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Abrogation of the Antifibrotic Effects of Natural Killer Cells/ Interferon- γ Contributes to Alcohol Acceleration of Liver Fibrosis

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Abstract

Background & Aims—Chronic alcohol drinking accelerates liver fibrosis in patients with viral hepatitis that cannot be fully explained by ethanol-enhanced liver damage. Here, we identified a novel mechanism by which alcohol accelerates liver fibrosis: inhibition of the antifibrotic effects of natural killer (NK) cells and interferon- γ (IFN- γ).

Methods—Alcohol administration was achieved by feeding mice with a liquid diet containing 5% ethanol for 8 weeks. Liver fibrosis was induced by administration of carbon tetrachloride (CCl₄) for 2 weeks. Hepatic stellate cells (HSCs) were also isolated and cultured for in vitro studies.

Results—CCl₄ treatment induced greater fibrosis and less apoptosis of HSCs in ethanol-fed mice compared with pair-fed mice. Polyinosinic-polycytidylic acid (Poly I:C) or IFN- γ treatment inhibited liver fibrosis in pair-fed but not in ethanol-fed mice. Poly I:C activation of NK cell cytotoxicity against HSCs was attenuated in ethanol-fed mice compared with pair-fed mice, which was due to reduced natural killer group 2 member D (NKG2D), tumor necrosis factor-related apoptosis-inducing ligand, and IFN- γ expression on NK cells from ethanol-fed mice. In vitro, HSCs from ethanol-fed mice were resistant to IFN- γ -induced cell cycle arrest and apoptosis compared with pair-fed mice. Such resistance was due to diminished IFN- γ activation of signal transducer and activator of transcription 1 (STAT1) in HSCs from ethanol-fed mice caused by the induction of suppressors of cytokine signaling proteins and the production of oxidative stress. Finally, HSCs from ethanol-fed mice were resistant to NK cell killing, which can be reversed by transforming growth factor- β 1 (TGF- β 1) neutralizing antibody.

Conclusions—Chronic ethanol consumption attenuates the antifibrotic effects of NK/IFN- γ /STAT1 in the liver, representing new and different therapeutic targets with which to treat alcoholic liver fibrosis.

Liver fibrosis is a common scarring response to virtually all forms of chronic liver injury and is characterized by an accumulation of extracellular matrix proteins in the liver.^{1–3} Increasing evidence suggests that several cell types contribute to liver fibrogenesis, including hepatic stellate cells (HSCs), myofibroblasts, bone marrow-derived progenitor cells, and hepatocytes.^{1–3} Among them, HSCs are believed to play a key role in the pathogenesis of liver fibrosis.^{1–3} HSCs are generally quiescent in normal healthy livers and become activated and differentiate during liver injury into myofibroblastic cells that are characterized by a loss of vitamin A

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(retinol) and enhanced collagen expression.^{1–3} Alcohol consumption, hepatitis C viral (HCV) infection, and nonalcoholic steatohepatitis are currently the 3 main causes of chronic liver injury leading to liver fibrosis.⁴ Interestingly, alcohol consumption was shown to accelerate liver fibrosis in patients with chronic HCV infection^{5–9} and in animals treated with hepatotoxins.¹⁰ The obvious underlying cause for this is enhanced liver injury induced by alcohol; however, this does not fully explain the acceleration of liver fibrosis observed. For example, several studies showed that most alcoholics with chronic HCV infection have histologic features similar to those in nonalcoholic patients with chronic HCV infection, with the exception that progression of liver fibrosis was much faster in alcoholics than in nonalcoholics.^{11,12} In addition to simply enhancing liver injury, alcohol may accelerate progression of liver fibrosis through several mechanisms.^{13–15} For instance, alcohol consumption may enhance hepatic HSC activation through an increase in gut-derived endotoxins, hypoxia (oxidative stress), or the formation of toxic and profibrogenic metabolites (eg, acetaldehyde or lipid peroxides).^{13–15} Recently, we and others have shown that activation of the innate immune system, natural killer (NK) cells and interferon- γ (IFN- γ), inhibits liver fibrosis.^{16–18} Moreover, the immunosuppressive effects of ethanol are well documented in alcoholics.^{19,20} Thus, we hypothesized that inhibition of NK cell/IFN- γ functioning as mediated by ethanol may be an important mechanism that contributes to ethanol acceleration of liver fibrosis.

