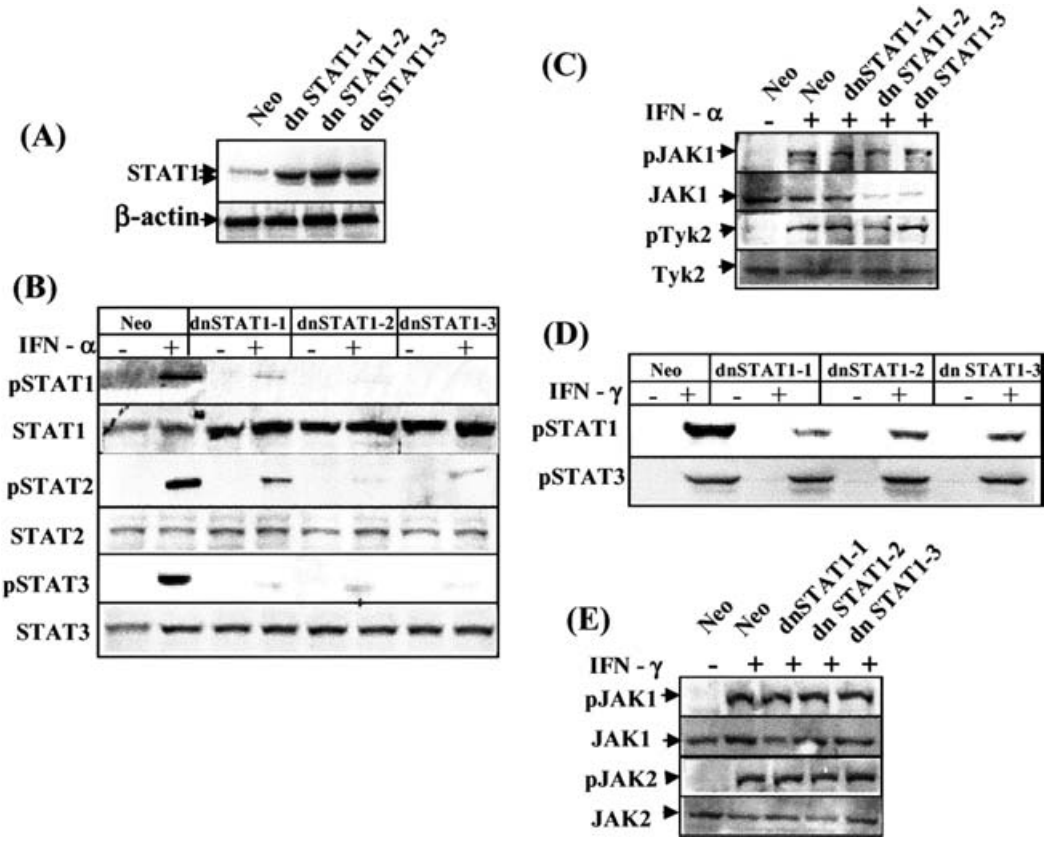

Figure 5 Overexpression of dominant-negative STAT1 cDNA by stable transfection attenuates IFN-α-activated STATs without affecting activation of JAKs

Hep3B cells were stably transfected with empty vector or dominant-negative (dn) STAT1 cDNA. Three positive clones were selected and named dnSTAT1-1, dnSTAT1-2 and dnSTAT1–3. Cells stably transfected with empty vectors are labelled Neo. (**A**) Total cell extracts were prepared from Neo or dnSTAT1 clones and immunoblotted with anti-STAT1 and anti-β-actin antibody. (**B**, **C**) Neo- and dnSTAT1-transfected cells were treated with IFN-α (500 units/ml) for 30 min (**B**) or 10 min (**C**), followed by Western blot analysis using antibodies as indicated. Determination of STAT2 phosphorylation was described in the Materials and methods section. (**D**, **E**) Neo- and dnSTAT1-transfected cells were treated with IFN-γ (1 ng/ml) for 30 min (**D**) or 10 min (**E**), followed by Western blot analysis using antibodies as indicated. p represents the tyrosine-phosphorylated form.

