

## Chronic alcohol consumption accelerates liver injury in T cell-mediated hepatitis: alcohol disregulation of NF- $\kappa$ B and STAT3 signaling pathways

Barbara Jaruga,<sup>1</sup> Feng Hong,<sup>1</sup> Won-Ho Kim,<sup>1</sup> Rui Sun,<sup>1</sup> Saijun Fan,<sup>2</sup> and Bin Gao<sup>1</sup>

<sup>1</sup>Section on Liver Biology, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892; <sup>2</sup>Laboratory of Molecular Oncology, Georgetown University Medical Center, Washington, District of Columbia 20007

Submitted 13 January 2004; accepted in final form 5 March 2004

**Jaruga, Barbara, Feng Hong, Won-Ho Kim, Rui Sun, Saijun Fan, and Bin Gao.** Chronic alcohol consumption accelerates liver injury in T cell-mediated hepatitis: alcohol disregulation of NF- $\kappa$ B and STAT3 signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 287: G471–G479, 2004. First published April 2, 2004; 10.1152/ajpgi.00018.2004.—Alcohol consumption is a major risk factor accelerating the progression of liver disease in patients with chronic hepatitis virus infection. However, the mechanism underlying the enhanced susceptibility of alcoholics to liver injury is not fully understood. Here, we demonstrate that chronic ethanol consumption increases the susceptibility of C57BL/6 mice to concanavalin A (Con A)-induced T cell-mediated hepatitis. Injection of a low dose of Con A (5  $\mu$ g/g) causes severe liver damage in ethanol-fed mice as evidenced by a significant elevation of serum alanine aminotransferase levels, massive necrosis, and infiltration of leukocytes but only slightly induces liver injury in control pair-fed mice. In ethanol-fed mice, the activation and cytotoxicity of natural killer T cells, cells that play key roles in Con A-induced T cell hepatitis, are not significantly enhanced relative to pair-fed mice. Moreover, Con A-induced activation of hepatic NF- $\kappa$ B is increased, whereas activation of STAT1 and STAT3 is attenuated in ethanol-fed mice. Consistent with this result, the expression of chemokines and adhesion molecules [such as ICAM-1, macrophage inflammatory protein (MIP)-1, MIP-2, and MCP-1] controlled by NF- $\kappa$ B is upregulated, whereas STAT1-controlled expression of chemokines (such as MIG and IP-10) is downregulated in ethanol-fed mice compared with pair-fed mice. In conclusion, chronic alcohol consumption accelerates T cell-mediated hepatitis via upregulation of the NF- $\kappa$ B signaling pathway and subsequently enhances expression of chemokines/adhesive molecules and recruitment of leukocytes into the liver. Downregulation of the antiapoptotic STAT3 signal may also contribute to alcohol potentiation of T cell hepatitis.

alcoholic liver injury; concanavalin A; nuclear factor- $\kappa$ B; STAT3

WORLDWIDE, ALCOHOL CONSUMPTION is a major cause of chronic liver disease. Interestingly, only ~15–20% heavy drinkers develop alcoholic liver injury, suggesting that alcohol is a cofactor for developing chronic liver disease (14, 17). Accumulating evidence indicates that many genetic and acquired factors are implicated in the susceptibility of individuals to alcohol-induced liver injury (5, 12, 14, 27, 30). These factors include chronic viral infection, nutritional factors, dose and duration of alcohol consumption, drinking patterns, age when drinking began, genetic polymorphisms of cytokines and alcohol-metabolizing enzymes, gender, histocompatibility antigens, immunological factors, genetic predisposition to alcohol addiction, and hepatic iron overload (2, 5, 12–14, 27, 30, 35,

39). In rodents, intragastric infusion of ethanol for 4–5 wk leads to steatosis, inflammation, and, to a lesser extent, fibrosis in the liver, whereas feeding Lieber-DeCarli liquid diet containing ethanol does not cause significant liver injury except steatosis in rats (15, 16, 28) but enhances endotoxin (26)- and galactosamine (41)-induced liver injury in mice and concanavalin A (Con A)- and endotoxin-liver injury in rats (6, 7). Here, we demonstrate that ethanol consumption significantly enhanced T cell-mediated hepatitis induced by Con A in mice. A low-dose injection of Con A induced significant elevations in serum transaminase levels, hepatic inflammation, and necrosis in ethanol-fed mice but not in pair-fed mice.

