STAT1 plays an essential role in LPS/d-galactosamine-induced liver apoptosis and injury

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Submitted 13 May 2003; accepted in final form 10 June 2003

Am J Physiol Gastrointest Liver Physiol 285: G761–G768, 2003. First published June 19, 2003; 10.1152/ajpgi.00224.2003.—Interferon-γ (IFN-γ) has been implicated in liver damage in animal models and chronic hepatitis C virus infection; however, the underlying mechanism is not clear. Here we examined the role of STAT1, a key signaling molecule for IFN-γ, in a model of murine hepatitis induced by the injection of LPS/d-galactosamine and in human hepatoma Hep3B cells. STAT1 is rapidly activated and highly induced after injection of LPS/d-galactosamine. Both overexpression of STAT1 and hepatocellular damage are located in the same pericentral region. Disruption of the STAT1 gene abolishes LPS/d-galactosamine-induced liver injury. Studies from IFN-γ-deficient mice indicate that IFN-γ is the major cytokine responsible for activation and hyperexpression of STAT1 in LPS/d-galactosamine-induced hepatitis. Hep3B cells overexpressing dominant negative STAT1 are resistant to IFN-γ and IFN-γ + TNF-α-induced cell death, whereas Hep3B cells overexpressing wild-type STAT1 are more susceptible to cell death. Taken together, these findings suggest that STAT1 plays an essential role in LPS/d-galactosamine-induced liver apoptosis and injury.

MATERIALS AND METHODS

Submitted 13 May 2003; accepted in final form 10 June 2003

Materials. Anti-STAT1 and anti-phospho-STAT1 (Tyr701) were obtained from New England Biolabs (Beverly, MA). Anti-Bax, anti-Bcl-2, and anti-ERK1/2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Murine and human IFN-γ and TNF-α were purchased from Bioroute International (Camarillo, CA). LPS and GalN were purchased from Sigma (St. Louis, MO). The Hep3B cell line
was purchased from the American Type Culture Collection (Rockville, MD).

**Murine hepatitis model induced by injection of LPS/GalN.** 129/SvEv-background STAT1 (−/−) mice and 129/SvEv wild-type-background mice were purchased from Taconic (Germantown, NY). BALB/c-background IFN-γ (−/−) and BALB/c wild-type-background mice were purchased from Jackson Laboratory (Bar Harbor, ME). Preliminary data showed that BALB/c-background mice exhibit more susceptibility to LPS/GalN-induced liver injury than 129/SvEv-background mice. Thus in this study, LPS (16 μg/kg) and GalN (800 mg/kg) were injected intraperitoneally into 129/SvEv-background mice, whereas LPS (6 μg/kg) and GalN (800 mg/kg) were injected intraperitoneally into BALB/c-background mice. For carbon tetrachloride (CCL4)-induced liver injury, mice were injected intraperitoneally with CCL4 serum and livers were collected for in vitro experiments. Carbon tetrachloride (400 mg/kg) was injected intraperitoneally into BALB/c wild-type-background mice, whereas LPS (6 μg/kg) and GalN (800 mg/kg) were injected intraperitoneally into BALB/c-background mice. For carbon tetrachloride (CCL4)-induced liver injury, mice were injected intraperitoneally with CCL4 (1.0 ml/kg) (Sigma, St. Louis, MO), which was diluted 1:1 in corn oil. All mice were killed within 120 h after injection, and serum and livers were collected for in vitro experiments.

**Stable transfection.** Hep3B cells were grown in six-well culture plates to ~50–60% confluence. The dominant negative point mutant of STAT1 (generous gifts from Dr. T. Hirano, Osaka University Graduate School of Medicine (Osaka, Japan)) (18) and vector control DNA were transfectioned into Hep3B cells by using a lipofectin reagent (GIBCO, Gaithersburg, MD). After 6 h, the medium was replaced with normal growth medium and cells were grown for an additional 48 h. Then the cells were subcultured and plated at 1:10 into selective medium containing geneticin (G418-sulfate; GIBCO) at a concentration (as determined by a killing curve) of 375 μg/ml. Growing, discrete colonies were isolated by using cloning cylinders (Fisher Scientific). The stably transfected cell lines were maintained under constant selective pressure in the continued presence of 375 μg/ml of geneticin.

**Western blotting.** Tissues or cells were homogenized in lysis buffer (in mM: 30 Tris, pH 7.5, 150 sodium chloride, 1 mM Tris-HCl buffer, 50 mM EDTA, pH 8.0; 30 min at 37°C). After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 16 h. Membranes were washed with TPBS [0.05% (vol/vol) Tween 20 in PBS (pH 7.4)] and incubated in a 1:4,000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

**Hematoxylin-eosin staining of liver sections.** After fixation of the livers with 4% formalin/PBS, livers were sliced and stained with hematoxylin-eosin.