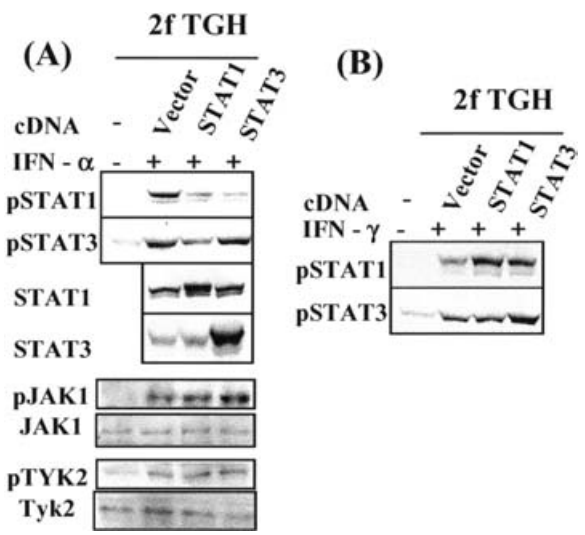

Figure 6 Overexpression of STAT1 or STAT3 by transient transfection attenuates IFN-α signalling

(**A**, **B**) 2fTGH cells were transiently transfected with empty vector, STAT1 cDNA or STAT3 cDNA plasmids for 48 h, followed by stimulation with IFN-α (500 units/ml; **A**) or IFN-γ (1 ng/ml; **B**) for 30 min. Cell extracts were prepared and immunoblotted with the indicated antibodies. p represents the tyrosine-phosphorylated form.

Overexpression of STAT1 suppressed IFN-α-mediated activation of STAT1 and STAT3, but did not affect IFN-α-mediated activation of JAK1 and Tyk2 (Figure 6A). Overexpression of STAT3 also markedly suppressed IFN-α-mediated activation of STAT1, and slightly inhibited STAT3 activation (Figure 6A). In contrast, transient transfection of STAT1 and STAT3 enhanced IFN-γ-mediated activation of STAT1 and STAT3, respectively (Figure 6B).

Stable overexpression of wild-type STAT1 or dominant-negative STAT1 prevents IFN-α-induced dissociation of IFNAR2–STAT2 complex

It has been demonstrated previously that STAT2 is essential for IFN-α-mediated activation of STAT1 [28,29]. In unstimulated cells, STAT2 is pre-associated with IFNAR2. Upon activation, the IFNAR1 subunit is phosphorylated on Tyr-466; STAT2 then dissociates from IFNAR2 and binds to IFNAR1, followed by STAT2 and STAT1 tyrosine phosphorylation. To explore the IFNAR2–STAT2 interaction in the cells overexpressing STAT1, a co-immunoprecipitation assay was performed. Cell lysates from overexpressing dnSTAT1 and wtSTAT1 clones and control Neo cells treated with or without IFN-α were immunoprecipitated with the IFNAR2 antibody and blotted with the anti-STAT2 antibody. In control Neo clones, STAT2 was clearly co-immunoprecipitated

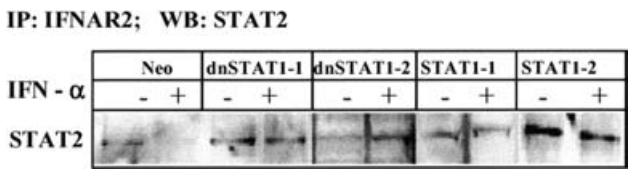

Figure 7 Overexpression of wild-type or dominant-negative STAT1 abolishes IFN-α-induced dissociation of IFNAR2 with STAT2

Neo-, dnSTAT1-, and wild-type STAT1-transfected Hep3B cells were stimulated with IFN-α (500 units/ml) for 30 min. Cell extracts were then immunoprecipitated (IP) with an anti-IFNAR2 antibody, followed by Western blotting (WB) with an anti-STAT2 antibody, followed by immunoblotting with STAT2 antibodies. A representative blot is shown. Similar results were obtained from three independent experiments.

with the anti-IFNAR2 antibody in IFN-α-untreated cells; such co-immunoprecipitation was abolished after IFN-α stimulation (Figure 7). These findings suggest that STAT2 dissociates from IFNAR2 after IFN-α stimulation. Interestingly, such IFNAR2–STAT2 dissociation was blocked in Hep3B cells overexpressing either dnSTAT1 cDNA or wtSTAT1 cDNA (Figure 7).