Con A injection has been widely used to study T cell-mediated hepatitis, whose manifestation closely resembles the pathology of a variety of human liver disorder (47). Accumulating evidence suggests that activation of a variety of cytokines and their downstream signals plays an important role in the development and progression of T cell-mediated liver injury in this model (46). TNF- $\alpha$  activation of the NF- $\kappa$ B signal (48, 50), IFN- $\gamma$  activation of signal transducer, and activator transcription factor 1 (STAT1) (21) and IL-4 activation of STAT4 (22) have all been shown to play crucial roles in the development of liver injury in Con A-induced hepatitis. In contrast, IL-6 activation of STAT3 is protective against liver injury (21). After activation of various signals, a wide variety of chemokines and adhesion molecules are induced and consequently attract infiltrating granulocytes, resulting in massive inflammation and necrosis (4, 22, 43). Recently, it has been shown that natural killer T (NKT) cells are essential for Con A-induced T cell hepatitis via expression of Fas ligand (FasL), which directly kills hepatocytes (45). To understand the molecular mechanism by which alcohol consumption enhances Con A-induced hepatitis, we compared Con A injection-induced activation of NKT cells, signaling pathways, and the expression of various chemokines and adhesion molecules in pair- and ethanol-fed mice. Our results show that in ethanol-fed mice, Con A-induced activation of STAT1 and STAT3 signaling pathways is inhibited, whereas activation of the NF- $\kappa$ B signaling pathway is enhanced compared with pair-fed mice. Consistent with the observed upregulation in NF- $\kappa$ B activation, the expression of various chemokines and adhesion molecules controlled by NF- $\kappa$ B is enhanced in ethanol-fed mice, which may be an important mechanism contributing to the increase in susceptibility of ethanol-fed mice to T cell-mediated hepatitis induced by Con A.

Address for reprint requests and other correspondence: B. Gao, Section on Liver Biology, NIAAA/NIH, Park Bldg. Rm. 120, 12420 Parklawn Dr., MSC 8115, Bethesda, MD 20892 (E-mail: bgao@mail.nih.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

## MATERIALS AND METHODS

**Materials.** Anti-STAT1, anti-phospho-STAT1 (Tyr<sup>701</sup>), anti-phospho-STAT3 (Tyr<sup>705</sup>), anti-STAT3, anti-phospho-ERK1/2, anti-I $\kappa$ B, and anti-phospho-I $\kappa$ B antibodies were obtained from Cell Signaling Technology (Beverly, MA).

**Mouse models of chronic ethanol consumption and Con A-induced hepatitis.** Male C57BL/6 mice weighing 20–25 g were fed a nutritionally adequate liquid diet containing 5% ethanol or a control diet in which ethanol was substituted isocalorically with dextrin maltose (BioServ, Frenchtown, NJ). Both diets were dispensed in glass liquid-diet feeding tubes (BioServ). Ethanol was introduced gradually by increasing the content by 1% (vol/vol) every 2 days until the mice were consuming diets containing 5% (vol/vol) ethanol for 2 or 4 wk. Subsequently, pair- and ethanol-fed mice were injected intravenously with Con A. After various time periods, mice were killed and liver injury was determined.

Initially, male C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME) were used in our studies. However, the ethanol-fed C57BL/6J mice gained less weight than pair-fed mice, and there was 15–20% mortality in the ethanol-fed group during the 4-wk feeding period. Similar mortality in ethanol-fed C57BL/6J mice was also reported previously (26). Although there was 15–20% mortality in the ethanol-fed C57BL/6J mice during the 4-wk feeding period, the surviving mice after 2- or 4-wk feeding with ethanol were more susceptible to Con A-induced liver injury compared with pair-fed mice (data not shown). Then we tested this model in male C57BL/6N mice from the National Cancer Institute (NCI) (Frederick, MD). Interestingly, ethanol-fed male C57BL/6N mice and pair-fed mice gained weight similarly, and none of them died during the 4-wk feeding period. Therefore, the data from the study of male C57BL/6N mice (NCI) were reported in this paper.

**Isolation and culture of primary mouse hepatocytes.** 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 subsequently perfused with an EGTA solution (in mM: 5.4 KCl, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 140 NaCl, 0.34 Na<sub>2</sub>HPO<sub>4</sub>, 0.5 EGTA, and 25 Tricine, pH 7.2) and DMEM (GIBCO-BRL, Gaithersburg, MD) and digested with 0.075% collagenase solution. The isolated mouse hepatocytes were then cultured in Hepato-ZYME-SFM media (GIBCO-BRL) on rat tail collagen-coated plates for 24 h, then in serum-free DMEM, and they were used to assay for NKT cytotoxicity.