NK cells are abundant in the liver, accounting for 10% of lymphocytes found in mouse livers and 30%–50% of lymphocytes from rat or human livers.^{21,22} Although activation of NK cells by double-stranded RNA, polyinosinic-polycytidylic acid (poly I:C), or IFN- γ inhibits liver fibrosis, depletion of NK cells enhances liver fibrosis.^{16,17} Amelioration of liver fibrosis by NK cells is accomplished through the direct killing of activated HSCs in natural killer group 2 member D (NKG2D)–, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–, and granzyme-dependent manners.^{16,17} NK cells can also inhibit liver fibrosis by production of IFN- γ .¹⁸ The antifibrotic effects of IFN- γ is well documented in animal studies, which show that IFN- γ inhibits HSC activation and stimulates NK cell killing of activated HSCs in vivo and in vitro.^{18,23,24} Furthermore, treatment with IFN- γ was shown to ameliorate liver fibrosis in patients with hepatitis B viral or HCV infection,^{25,26} but a recent clinical trial showed that IFN- γ treatment had no beneficial effect in patients with advanced liver disease.²⁷ The difference between these studies may be due to the selection of patients with differing stages of liver disease. It is possible that IFN- γ may have beneficial effects when treating early stages of liver fibrosis but not the later stage of cirrhosis. The actions of IFN- γ are mediated through binding to IFN- γ receptors, followed by phosphorylation of Janus kinases (JAKs) and the signal transducer and activator of transcription 1 (STAT1). Phosphorylated STAT1 proteins form dimers and translocate into the nucleus to induce transcription of a variety of genes, including a family of suppressor of cytokine signaling protein (SOCS). The SOCS proteins then in turn bind to JAKs and turn off IFN- γ signaling in a feedback loop.²⁸

In this report, we explored the effects of chronic ethanol consumption on NK cells/IFN- γ –mediated antifibrotic effects in the liver in vivo and in vitro. Our results suggest that abrogation of the antifibrotic effects of NK cells/IFN- γ may be an important mechanism responsible for ethanol acceleration of liver fibrosis.

Methods and Materials

Mice

C57BL/6N mice were purchased from the National Cancer Institute (Frederick, MD). All mice used in the present study were housed in a specific pathogen-free facility and were cared for in accordance with National Institutes of Health guidelines.

Chronic Ethanol Consumption and Liver Fibrosis Induced by CCl₄

Male C57BL/6N mice weighing ≈ 25 g were fed a nutritionally adequate liquid diet containing 5% ethanol or a pair-fed diet in which ethanol was substituted isocalorically with dextrin maltose (BioServ, Frenchtown, NJ). Ethanol was introduced gradually by increasing the content by 1% (vol/vol) every day until the mice were consuming diets containing 5% ethanol for 8 weeks. After 8 weeks of feeding, mice continued on the same diets and were injected (intraperitoneally, 3 times a week) with 1 mL/kg or 2.5 mL/kg body weight of 10% pure carbon tetrachloride (CCl₄; Sigma, St Louis, MO) dissolved in olive oil (Sigma) for 2 weeks. Mice were then killed, and liver tissues were collected.

Cotreatment With CCl₄ Plus Poly I:C or CCl₄ Plus IFN- γ in Mice

Eight-week ethanol- or pair-fed mice were injected with CCl₄ (0.1 mL/kg, 3 times a week) and poly I:C (5 μ g/g, 3 times a week, intraperitoneally; Sigma) or murine IFN- γ (2000 IU/g, subcutaneous injection, 7 times a week; R&D Systems, Minneapolis, MN) for an additional 2 weeks. Poly I:C was administered 12 hours before the CCl₄ injection.

Isolation, Culture, and Treatment of Hepatic Stellate Cells, and Preparation of D4 HSCs

Mouse liver hepatic stellate cells (HSCs) were isolated by in situ collagenase perfusion and differential centrifugation on Optiprep (Sigma) density gradients as described previously.¹⁸ The isolated HSCs were resuspended in RPMI 1640 medium containing 20% fetal bovine serum and then plated onto 24-well plates at a density of 1×10^4 cells/well or onto 6-well plates at a density of 1×10^5 cells/well as described previously.¹⁸ On day 3, HSCs were cultured in serum-free medium for an additional 24 hours. These cells were designated as D4 HSCs. The D4 HSCs were then treated with IFN- γ for 30 minutes for immunohistochemistry experiments or 24 hours for cell proliferation assays.