**Analysis of alanine transaminase activity.** Liver injury was quantified by measurement of plasma alanine transaminase activity (ALT) by using a kit from Sigma.

**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, pH 8.0; 30 min at 37°C). Sections were then incubated in 0.3% H2O2 in methanol to block endogenous peroxidase activity. Nonspecific 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 instruction (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA). Color development was induced by using 3,3'-diaminobenzidine substrate during a 5- to 10-min incubation period. With the use of this substrate, specific staining can be visualized by a light microscopy.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.** Liver tissues were removed and fixed for 24 h in 4% paraformaldehyde, dehydrated in graded ethanol, and embedded in paraffin. Paraffin sections of 4 μm were adhered to slides pretreated with a 0.01% aqueous solution of poly-L-lysine (mol wt 300,000; Sigma) and then processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to manufacturer’s instructions (Upstate Biotechnology, Lake Placid, NY). Brieﬂy, sections were incubated with terminal transferase and avidin-labeled dUTP, followed by incubation with a FITC-labeled anti-avidin antibody. The slides were then examined under a fluorescence microscope.

**Flow cytometric analysis of apoptosis.** After trypsinization, ~10⁶ cells were collected by centrifugation at 1,000 g for 5 min. Cells were then washed in PBS followed by resuspension and fixation in 70% ethanol for ~2 h. Cells were then washed with PBS and resuspended in 500 μl PBS containing 100 μg RNase, followed by a 30-min incubation at 37°C. Cellular DNA was then stained by the addition of 10 μg propidium iodide, and cells were analyzed on a FACSscan by using Cellquest software (Becton Dickinson).

**DNA fragmentation.** Cultured hepatocytes were washed twice with PBS and lysed in buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 μg/ml proteinase K) at 37°C for 18 h. DNA was extracted with an equal volume of phenol/chloroform (1:1) and precipitated at ~70°C. DNA pellets were resuspended in 10 μl of 10 mM Tris (pH 7.8) and 1 mM EDTA buffer and incubated for 1 h at 37°C with 1 μl/ml RNase (Roche Molecular Biochemicals) to remove RNA. DNA pellets were electrophoresed for 2–3 h at 90 V on 1.8% agarose gels. The gel was stained with ethidium bromide, and the DNA fragments were visualized under ultraviolet light.

**Primary mouse hepatocyte isolation and culture.** Mice weighing 20–25 g were anesthetized with pentobarbital sodium (30 mg/kg ip), and the portal vein was cannulated under aseptic conditions. The liver was perfused with EGTA solution (in mM: 5.4 KCl, 0.44 KH2PO4, 140 NaCl, 0.34 Na2HPO4, 0.5 EGTA, and 25 Tricine, pH 7.2) and DMEM (GIBCO, Gaithersburg, MD) and digested with 0.075% collagenase solution. The isolated mouse hepatocytes were then cultured in hepato-ZYMESFM media (GIBCO) in rat tail collagen-coated plates for 24 h followed by drug treatment.

**Statistical analysis.** The association between protein levels and ALT levels was analyzed by Spearman’s rank correlation test. For comparing values obtained in three or more groups, one-factor analysis of variants was used followed by Turkey’s post hoc test; P < 0.05 was taken to imply statistical significance.

**RESULTS**

**Activation and hyperexpression of STAT1 in LPS/GalN-induced murine hepatitis.** To understand the role of IFN-γ in liver injury in LPS/GalN-induced murine hepatitis, activation and expression of STAT1, a key-signaling molecule for IFN-γ, were examined. As shown in Fig. 1A, injection of LPS/GalN rapidly induced peak STAT1 tyrosine phosphorylation within 4 h, which returned to basal levels by 24 h. Expression of STAT1 proteins was also significantly elevated, beginning at 24 h postinjection and lasting for 120 h. On
the contrary, STAT1 activation was not detectable and STAT1 protein expression was only slightly induced in the CCl4-induced liver injury model. Expression of STAT1 protein in the LPS/GalN-treated mouse livers was also examined by immunohistochemistry. In normal mouse liver, very weak STAT1 staining was detected in hepatocytes, but significant STAT1 staining was detected in nonparenchymal cells in both pericentral and periportal areas (Fig. 1B). In contrast, strong STAT1 immunochemical staining was observed not only in nonparenchymal cells but also in hepatocytes in the LPS/GalN-treated mouse livers (Fig. 1C). Interestingly, STAT1 expression showed a mosaic pattern with prominent diffuse cytoplasmic staining in the pericentral region, whereas the periportal hepatocytes were not significantly stained. The observed staining pattern was specific for STAT1 protein, because control sections incubated in the absence of primary antibody failed to reveal STAT1 staining (data not shown).