**Western blot analysis.** Liver tissue extracts were prepared by homogenation in lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol) at 4°C, followed by centrifugation at 16,000 rpm at 4°C for 10 min. After protein concentrations were quantified, the supernatants were mixed in Laemmli loading buffer, boiled for 4 min, and then subjected to Western blot analysis. Membranes were blotted against primary antibodies for 16 h, washed with 0.05% (vol/vol) Tween 20 in PBS (pH 7.4), and incubated with a 1:4,000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by the enhanced chemiluminescence reaction method (Amersham Pharmacia Biotech, Piscataway, NJ).

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

**Analysis of alanine aminotransferase and aspartate aminotransferase activities.** Liver injury was quantified by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a kit purchased from DREW Scientific (Cumbria, UK).

**Immunohistochemistry.** Neutrophils and macrophages in the liver were detected immunohistochemically using, respectively, anti-neutrophil MPO antibody (Lab Vision, Fremont, CA) and anti-F4/80 antibody (Novus Biologicals, Cambridge, UK) as described previ-

ously (22). The numbers of neutrophils in the liver were counted in 10 randomly chosen visual fields (magnification,  $\times 200$ ), and the average of 10 selected microscopic fields was calculated.

**Assay of hepatic eosinophil peroxidase activity.** Hepatic eosinophil peroxidase (EPO) activity was measured as described previously (40). EPO enzymatic activities in hepatic tissues were calculated by subtracting the mean background absorbance and are expressed as a change at 490 nm absorbance.

## RT-PCR

RT-PCR analysis and the sequences of the primers used in the study were described previously (22, 36). The  $\beta$ -actin gene was amplified and used as an internal control. The PCR bands were scanned using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**EMSA.** EMSAs were performed in 20- $\mu$ l volumes with 20 mM Tris·HCl, pH 7.9, 1.5% glycerol, 50  $\mu$ g/ml BSA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g poly(dI-dC), 1 ng <sup>32</sup>P-labeled probe, and 10  $\mu$ g nuclear extract. Reactions were incubated at 25°C for 20 min and subsequently analyzed by electrophoresis through nondenaturing stock polyacrylamide gels (6 or 10%) in 0.5  $\times$  TBE buffer containing 44.5 mM Tris·HCl, pH 8.2, 44.5 mM boric acid, and 1 mM EDTA. After the gel was prerun at 100 V for 2 h, electrophoresis was performed at 270 V for 2 h at 4°C. The gels were then analyzed using the PhosphorImager ImageQuant program (Molecular Dynamics). The NF- $\kappa$ B binding site of the double-stranded oligonucleotide 5'-AGT TGA GGG GAC TTT CCC AGG-3' was used as a probe to determine NF- $\kappa$ B binding.

**Isolation of liver mononuclear cells and adoptive transfer.** Pair- and ethanol-fed mouse livers were removed and pressed through a 200-gauge stainless steel mesh. The liver cell suspension was collected and suspended in RPMI 1640 media (GIBCO-BRL). After centrifugation at 50 g for 5 min, mononuclear cells (MNC supernatant) were separated from parenchymal cells (pellet). MNCs were then washed in PBS and resuspended in 40% Percoll (Sigma) in RPMI 1640. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 750 g. MNCs were collected from the interphase, washed twice in PBS, and resuspended in RPMI 1640.

Adoptive transfer of hepatic MNCs into the liver was performed as described previously (22).

**Cytotoxicity assay.** The cytotoxicity of hepatic MNCs against primary mouse hepatocytes was measured using a 4-h AST release assay as described previously (22). Briefly, mice were treated with Con A for 2 h, then hepatic MNCs were isolated and subsequently added to cultured mouse hepatocytes at the indicated effector-to-target (E/T) ratios. After 4 h, the supernatant was harvested and AST activity was measured. The specific cytotoxicity was calculated as  $\text{AST}_{\text{experimental}} - \text{AST}_{\text{spontaneous}} / \text{AST}_{\text{maximum}} - \text{AST}_{\text{spontaneous}} \times 100\%$ .