For coculture experiments, D4 HSCs were cocultured with hepatocytes for 24 hours with cell-culture inserts of 3- μ m pore size (Corning, Acton, MA) to separate both cell populations as described previously.^{29,30} After various time points, HSCs were incubated with IFN- γ , TGF- β 1, or both. For blocking oxidative stress of hepatocytes, medium was added with 100- μ mol/L Trolox, 1-mmol/L ascorbic acid, and 5-mmol/L N-acetyl cysteine (Sigma) as described with some modifications.³¹

Other Methods

RT-PCR, Western Blotting, Cell Proliferation, TUNEL (transferase-mediated dUTP nick-end labeling) Assay, Analysis of Caspase-3 Activity, Flow Cytometric Analysis, Histology, Immunohistochemistry, Immunocytochemistry, Measurement of Hepatic Hydroxyproline Contents, Isolation of Hepatic Mononuclear Cells (MNCs) and NK Cells, and Cytotoxicity Assay, and Transforming Growth Factor β 1 (TGF- β 1) Treatment

Details about these methods are described in the Supplementary Materials and Methods (see supplemental material online at www.gastrojournal.org). All Western blotting, reverse transcription–polymerase chain reaction (RT-PCR), and immunocytochemistry experiments were repeated at least 2 times with 5–10 different mice or samples per group, and only representative blots are shown in the figures. The details of density quantification are shown in the figures or in the supporting materials.

Statistical Analysis

Data are expressed as means $\pm$ SEMs. To compare values obtained from ≥ 2 groups, the Student *t* test or one-way analysis of variance was performed. A value of $P < .05$ was considered significant.

Results

Ethanol Feeding Decreases HSC Apoptosis and Accelerates Liver Fibrosis During CCl₄-Induced Liver Fibrogenesis

Figure 1A shows that injection with 0.1 mL/kg CCl₄ induced higher levels of serum ALT in ethanol-fed mice than in pair-fed mice. To generate similar levels of liver injury in both groups, we injected pair-fed mice with a higher dose of CCl₄ (0.25 mL/kg). Our results showed that injection with 0.25 mL/kg CCl₄ in pair-fed mice caused a similar elevation of serum ALT levels compared with injecting 0.1 mL/kg CCl₄ in ethanol-fed mice (Figure 1A). Next, we compared liver fibrosis in these mice (Figure 1B–D). As expected, injection of the same dose of CCl₄ (0.1 mL/kg) induced a greater increase in α -smooth muscle actin (α -SMA) protein, hydroxyproline, collagen fibers, and α -SMA–positive cells in the livers of ethanol-fed mice than of pair-fed mice. Interestingly, despite similar degrees of liver injury, there was remarkably more hydroxyproline and collagen deposition and α -SMA staining in ethanol-fed mice treated with 0.1 mL/kg CCl₄ compared with pair-fed mice treated with 0.25 mL/kg CCl₄. This finding indicates that liver fibrosis is accelerated in ethanol-fed mice compared with pair-fed mice despite similar levels of liver injury.

Next, we compared HSC apoptosis in ethanol- and pair-fed mice challenged with CCl₄. As shown in Figure 1D and E, the number of α -SMA⁺TUNEL⁺ cells/per field was lower in ethanol-fed mice treated with 0.1 mL/kg CCl₄ than in pair-fed mice treated with 0.25 mL/kg CCl₄. Furthermore, the percentage of α -SMA⁺TUNEL⁺ cells/total α -SMA⁺ cells in ethanol-fed mice treated with 0.1 mL/kg CCl₄ was lower than in pair-fed mice treated with either 0.1 or 0.25 mL/kg CCl₄.

Ethanol Consumption Abolishes the Antifibrotic Effects of Poly I:C in the Liver

The above findings suggested that during fibrogenesis HSCs in ethanol-fed mice were more resistant to apoptosis than those from pair-fed mice. Recently, we showed that activation of NK cells by poly I:C played an important role in inducing HSC apoptosis and inhibiting liver fibrosis.^{16,18} Thus, we wondered whether ethanol feeding affects the antifibrogenic properties of NK cells activated by poly I:C. As shown in Figure 2A–C, poly I:C treatment inhibited liver fibrosis in pair-fed mice as shown by inhibition of hepatic contents of α -SMA and hydroxyproline; however, such inhibition was less evident in ethanol-fed mice.