Elevated STAT1 protein expression in chronic hepatitis C virus-infected livers

The above data clearly suggest that high levels of IFN-γ and STAT1 negatively regulate IFN-α-activated signals; thus it will be interesting to examine the levels of IFN-γ and STAT1 in patients with chronic hepatitis C infection. It has been reported previously that high levels of IFN-γ are always associated with chronic hepatitis C patients [22–25]. Here we examined the expression of STAT1 in the livers of 39 patients with chronic hepatitis C virus infection and seven healthy controls. As shown in Figure 8(A), expression of the STAT1 protein was markedly elevated (up to 5-fold) in about 60% of these hepatitis C virus-infected livers compared with normal healthy controls (Figure 8A). Expression of the proapoptotic Bax protein was upregulated, whereas expression of ERK1/2 remained unchanged in chronic hepatitis C virus-infected livers compared with healthy controls.

Expression of STAT1 protein in chronic hepatitis C virus-infected livers was also examined by immunohistochemistry. Consistent with our Western blot analyses, very weak STAT1 immunostaining was detected in healthy livers, whereas strong heterogeneous STAT1 immunohistochemical staining was detected in the majority of chronic hepatitis C virus-infected livers. Notably, overexpressed STAT1 was detected in hepatocytes in the vicinity of the septa or within inflammatory areas. Staining appeared as separate, relatively large brown granules located in the cytoplasm of the hepatocytes. The remaining hepatocytes showed diffuse cytoplasmic staining of mild to moderate intensity with occasional small cytoplasmic brown granules. In addition, positive staining was observed in some small inflammatory cells and non-parenchymal cells. A representative immunostaining of STAT1 in the liver from chronic hepatitis C virus-infected case #3 is shown in Figure 8(B).

DISCUSSION

In the present study, we demonstrate that long-term (3 and 6 days) but not short-term exposure of human hepatoma cells to IFN-γ suppresses IFN-α-induced activation of STAT1, STAT2 and STAT3. Furthermore, we provide evidence suggesting that IFN-γ-mediated inhibition of the IFN-α signalling pathway is probably mediated, at least in part, via the induction of STAT1 protein expression. Finally, significantly higher levels of STAT1 protein expression were detected in about 60% of chronic hepatitis C virus-infected livers compared with normal healthy controls. Such high levels of STAT1 protein expression in the liver could negatively regulate IFN-α therapy and partly contribute to IFN-α treatment failure in the majority of patients with chronic hepatitis C infection.

Since resistance to IFN-α treatment remains a serious problem, different types of combination therapies have been introduced. In particular, it was expected that the combination of IFN-α with agents known to upregulate the expression or activity of STAT1 protein may restore the responsiveness of cells to IFN-α, because several recent findings suggest that IFN-α resistance is associated with defects in STAT1 protein expression [30–33]. Gene-targeting studies demonstrated that STAT1-deficient mice were completely unresponsive to IFNs and were highly sensitive to viral infection [32,33]. Furthermore, since downregulation of STAT1 in some cancer cell lines are correlated with resistance to IFNs, increased levels of this crucial regulator could possibly restore the IFN response [30,31]. A clinical study suggested that priming with IFN-γ before initiating IFN-α treatment had a beneficial effect on antiviral therapy in a small percentage of patients [10,11]. Wong et al. [34] have previously shown that IFN-γ priming for 16 h enhances IFN-α-activated signals and genes in melanoma cells. In contrast, we demonstrate here that prolonged treatment with IFN-γ for 3 and 6 days markedly inhibits IFN-α-activated STAT1, STAT2 and STAT3. The discrepancy between our study and this previous study [34] may be due to different doses of IFN-γ used and different lengths of treatment. In the present study, a much lower and more physiological dose of IFN-γ (10 i.u./ml) was used, whereas a very high dose (1000 i.u./ml) was used in the previous study [34]. Furthermore, our results showed that the inhibitory effect of IFN-γ on IFN-α signalling was only observed after long-term treatment (3 and 6 days) with IFN-γ, whereas short-term treatment (up to 1 day) with IFN-γ slightly enhanced IFN-α-activated STAT1. The latter is consistent with the previous study [34].