**Flow cytometric analysis of NKT cell activation in the liver after administration of Con A.** To compare activation of NKT cells in pair- and ethanol-fed mice, hepatic MNCs was stained with anti-NK1.1, anti-CD3, and anti-Fas antibodies (PharMingen, San Diego, CA) and analyzed through a fluorescent-activated cell sorter (FACScalibur, Becton Dickinson, Mountain View, CA).

**Statistical analysis.** For comparing values obtained in three or more groups, one-factor analysis of variance was used followed by Tukey's post hoc test. Statistical significance was taken at the  $P < 0.05$  level.

## RESULTS

**Chronic ethanol consumption potentiates T cell-mediated hepatitis induced by Con A.** As shown in Fig. 1, injection of a low dose of Con A (5  $\mu$ g/g) failed to induce significant liver injury in 2- or 4-wk pair-fed groups (Fig. 1, A and B). However, the same treatment caused severe liver damage in both 2- and 4-wk ethanol-fed groups, as evidenced by signif-

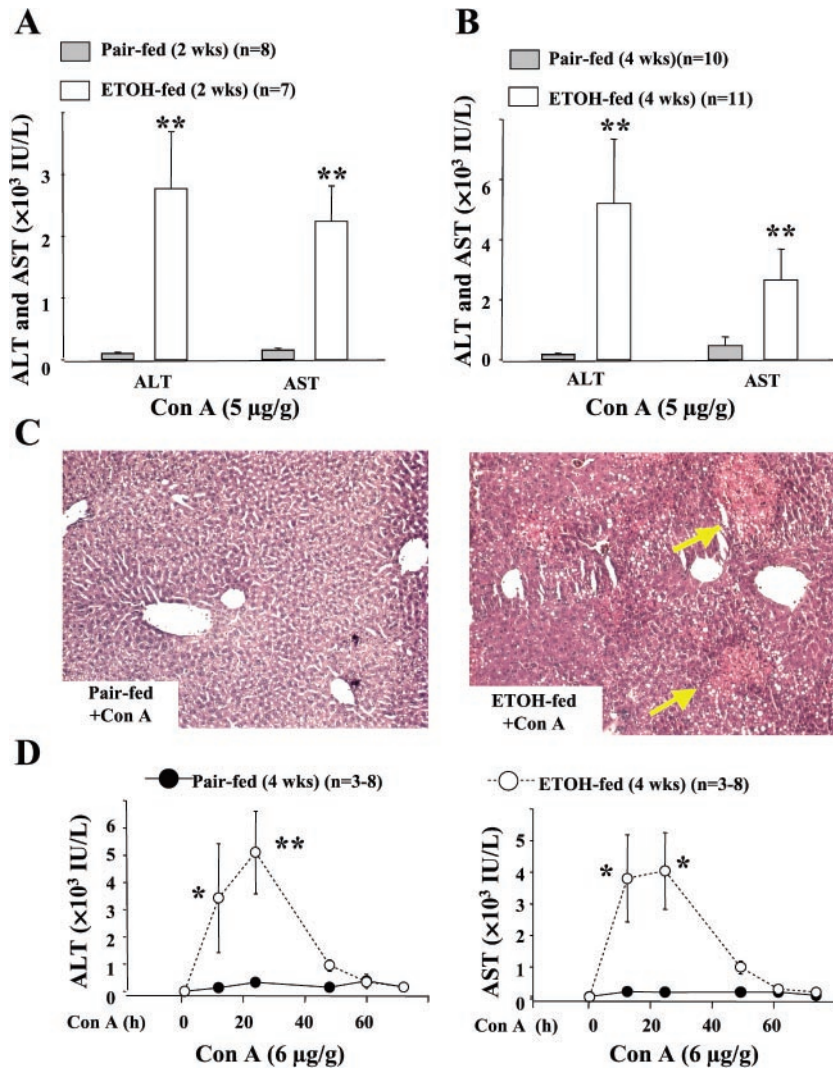


Fig. 1. Chronic ethanol (ETOH) consumption potentiates conconavalin A (Con A)-induced T cell-mediated hepatitis. A and B: 2-wk pair- and ETOH-fed mice (A), 4-wk pair- and ETOH-fed mice (B) were injected with Con A ( $5 \mu\text{g/g}$ ). After 12 h, serum were collected for alanine aminotranferase (ALT) and aspartate aminotranferase (AST) assay. C: livers collected from A were stained with hematoxylin and eosin. Yellow arrows indicate massive necrosis observed in the liver. D: 4-wk pair- and ETOH-fed mice were injected with Con A ( $6 \mu\text{g/g}$ ). After various time periods, serum were collected for ALT and AST assays. Values in A, B and D represent means  $\pm$  SE from 3–11 mice at each time point. Significant difference from the corresponding pair-fed group is indicated by \* ( $P < 0.05$ , \*\* $P < 0.01$ ).