Ethanol Feeding Inhibits Hepatic NK Cell Killing of HSCs via Down-regulation of NKG2D and TRAIL Expression on NK Cells

In cytotoxicity assays, liver MNCs and NK cells from poly I:C–treated pair-fed mice produced approximately 43% and 49% cytotoxicity against activated HSCs (4-day cultured HSCs), respectively, whereas the cells from poly I:C–treated ethanol-fed mice produced only $\approx$ 15% and 13% cytotoxicity, respectively (Figure 3A). RT-PCR in Figure 3B and C revealed that treatment with poly I:C induced significant expression of NKG2D, TRAIL, perforin, Fas L, and IFN- γ in pair-fed mouse liver MNCs. This induction was much lower in ethanol-fed mice. Results from fluorescence-activated cell sorting analyses showed that poly I:C treatment yielded a significant elevation of NK cells and expression of NKG2D, TRAIL, and IFN- γ on liver NK1.1⁺ cells from pair-fed mice, whereas such induction was diminished in ethanol-fed mice (Figure 3D).

Ethanol Feeding Abolishes the Antifibrotic Effects of IFN- γ by Inhibition of IFN- γ –Activated STAT1 Signaling in HSCs

Treatment with poly I:C inhibits liver fibrosis by an IFN- γ –dependent mechanism.¹⁶ Thus, we investigated whether ethanol suppression of the antifibrotic effects of poly I:C is mediated

through blocking IFN- γ activity. Immunohistochemical analyses, Western blotting, and measurement of hepatic hydroxyproline contents showed that IFN- γ treatment inhibited liver fibrosis in pair-fed mice but not in ethanol-fed mice (Figure 4A–D). These data suggest that the antifibrotic effects of IFN- γ become invalid with chronic ethanol consumption. In vivo treatment with IFN- γ inhibited pSmad3 expression in pairfed mice, which was likely due to induction of Smad7 (Figure 4B and C). In contrast, in ethanol-fed mice, IFN- γ treatment only slightly induced Smad7 protein expression and did not inhibit pSmad3 expression (Figure 4B and C). In addition, IFN- γ treatment induced STAT1 activation in pair-fed mice; such activation was diminished in ethanol-fed mice (Figure 4B and C).

Next, we compared IFN- γ signaling and activity in HSCs from pair- (pair HSCs) and ethanol-fed mice (ethanol HSCs). As shown in Figure 5A, IFN- γ treatment inhibited pair HSC proliferation at doses ranging from 1 to 100 ng/mL, but only high doses of IFN- γ (50 and 100 ng/mL) inhibited ethanol HSC proliferation. Treatment with IFN- γ induced elevated levels of caspase-3 activity in pair HSCs but not ethanol HSCs (Figure 5B). Furthermore, immunocytochemical analyses showed that positive staining of pSTAT1 was detected in the nucleus of cultured pair HSCs after treatment with 5 or 50 ng/mL IFN- γ , whereas only the 50 ng/mL dose of IFN- γ induced pSTAT1 activation in ethanol HSCs (Figure 5C). Western blotting revealed that IFN- γ treatment induced phosphorylation of JAK1, JAK2, and STAT1 in pair HSCs. Such activation was suppressed in ethanol HSCs (Figure 5D and E). Furthermore, expression of SOCS1 protein was detected at higher levels in freshly isolated (day 0) or 4-day-cultured ethanol HSCs compared with pair HSCs (Figure 5F).

IFN- γ /STAT1 Signaling Is Disrupted in HSCs Cocultured With Hepatocytes From Ethanol-Fed Mice

To investigate the mechanism by which IFN- γ signaling was suppressed in ethanol HSCs, we examined the effects of hepatocytes from pair- (pair hepatocytes) or ethanol-fed mice (ethanol hepatocytes) on IFN- γ signaling in HSCs. As shown in Figure 6A, IFN- γ treatment induced strong STAT1 activation in HSCs cocultured with pair hepatocytes but only induced weak STAT1 activation in HSCs cocultured with ethanol hepatocytes, suggesting that ethanol hepatocytes inhibit IFN- γ signaling in HSCs. This conclusion was further confirmed by Western blot analyses in Figure 6B, which showed that IFN- γ activation of pSTAT1 was observed in HSCs cocultured with pair hepatocytes but not with ethanol hepatocytes. Interestingly, IFN- γ activation of STAT1 in HSCs cocultured with ethanol hepatocytes was recovered by pretreatment with antioxidants such as ascorbic acid, Trolox, and N-acetyl cysteine (Figure 6B). Furthermore, treatment with an oxidant such as hydrogen peroxide (H₂O₂) blocked IFN- γ activation of pSTAT1 and induction of interferon regulatory factor 1 (IRF-1) expression in HSCs (Figure 6C and D).