**Essential role of STAT1 in LPS/GalN-induced liver apoptosis and injury.** To define the role of STAT1 in hepatitis, we examined the liver injury induced by injection of LPS/GalN in STAT1 (-/-) mice by measuring the serum levels of ALT. As shown in Fig. 2A, ALT levels were dramatically elevated in STAT1 (+/+) mice but not significantly increased in STAT1 (-/-) mice after the administration of LPS/GalN. In contrast, similar elevation of ALT activity was observed in both STAT1 (+/+) and STAT1 (-/-) mice in the CCl4-induced liver injury model (Fig. 2A) in which STAT1 was not activated (Fig. 1A). Examination of liver pathology showed that massive necroses, apoptosis, and inflammation were mainly observed in the pericentral region in STAT1 (+/+) mice after the injection of LPS/
GalN (Fig. 2B), whereas no significant liver damage and inflammation were observed in LPS/GalN-treated STAT1 (−/−) mice (Fig. 2C). Apoptosis in the liver of STAT1 (+/+ ) mice but not in STAT1 (−/− ) mice after injection of LPS/GalN was further confirmed by TUNEL assays and FASC analysis. As shown in Fig. 3A, significant TUNEL-positive staining was detected in the livers of STAT1 (+/+ ) mice but not in STAT1 (−/− ) mice after the administration of LPS/GalN. To further confirm the apoptosis, hepatocytes were isolated from the LPS/GalN-treated mice and subjected to FACS analysis. As shown in Fig. 3, B and C, significant apoptotic cells (M1 phase, sub-G1 peak) were detected in the liver of STAT1 (+/+ ) mice but not in STAT1 (−/− ) mice at 3 and 7 h after the administration of LPS/GalN.

IFN-γ is the major cytokine responsible for activation and induction of STAT1 in vivo in the murine hepatitis model, IFN-γ-deficient mice were used. As shown in Fig. 4, A and B, injection of LPS/GalN induced significant STAT1 tyrosine phosphorylation and protein expression in wild-type mice, whereas such activation and induction were markedly attenuated in IFN-γ-deficient mice, suggesting that activation and induction of STAT1 after injection of LPS/GalN are predominantly induced by IFN-γ in vivo.

To further define which factor(s) is responsible for activation and induction of STAT1, we examined the effects of nine different cytokines on STAT1 activation and protein expression in Hep3B cells. As shown in Fig. 4C, among nine cytokines tested, IFN-γ and IFN-α significantly induced STAT1 tyrosine phosphorylation and protein expression and IL-6 also weakly stimulated STAT1 tyrosine phosphorylation. IFN-γ induced much higher levels of STAT1 tyrosine phosphorylation and protein expression than did IFN-α. As shown in Fig. 4D, a low dose of IFN-γ (5 U/ml) stimulated ~30-

Fig. 2. Essential role of STAT1 in LPS/GalN-induced liver injury. A: STAT1 (+/+ ) and STAT1 (−/− ) mice (5 per group) were injected with LPS (16 μg/kg ip) and GalN (800 mg/kg ip) for 24 h or CCl4 (1.0 ml/kg ip) for 24 h followed by determination of alanine transaminase (ALT) activity. B and C: photomicrographs of representative mouse livers treated with LPS/GalN for 24 h with hematoxylin and eosin staining are shown. In STAT1 (+/+ ) mouse livers (B), massive necrosis and apoptosis were detected in the pericentral area but not in the periportal area. No significant necrosis and apoptosis were detected in STAT1 (−/− ) mouse livers (C). Magnification, ×100, ×400.
fold induction of STAT1 protein expression, whereas the same concentration of IFN-α only caused approximately twofold induction. To determine the rate of onset of the stimulatory effect of IFN-γ on STAT1 protein expression, Hep3B cells and primary mouse hepatocytes were treated with IFN-γ for various time periods. Induction was evident at 8 h and reached its peak at 12 h after treatment with IFN-γ in primary mouse hepatocytes but reached its peak at 24 h in Hep3B cells (Fig. 4E). Taken together, these findings suggest that IFN-γ is the strongest stimulator for induction of STAT1 tyrosine phosphorylation and protein expression in vitro.