icant elevations in serum ALT and AST levels (Fig. 1, A and B) and massive necrosis in the liver (Fig. 1C). Similarly, injection of Con A ( $6 \mu\text{g/g}$ ) did not induce significant liver injury in 4-wk pair-fed mice but significantly elevated serum ALT and AST levels in 4-wk ethanol-fed mice (Fig. 1D).

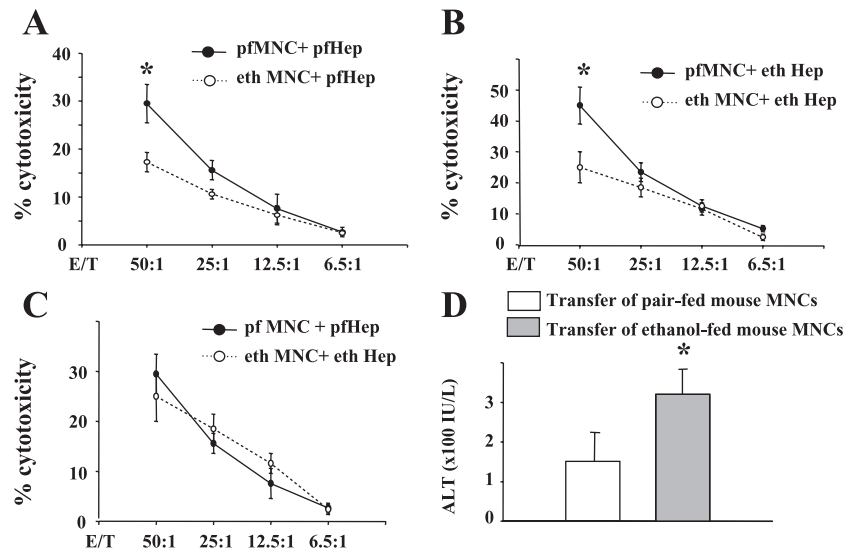
*Cytotoxicity of hepatic MNCs against primary hepatocytes is not enhanced in ethanol-fed mice.* The cytotoxicity of hepatic NKT cells against hepatocytes has been suggested to play an important role in Con A-induced liver injury (45). Therefore, we examined whether the cytotoxicity of hepatic MNCs against hepatocytes was enhanced in ethanol-fed mice as a potential mechanism for alcohol potentiation of T cell-mediated hepatitis. Three different groups of effectors/targets were compared. As shown in Fig. 2, MNCs from Con A-treated pair-fed mice, compared with MNCs from Con A-treated ethanol-fed mice, showed significantly higher cytotoxicity against both pair-fed (Fig. 2A) and ethanol-fed mouse hepatocytes (B) at a 50:1 E/T ratio. This indicates that the cytotoxicity of hepatic MNCs from ethanol-fed mice was reduced compared with MNCs from pair-fed mice. Figure 2, A and B, also showed that hepatocytes from ethanol-fed mice were more susceptible to MNC killing than from pair-fed mice. As shown in Fig. 2, A and B, pair-fed MNCs were cytotoxic against 45%

of hepatocytes from ethanol-fed mice compared with only 29% of pair-fed mouse hepatocytes. Similarly, ethanol-fed liver MNCs caused cytotoxicity in 25% of ethanol-fed mouse hepatocytes and only 17% of pair-fed mouse hepatocytes. In Fig. 2C, the cytotoxicity of pair-fed mouse MNCs against pair-fed mouse hepatocytes and ethanol-fed mouse MNCs against ethanol-fed mouse hepatocytes was compared, indicating that the cytotoxicity between these two groups of effectors/targets was comparable. Taken together, these findings show that increased hepatic MNC cytotoxicity is not likely responsible for ethanol-induced potentiation of T cell-mediated hepatitis.