Previously, it was reported that IFN- γ inhibits TGF- β 1 signaling in HSCs.³² We wondered whether coculture with ethanol hepatocytes was able to abolish IFN- γ inhibition of TGF- β 1 signaling in HSCs. As shown in Figure 6E, TGF- β 1 treatment induced Smad 3 phosphorylation in HSCs cocultured with ethanol hepatocytes or pair hepatocytes. Such activation was inhibited by IFN- γ in HSCs cocultured with pair hepatocytes but not with ethanol hepatocytes. Moreover, RT-PCR analyses revealed that TGF- β 1 treatment induced expression of collagen type 1 α 2 (COL1A2) in HSCs cocultured with pair hepatocytes, such induction was much lower in HSCs cocultured with ethanol hepatocytes (Figure 6F). IFN- γ pretreatment inhibited TGF- β 1 induction of COL1A2 in HSCs cocultured with pair hepatocytes but not in HSCs cocultured with ethanol hepatocytes (Figure 6F).

Ethanol HSCs Are Resistant to NK Cell Killing by a TGF- β 1-Dependent Mechanism

In cytotoxicity assays, 4-day cultured ethanol HSCs were resistant to NK cell killing compared with pair HSCs (Figure 7A). Because TGF- β 1 was implicated in suppression of NK cell activity³³ and HSCs are an important producer of TGF- β 1 during HSC activation,^{14,15} we investigated whether TGF- β 1 was involved in the resistance to NK cell killing of ethanol HSCs. The RT-PCR analyses showed that expression of TGF- β 1 mRNA was much stronger in ethanol HSCs than in pair HSCs (Figure 7B), suggesting that ethanol HSCs produce more TGF- β 1. Furthermore, the addition of TGF- β 1 decreased the cytotoxicity of NK cells against pair and ethanol HSCs. In contrast, the addition of TGF- β 1-neutralizing antibody slightly enhanced the cytotoxicity of NK cells against pair HSCs but markedly enhanced the cytotoxicity against ethanol HSCs (Figure 7C). To elucidate the effect of TGF- β 1 on NK cells, MNCs isolated from poly I:C-treated mice were incubated with TGF- β 1. Basal expression levels of NKG2D, TRAIL, and IFN- γ in MNCs from poly I:C-treated mice were greater than those from poly I:C-untreated mice, which was consistent with our previous reports.^{16,18} Interestingly, incubation with TGF- β 1 reduced expression of these genes (Figure 7D). These findings suggest that TGF- β 1 secreted by activated HSCs may serve to diminish NK cytotoxicity against HSCs through down-regulating NKG2D, TRAIL, and IFN- γ expression on NK cells.

Discussion

Chronic alcohol consumption is one of the main causes of accelerated liver fibrosis in patients with viral hepatitis.^{5–9} In rodents, ethanol feeding was also shown to accelerate CCl₄-induced liver fibrosis.¹⁰ It is generally believed that ethanol feeding enhances CCl₄ metabolism by induction of cytochrome P450 2E1 expression and, subsequently, enhances CCl₄-induced liver injury. Here, we also confirmed that injection of the same dose of CCl₄ induced more fibrosis in ethanol-fed mice than in pair-fed mice (Figure 1A). Interestingly, the progression of liver fibrosis was still faster in ethanol-fed mice than in pair-fed mice even when similar levels of liver injury (serum levels of ALT) were achieved in both groups by injection of a higher dose of CCl₄ in pair-fed mice (Figure 1). This suggests that, in addition to enhanced liver injury, other mechanisms also contribute to ethanol acceleration of liver fibrosis. Our further studies indicate that attenuation of the antifibrotic effects of NK cells/IFN- γ is another important mechanism that contributes to ethanol acceleration of liver fibrosis. Mechanisms of chronic ethanol consumption-induced acceleration of fibrosis includes (1) inhibition of NK cell killing of activated HSCs, (2) induction of HSC resistance to NK cell killing in a TGF- β 1-dependent manner, (3) interruption of IFN- γ /STAT1 signaling in HSCs by induction of SOCS proteins, and (4) inhibition of IFN- γ /STAT1 signaling in HSCs by induction of oxidative stress. We have integrated these findings in Figure 8.