Although the antiproliferative and proapoptotic effects of IFN-γ/STAT1 have been well-documented, we provide evidence suggesting that suppression of IFN-α signalling by IFN-γ and high STAT1 levels were not due to inducing changes in cell-cycle distribution or changes associated with apoptosis activation. First, the dose of IFN-γ we used in this study was low (10 i.u./ml), which only slightly induced cell-cycle arrest and did not induce apoptosis of Hep3B cells (results not shown). Secondly, overexpression of STAT1 did not significantly cause cell-cycle changes and apoptosis of Hep3B cells [26], which is probably because Hep3B cells are very resistant to apoptosis. Finally, more interestingly, prolonged treatment with IFN-γ for 3 and 6 days, or overexpression of STAT1, inhibited IFN-α signalling but did not inhibit rather enhanced IFN-γ and IL-6 signalling in Hep3B cells. Taken together, these findings suggest that inhibition of IFN-α signalling by prolonged IFN-γ treatment or overexpression of STAT1 is specific and not due to changes in cell functions.

Two major mechanisms responsible for inhibition of IFN-α signalling have been proposed. These include SOCS and cytoplasmic phosphatases [35–39]. Only SOCS1 mRNA was detected within 6 h and no other SOCSs were detected 1–6 days after IFN-γ stimulation (Figure 2). However, we cannot rule out the possible involvement of low levels of SOCS proteins in the inhibitory effect of long-term IFN-γ on IFN-α signalling. Accumulating evidence suggests that protein tyrosine phosphotases play an important role in regulation of IFN-α signalling [37–39]. Particularly, ligand-stimulated tyrosine phosphorylation of IFNAR