**Human Hep3B cells overexpressing STAT1 are more susceptible to IFN-γ-induced cell death; disruption of the STAT1 gene abolishes such susceptibility.** To further define the role of hyperexpression of STAT1 in cell death, Hep3B cells were stably transfected with STAT1 cDNA. Three vector-transfected and three STAT1-transfected clones were selected. As shown in Fig. 5A, high levels of STAT1 proteins were detected in three STAT1-transfected clones compared with vector-transfected clones. STAT1 tyrosine phosphorylation induced by IFN-γ-treatment was significantly increased in STAT1-transfected Hep3B cells (Fig. 5B). Densitometry analysis shows that pSTAT1 in STAT1 transfected cells at 0.5, 1, and 2 h after IFN-γ stimulation is about twofold that in control cells. We then compared IFN-γ- or IFN-γ + TNF-α-induced apoptosis in vector-transfected and STAT1-transfected Hep3 cells by measuring DNA fragmentation and FASC analysis. As shown in Fig. 5C, treatment with IFN-γ for 24 h induced significant DNA fragmentation in STAT1-transfected Hep3B cells but not in vector-transfected cells, which was also confirmed by FASC analysis in Fig. 5, D and E (apoptotic cells (M₁; sub-G₁ peak): 9.5 ± 0.6% in IFN-γ-treated vector-transfected cells vs. 19.8 ± 1.4% in IFN-γ-treated STAT1-transfected cells (Fig. 5E)). Treatment with TNF-α did not induce significant apoptosis in either vector-transfected cells or STAT1-transfected cells, whereas a combination of IFN-γ and TNF-α treatment induced significant apoptosis in both vector- and STAT1-transfected cells (Fig. 5, C–E). Much higher apoptosis was detected in STAT1-transfected cells than vector-transfected cells [apoptotic cells (M₁; sub-G₁ peak): 21.5 ± 0.9% in IFN-γ + TNF-α-treated vector-transfected cells vs. 54.0 ± 11% in IFN-γ + TNF-α-treated STAT1-transfected cells (Fig. 5E)]. These findings suggest that Hep3B cells overexpressing STAT1 are more susceptible to IFN-γ or IFN-γ + TNF-α-induced cell death.

To further confirm the essential role of STAT1 in IFN-γ- or IFN-γ + TNF-α-induced hepatocyte death, Hep3B cells were stably transfected with dominant negative STAT1 cDNA, followed by treatment with cytokines. As shown in Fig. 6A, stable transfection of dominant negative STAT1 abolished IFN-γ + TNF-α-induced hepatocyte death. The role of STAT1 in IFN-γ or IFN-γ + TNF-α-induced hepatocyte death was also examined in hepatocytes isolated from STAT1 (+/+) and STAT1 (−/−) mice. As shown in Fig. 6B, treatment with IFN-γ or IFN-γ + TNF-α for 24 h induced significant apoptosis in STAT1 (+/+) mouse hepatocytes but not in STAT1 (−/−) mouse hepatocytes. Treatment with TNF-α did not induce marked apoptosis in either of them.

**DISCUSSION**

IFN-γ has been implicated in the pathogenesis of acute and chronic liver disease in humans (19, 20, 22)
and immunohistochemistry shows that both overexpression of STAT1 and hepatocellular damage are located in the same pericentral region (Figs. 1 and 2). These findings suggest that hyperexpression of STAT may also be implicated in the pathogenesis of LPS/GalN-induced apoptosis and liver injury.

Although it is clear that IFN-γ-activation of STAT1 is essential for development of liver apoptosis and injury, the underlying mechanism is still unclear. Several molecular pathways are plausible to consider. First, IFN-γ alone or synergistically with TNF-α induces hepatocyte apoptosis via a STAT1-dependent mechanism (Fig. 6). Second, IFN-γ-induced activation of STAT1 induces the expression of several chemokines, including IP-10 and Mig (5), which could attract leukocytes into the liver. Third, STAT1 also controls IRF-1 induction, a key proapoptotic factor that plays an important role in hepatocyte apoptosis (13), as well as ConA- and LPS/GalN-induced liver injury (21). Fourth, STAT1 is involved in the induction of iNOS (6), a key factor that plays an important role in liver injury induced by hepatitis (21). Finally, IFN-γ significantly induces STAT1 protein expression, which further enhances IFN-γ signaling and the susceptibility of hepatocytes to IFN-γ- or IFN-γ + TNF-α-induced apoptosis.