In the investigation, we obtained several lines of evidence, suggesting that chronic ethanol consumption inhibits the antifibrotic properties of NK cells. First, we and others have shown that NK cells kill activated HSCs, which contributes to HSCs apoptosis during liver fibrogenesis.^{16,17} Here, we showed that liver NK cells isolated from ethanol-fed mice had less killing of activated HSCs than those from pair-fed mice (Figure 3), which may contribute to less HSC apoptosis observed in ethanol-fed mice after administration of CCl₄ (Figure 1). Second, activation of NK cells by poly I:C or IFN- γ inhibited liver fibrosis in pair-fed mice but not in ethanol-fed mice (Figures 2 and 4). Third, it has been well documented that alcohol consumption decreases NK cell activity and count in animal experiments^{34,35} and in human alcoholics.³⁶ Down-regulation of hepatic NK cell number and activity after alcohol feeding was also confirmed in this study (Figure 3). Further studies suggest that ethanol inhibition of NK cell killing of HSCs is likely mediated by down-regulation of NKG2D, TRAIL, Fas L, and IFN- γ expression on NK cells (Figure 3). Although our findings clearly showed that ethanol feeding inhibits hepatic NK cell activity, the underlying mechanism remains unclear.

Investigational findings on the effect of ethanol on peripheral and splenic NK cells suggest that chronic ethanol consumption inhibits NK cell activity by multiple mechanisms, including elevation of corticosterone³⁷ and reduction of opioid peptide β -endorphin.³⁸ In addition, Pruett et al³⁹ recently reported that ethanol inhibited poly I:C activation of Toll-like receptor 3 (TLR3) signaling in macrophages. It is plausible then to speculate that ethanol may also inhibit TLR3 signaling in NK cells, contributing to ethanol inhibition of NK cell activity.

In addition to inhibiting NK cell cytotoxicity against activated HSCs, ethanol feeding also enhanced HSC resistance to NK killing. As shown in Figure 7, liver MNCs showed $\approx 40\%$ cytotoxicity against HSCs isolated from pair-fed mice, but only $\approx 20\%$ against HSCs from ethanol-fed mice. Previously, several studies reported that TGF- $\beta 1$ secreted by tumor cells and dendritic cells were responsible for poor NK cytotoxicity against tumor cells by down-regulation of the NK-activating receptor, NKG2D.³³ In addition, up-regulation of the TGF- $\beta 1$ gene and of its cell membrane receptors are features of activated HSCs in vitro and in vivo.⁴⁰ Therefore, we hypothesized that resistance of HSCs from ethanol-fed mice to NK killing was mediated by overproduction of TGF- $\beta 1$. Indeed, our findings support this notion. As shown in Figure 7, RT-PCR analyses confirmed that up-regulation of TGF- $\beta 1$ mRNA expression in HSCs of ethanol-fed mice and that blocking TGF- $\beta 1$ with a neutralizing antibody increased the susceptibility of HSCs to NK killing whereas treatment with TGF- $\beta 1$ decreased it. Until now, TGF- $\beta 1$ was considered as one of the most important survival factors of activated HSCs through the suppression of CD95L expression on HSCs.⁴¹ Our findings in this study suggest that inhibition of NK cell killing of HSCs may also contribute to the important role of TGF- $\beta 1$ in HSC survival.

NK cells ameliorate liver fibrosis not only by direct killing of activated HSCs but also by production of IFN- γ . The antifibrotic effects of IFN- γ is mediated by direct induction of HSC cell cycle arrest and apoptosis, stimulation of NK cell cytotoxicity against HSCs, and inhibition of TGF- $\beta 1$ signaling in a STAT1-dependent manner.^{16,18,23,24} In this study, we provided in vivo and in vitro evidence to suggest that chronic ethanol feeding diminishes the antifibrotic effects of IFN- γ in the liver. As shown in Figure 4A, IFN- γ treatment inhibited liver fibrosis in pair-fed mice but not in ethanol-fed mice. This is likely due to inhibition of IFN- γ -mediated pSTAT1 activation and Smad7 induction in ethanol-fed mice (Figure 4B). In vitro evidence also showed that the antiproliferative and proapoptotic effects of IFN- γ were diminished in HSCs isolated from ethanol-fed mice compared with those from pair-fed mice, which is likely due to ethanol inhibition of IFN- γ /STAT1 signaling in these cells. Previously, we and others have shown that ethanol inhibits the IFN- γ /STAT1 signaling pathway in hepatocytes by induction of protein kinase C, oxidative stress, and SOCS1.^{42,43} The latter 2 mechanisms likely also contribute to ethanol inhibition of IFN- γ activation of STAT1 in HSCs because antioxidant treatment reversed the inhibitory effect of IFN- γ (Figure 6) and SOCS1 protein expression was much higher in ethanol HSCs than in pair HSCs (Figure 5F). Interestingly, Nieto et al^{29,30} reported that reactive oxygen species produced by hepatocytes can also stimulate HSC activation. Taken together, alcohol consumption can induce hepatocytes to produce oxidative stress, which will not only induce HSC activation but also protect HSCs from IFN- γ -induced cell cycle arrest and apoptosis, resulting in acceleration of liver fibrosis.