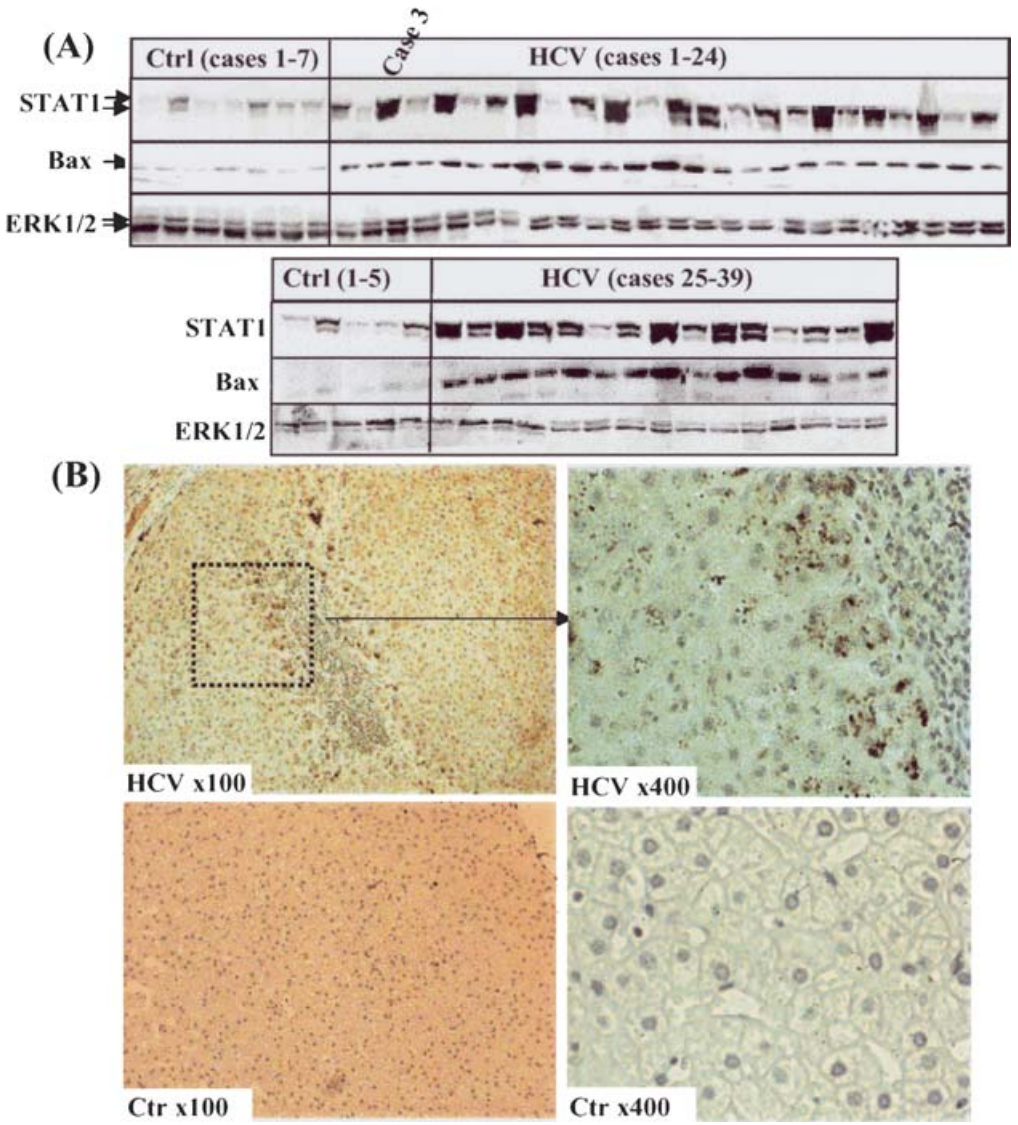

Figure 8 STAT1 is hyperexpressed in the livers of patients with chronic hepatitis C virus infection

(**A**) Liver extracts from seven normal controls (Ctrl) and 39 patients with chronic hepatitis C virus (HCV) infection were immunoblotted with STAT1, Bax and ERK1/2 antibodies. (**B**) Photomicrographs of representative STAT1 immunostaining in the liver from chronic hepatitis C virus-infected case #3 is shown (magnifications are shown on the panels). STAT1 was stained as separate, relatively large brown granules in the cytoplasm of the hepatocytes, which were aligned along inflammatory cells. Diffuse cytoplasmic STAT1 staining of mild to moderate intensity with occasional small cytoplasmic brown granules was observed in remaining hepatocytes.