In this paper, we also provide evidence suggesting that IFN-γ is mainly responsible for not only activation of STAT1 but also induction of STAT1 protein expression in LPS/GalN-induced hepatitis, because disruption of the IFN-γ gene almost completely abolished the activation (Fig. 4A) and hyperexpression of STAT1 (Fig. 4B). We have also previously shown that IFN-γ is a major cytokine responsible for STAT1 activation and induction in the liver of ConA-induced hepatitis (9). Among nine different cytokines tested, IFN-γ, even at very low concentrations, is the strongest activator of STAT1 protein expression in mouse and human hepatocytes (Figs. 4, C and D), further suggesting that IFN-γ is a major cytokine contributing STAT1 activation and induction in vivo. Interestingly, hyperexpression of STAT1 protein was detected in the livers of chronic HCV infection (unpublished data), indicating that STAT1 may also be implicated in the pathogenesis of chronic hepatitis C infection. High levels of IFN-γ have been reported in the plasma and the livers of chronic HCV infection patients (19, 20, 22), and this is likely responsible for high levels of STAT1 protein expression in the livers of these patients. Taken together, IFN-γ-mediated activation and induction of STAT1 play an essential role in LPS/GalN- and ConA-induced liver apoptosis and injury (9) and are likely involved in the pathogenesis of chronic hepatitis C infection. In contrast, STAT1 is not significantly activated and plays a minor role in CCl4-induced liver injury (Figs. 1A and 2A). CCl4 is a classic hepatotoxicant that causes injury initially from the metabolism of CCl4 to the trichloromethyl radical, which directly damages hepatocytes. CCl4 also initiates secondary liver injury through activation of Kupffer cells and subsequently releasing TNF-α, which influences the extent of liver damage or its repair (17, 27). The finding
that injection of CCl₄ did not cause significant STAT1 activation in the liver (Fig. 1) suggested that IFN-γ was not significantly elevated after injection of CCl₄. On the contrary, serum levels of IFN-γ are significantly elevated in ConA-mediated hepatitis and LPS/GalN-induced liver damage (2, 9), and elevated IFN-γ subsequently activates STAT1 and promotes hepatocyte apoptosis in these models.

![Image](image_url)

**Fig. 5.** Overexpression of STAT1 increases the susceptibility of Hep3B cells to IFN-γ- or IFN-γ/TNF-α-induced apoptosis. A: Hep3B cells were stably transfected with empty vector or STAT1 expression vector as described in MATERIALS AND METHODS. Three empty vector-transfected (Neo1, Neo2, Neo3) and three STAT1-transfected clones (STAT1-1, STAT1-2, STAT1-3) were selected and subjected to Western blot analysis using anti-STAT1 and β-actin antibodies. B: vector-transfected (Neo1) and a STAT1-transfected clone (STAT1-2) were stimulated with IFN-γ (5 U/ml) for various time periods, followed by Western blot analysis using by using anti-phospho-STAT1 and anti-STAT1 antibodies. A representative of 3 independent experiments is shown. C and D: vector-transfected (Neo1) and a STAT1-transfected clone (STAT1-2) were treated with IFN-γ (10 U/ml), TNF-α (10 ng/ml), or IFN-γ (10 U/ml) + TNF-α (10 ng/ml) for 24 h. Apoptosis was then analyzed by DNA fragmentation (C) or FACS (D). Similar data were obtained from other clones. A representative of 4 independent experiments is shown. E: apoptotic cell numbers (M₁, sub-G₁ peak) were obtained from 3 different clones by using FACS analysis. *P < 0.05, **P < 0.001 compared with corresponding treated-vector-transfected clones.

**Fig. 6.** STAT1 is essential for IFN-γ- or IFN-γ + TNF-α-induced apoptosis. A: Hep3B cells stably transfected with dominant negative STAT1 cDNA were treated with IFN-γ (10 U/ml), TNF-α (10 ng/ml), or IFN-γ (10 U/ml) + TNF-α (10 ng/ml) for 24 h. Apoptosis was determined by DNA fragmentation. Similar IFN-γ + TNF-α treatment caused apoptosis in vector-transfected or wild-type STAT1-transfected Hep3B cells (see Fig. 5C). B: hepatocytes from STAT1 (+/+) and STAT1 (-/-) mice were treated with IFN-γ (10 U/ml), TNF-α (10 ng/ml), or IFN-γ (10 U/ml) + TNF-α (10 ng/ml) for 24 h, followed by FACS analysis. Apoptosis (M₁, sub-G₁ peak) was measured by propidium iodide staining. A and B are representatives of 4 independent experiments. C: apoptotic cells (M₁, sub-G₁ peak) were obtained from 4 independent experiments as shown in B. *P < 0.05, **P < 0.01 compared with corresponding treated STAT1 (+/+) group.
REFERENCES