In summary, chronic ethanol consumption stimulates overproduction of oxidative stress and TGF- $\beta 1$, desensitization of HSCs from NK cytotoxicity, induction of HSC resistance to NK cell killing, and inhibition of the anti-fibrotic effects of IFN- γ , collectively resulting in acceleration of liver fibrosis. Antioxidant and neutralizing TGF- $\beta 1$ antibody treatment may have beneficial effects in slowing down alcohol acceleration of liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

α -SMA	α -smooth muscle actin
ALT	alanine aminotransferase
CCl ₄	carbon tetrachloride
COL1A2	collagen type 1 α 2
HCV	hepatitis C viral
HSCs	hepatic stellate cells
IFN- γ	interferon- γ
IRF-1	interferon regulatory factor 1
JAK	Janus kinase
MNC	mononuclear cell
NK cells	natural killer cells
NKG2D	natural killer group 2 member D
poly I:C	polyinosinic-polycytidylic acid
RT-PCR	reverse transcription–polymerase chain reaction
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TGF- β 1	transforming growth factor- β 1
TLR3	Toll-like receptor 3
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TUNEL	transferase-mediated dUTP nick-end labeling

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Figure 1.

Ethanol feeding decreases HSC apoptosis and accelerates liver fibrosis during CCl₄-induced liver fibrogenesis. Mice were fed either an ethanol diet or pair-fed a control diet for 8 weeks and then injected with CCl₄ for an additional 2 weeks while continuing the same diet. Killed at week 10, the sera were collected for measuring ALT (A) and the livers were collected for Western blotting (B) or for measuring hepatic hydroxyproline (C) or immunohistochemistry analyses (D). (D) Trichrome staining for collagen fibers, α-SMA staining for activated HSCs, and TUNEL staining for apoptosis. Arrows indicate α-SMA⁺ TUNEL⁺ cells, which were counted per field of magnification (×200) and shown in panel E. Values in panels A, C, and E are means ± SEMs (n = 7). ***P* < .01, ****P* < .001 vs pair-fed + 0.25 mL/kg CCl₄.

Figure 2.

Ethanol feeding abolishes the antifibrotic effects of poly I:C. Eight-week ethanol- and pair-fed mice were maintained on the same diet while receiving injections of CCl₄ (0.1 mL/kg) plus poly I:C or saline for an additional 2 weeks. Liver tissues were stained with anti- α -SMA antibody to detect activated HSCs (A) or subject to Western blotting (B) or measuring for hepatic hydroxyproline (C). Values in panel C are means $\pm$ SEMs (n = 6). **P* < .05 vs corresponding saline group.

Figure 3.

Ethanol feeding inhibits hepatic NK cell killing of HSCs and down-regulates NKG2D and TRAIL expression on NK cells. Eight-week ethanol- and pair-fed mice were treated with poly I:C (5 μ g/g, intraperitoneal). After 16 hours, liver MNCs or NK cells were isolated and used in cytotoxicity assays against 4-day cultured HSCs (A). Liver MNCs were also subject to RT-PCR analyses (B). The densities of bands in *panel B* were quantified and shown in *panel C*. Liver MNCs were also analyzed by fluorescence-activated cell sorting (D). Values in *panels A, C, and D* are means $\pm$ SEMs (n = 6). * P < .05, ** P < .01 vs corresponding pair-fed group.

Figure 4.

Ethanol feeding abolishes the antifibrotic effects of IFN- γ . Eight-week ethanol- and pair-fed mice were maintained on the same diet and injected with CCl₄ (0.1 mL/kg) plus IFN- γ or saline for an additional 2 weeks. Liver tissues were stained with anti- α -SMA antibody to detect activated HSCs (A) or subject to Western blotting (B and C) or measuring hepatic hydroxyproline (D). The densities of bands in *panel B* were quantified and shown in *panel C*. Values in *panels C* and *D* are means $\pm$ SEMs (n = 4). **P* < .05, ***P* < .01 vs corresponding IFN- γ nontreated groups.

Figure 5.