can be selectively inhibited by the PTP1 phosphatase, resulting in dephosphorylation and inactivation of JAK1 [39]. Our findings that IFN-α activation of JAKs was not affected after prolonged IFN-γ treatment or overexpression of STAT1 (Figures 1, 4, 5 and 6) suggest that IFN-γ downregulation of IFN-α activation of STATs does not occur at the IFNAR level, and protein tyrosine phosphatases targeting JAKs are unlikely to be involved in suppression of IFN-α signalling. However, we cannot eliminate a role for protein phosphatases targeting activated STATs in IFN-γ inhibition of IFN-α signalling. Here we provide evidence suggesting that IFN-γ-mediated inhibition of IFN-α signalling is via a novel mechanism, such as induction of high levels of STAT1 protein expression, as demonstrated by overexpression of STAT1 via either stable transfection or transient transfection, which markedly inhibits IFN-α activation of STAT1, STAT2 and STAT3 (Figures 4–6). Overexpression of wild-type STAT1 in Hep3B cells, as expected, potentiates IFN-γ activation of STAT1, but surprisingly inhibits IFN-α-activation of STAT1, STAT2 and STAT3 (Figure 4). Although both IFN-α and IFN-γ pathways share STAT1, they act through different specific receptors and employ different mechanisms to activate STAT1. Upon type I IFN binding, type I IFN-receptor-associated tyrosine kinases (JAK1 and Tyk2) are activated, leading to phosphorylation of Tyr-466 of the IFNAR1. This in turn causes translocation of IFNAR2-associated STAT2 to IFNAR1, phosphorylates STAT2 and STAT1 on Tyr-690 and Tyr-701, respectively [12–16]. Pre-activation of STAT2 is essential for IFN-α activation of STAT1 because deletion of the STAT2 gene abolishes IFN-α-induced STAT1 activation [28,29]. In contrast, activation of STAT1 in IFN-γ signalling pathway is STAT2 independent. The IFN-γ receptor is composed of two distinct subunits, IFNGR1 and IFNGR2. Upon ligand binding, IFNGR-associated JAK1 and JAK2 are activated, followed by phosphorylation of IFNGR1 on Tyr-440. The activated IFNGR–JAK complex then recruits and phosphorylates

STAT1 [12–16]. Therefore, it is easy to understand how overexpression of STAT1 potentiates IFN-γ activation of STAT1 (Figure 4). However, the molecular mechanism by which overexpression of STAT1 inhibits IFN-α-induced STAT2 and STAT1 activation is not clear. Blocking the dissociation of STAT2 from IFNAR2 as demonstrated in Figure 7 could be an important mechanism because such dissociation has been suggested as a critical step for IFN-α-induced STAT2 and STAT1 tyrosine phosphorylation and activation [40,41]. Further studies are required to clarify the underlying mechanism by which overexpression of STAT1 blocked dissociation of STAT1 and IFNAR2. Taken together, our findings suggest that high levels of STAT1 protein expression induced by IFN-γ probably contribute, at least in part, to IFN-γ suppression of IFN-α signalling. Finally, although both short-term (up to 1 day) and long-term (3 and 6 days) IFN-γ treatment induced STAT1 protein expression (Figure 1), the former treatment did not inhibit, but rather slightly enhanced, IFN-α signalling. This may be due to the ongoing IFN-γ activity after short treatment because significant activation of STAT1 and JAKs was still detected after 1 day treatment with IFN-γ (Figures 1A and 1E).

In the present study, we also demonstrate that STAT1 protein expression in the liver is much higher in about 60% of patients with chronic hepatitis C infection than normal healthy controls. Elevation of STAT1 protein expression in the liver is also detected in other liver injury models such as concanavalin A- [42] and lipopolysaccharide/D-galactosamine-induced hepatitis [26], and IFN-γ is likely to be responsible for such elevation because disruption of the IFN-γ gene almost completely abolished the elevation of STAT1 protein expression in these models [26,42]. High levels of IFN-γ have been reported in the plasma and the livers of these patients [22–25], suggesting that IFN-γ may also be the major cytokine contributing to the elevation of STAT1 protein in chronic hepatitis C virus-infected livers.

In summary, we demonstrate that long-term IFN-γ treatment suppression of IFN-α signalling is mediated partly through elevation in STAT1 protein expression. This study can significantly impact on clinical practice because high levels of both IFN-γ [22–25] and STAT1 (Figure 8) are associated with the majority of patients with chronic hepatitis C infection. These high levels of IFN-γ and STAT1 protein may inhibit IFN-α therapy and contribute to IFN-α treatment failure in these patients. Further clinical studies are required to clarify the correlation between IFN-α treatment failure and high STAT1 in patients with chronic hepatitis C infection. IFN-γ/STAT1 could be new therapeutic targets for improving the efficacy of IFN-α therapy. For example, anti-IFN-γ antibody or antisense oligonucleotides against IFN-γ or STAT1 could be used in non-responders with high levels of IFN-γ and STAT1 expression. Combination of IFN-γ and IFN-α treatment for viral hepatitis therapy should be carefully designed.

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