HSCs from ethanol-fed mice are resistant to IFN- γ stimulation. (A) HSCs isolated from 8-week pair- or ethanol-fed mice were cultured for 4 days (D4 HSCs) and treated with IFN- γ for 24 hours. Cell proliferation was then measured by [³H] thymidine incorporation assay. (B) D4 HSCs were stimulated with IFN- γ (5 ng/mL) for 6 or 12 hours. Caspase-3 activity was measured. (C–E) D4 HSCs were stimulated with IFN- γ for 30 minutes, and STAT1 activation was detected by immunostaining with anti-pSTAT1 antibody (C) or Western blotting (D). The densities of bands in *panel D* were quantified and shown in *panel E*. In *panel F*, HSCs isolated from 8-week pair- and ethanol-fed mice were cultured for 4 or 7 days. The SOCS1 protein was then examined by Western blotting. Values in *panels A, B, and E* are means $\pm$ SEMs from 3 to 4 independent experiments. * P < .05, ** P < .01 vs corresponding pair-fed groups.

Figure 6.

Coculture of HSCs with hepatocytes from ethanol-fed mice attenuates IFN- γ /STAT1 signaling in HSCs. (A) D4 HSCs were cocultured with hepatocytes from pair- or ethanol-fed mice for 24 hours and subsequent treatment with IFN- γ (5 ng/mL) for 30 minutes, followed by immunostaining with anti-pSTAT1 antibody. (B) D4 HSCs and hepatocytes were cocultured with or without antioxidants for 24 hours, then treated with IFN- γ (5 ng/mL) for 30 minutes, followed by Western blot analyses. The densities of bands were quantified and shown in the right panel. Values represent means $\pm$ SEMs from 4 independent experiments. *** P < .001 vs ethanol group without antioxidants. (C and D) D4 HSCs were incubated with or without 0.5 mmol/L H₂O₂ for 5 minutes, then stimulated with IFN- γ (5 ng/mL) for 30 minutes, followed by immunostaining with anti-pSTAT1 antibody (C) or RT-PCR analyses of IRF-1 expression (D). (E and F) D4 HSCs were cocultured with hepatocytes from pair- or ethanol-fed mice for 24 hours, then treated with TGF- β 1 (2.5 ng/mL for 45 minutes) or IFN- γ + TGF- β 1 (pretreatment with 50 ng/mL IFN- γ for 45 minutes first then 2.5 ng/mL TGF- β 1 for an additional 45 minutes), followed by immunostaining with anti-pSmad3 antibody (E) or RT-PCR analyses of COL1A2 expression (F). The densities of bands in panel F were quantified and shown in the upper panel. Values represent means $\pm$ SEMs from 5 independent experiments. *** P < .001 vs the corresponding IFN- γ (-)/TGF- β (+) group. (A, C, and E, black arrow shows positive reaction and blank arrow shows very weak reaction). (Pair-Hep: pair-fed hepatocytes; EtOH-Hep: ethanol-fed hepatocytes).

Figure 7.

HSCs from ethanol-fed mice are resistant to NK cell killing by a TGF-β1– dependent mechanism. (A) D4 HSCs from pair- or ethanol-fed mice were used as target cells, and liver MNCs from poly I:C-treated normal mice were used as effector cells. The cytotoxicity of liver MNCs against D4 HSCs was measured. Values are means ± SEMs from 3 independent experiments. ** $P < .01$ vs corresponding pair-fed groups. (B) HSCs from pair- or ethanol-fed mice were cultured for various days and then subject to RT-PCR analyses. (C) D4 HSCs from pair- or ethanol-fed mice were incubated with murine recombinant TGF-β1 protein or TGF-β1 antibody for 20 minutes, then cytotoxicity assays similar to *panel A* were performed. Values are means ± SEMs from 6 independent experiments. * $P < .05$; ** $P < .01$ vs corresponding 0 groups. (D) Poly I:C treated MNCs were incubated with or without TGF-β1 for 1 hour, then expression of NKG2D, TRAIL, or IFN-γ were measured by RT-PCR analyses. (P, pair-fed; Et, EtOH-fed).

Figure 8.

A model for chronic alcohol acceleration of liver fibrosis by inhibition of NK cell killing of HSCs and suppression of IFN- γ signaling in HSCs. (1) Ethanol directly inhibits NK cytotoxicity against HSCs by down-regulating expression of NKG2D (NK cell activating receptor), TRAIL, and IFN- γ . (2) Ethanol stimulates HSCs to produce TGF- β , followed by inhibiting NK cell killing of HSCs; (3) ethanol induces expression of SOCS1 protein, followed by inhibiting IFN- γ signaling in HSCs; (4) ethanol stimulates hepatocytes to produce oxidative stress, which subsequently inhibits IFN- γ signaling in HSCs.