Next, the activity of liver MNCs from pair- and ethanol-fed mice via adoptive transfer was compared. As shown in Fig. 2D, adoptive transfer of liver MNCs from ethanol-fed mice induced higher levels of ALT elevation after Con A injection relative to adoptive transfer of pair-fed mouse liver MNCs. These findings suggest that chronic ethanol consumption enhances the sensitivity of liver MNCs to the effects of Con A administration ( $P < 0.05$ ).

*Effects of chronic ethanol consumption on NKT cells in Con A-induced hepatitis.* Activation of NKT cells has been suggested to play an important role in Con A-induced liver injury (45). We wished to examine Con A activation of NKT cells in

Fig. 2. Effects of chronic ETOH consumption on the cytotoxicity of hepatic lymphocytes against primary hepatocytes. *A*, *B* and *C*: hepatic mononuclear cells (MNCs) were isolated from pair-fed (pfMNC) or ETOH-fed (ethMNC), and their cytotoxicity was tested against pair-fed mouse hepatocytes (pfHep) or ETOH-fed mouse hepatocytes (ethHep) at the indicated effector-to-target (E/T) ratios as described in MATERIALS AND METHODS. Values are shown as means  $\pm$  SE from 3 mice at each time point. \* $P < 0.05$  compared with corresponding ETOH. MNC groups. *D*: adoptive transfer of ETOH-fed mouse MNCs potentiates Con A-induced liver injury compared with transfer of pair-fed mouse MNCs. Total hepatic MNCs ( $5 \times 10^6$  cells) isolated from pair- or ETOH-fed mice were injected into the livers of C57BL/6 mice, followed by intravenous injection of Con A ( $5 \mu\text{g/g}$ ). Serum ALT levels were measured 9 h later. Values are shown as means  $\pm$  SE from 3 independent experiments. \* $P < 0.05$  compared with the group transferred with pair-fed mouse MNCs.



pair- and ethanol-fed mice. As shown in Fig. 3, *A* and *B*, lymphocytes from pair-fed C57BL/6 mouse livers contained  $\sim 31.56\%$  NKT cells (gate R2, *top-right* quadrant), similar to levels found in normal C57BL/6 mice. The percentage of NKT cells was slightly but not significantly elevated in ethanol-fed C57BL/6 mouse livers compared with pair-fed mice. The total number of NKT cells was similar in ethanol- and pair-fed mice due to the comparable overall number of hepatic lymphocytes between these two groups (data not shown). After Con A was injected, the percentage and number of NKT cells similarly declined in both groups of pair- and ethanol-fed mice (Fig. 3, *A* and *B*).

Next we examined the expression of FasL on NKT cells, which has been implicated in Con A-induced T cell hepatitis (45). As shown in Fig. 3C, 3.83% FasL-positive cells (gate R2, *right panel*) were detected in pair-fed mouse liver lymphocytes, which was comparable with 2.78% in ethanol-fed group. After injection of Con A, FasL expression was rapidly elevated in pair-fed mice and reached to 22.90% 12 h postinjection. FasL expression was also increased in ethanol-fed mice after injection of Con A but was not less evident compared with pair-fed group. These results are consistent with the findings shown above demonstrating a decrease in cytotoxicity from hepatic MNCs in ethanol-fed mice (Fig. 2).

**Effects of chronic ethanol consumption on infiltration of leukocytes in T Cell-mediated hepatitis.** To further understand the mechanism responsible for ethanol potentiation of Con A-induced hepatitis, infiltration of leukocytes into the liver was compared between pair- and ethanol-fed mice. As shown in Fig. 4, significant numbers of Kupffer cells were detected in normal livers. Previously, it was shown (22) that injection of a high dose of Con A ( $16 \mu\text{g/g}$ ) rapidly and markedly caused Kupffer cell death. Injection of a low dose of Con A ( $5 \mu\text{g/g}$ ) failed to cause Kupffer cell death in pair-fed mice (Fig. 4A). In contrast, the same treatment caused massive Kupffer cell death in ethanol-fed mice (Fig. 4A).

Before Con A stimulation, there were no significant neutrophils detected in the livers of pair- and ethanol-fed mice (Con A, 0 h in Fig. 4B). Con A administration caused significant infiltration of neutrophils in the livers 12 h postinjection. Such infiltration was significantly higher in ethanol-fed mice compared with pair-fed

mice (Fig. 4B;  $P < 0.05$ ). Injection of Con A also caused significant elevation of hepatic EPO activity (Fig. 4C), suggesting eosinophils infiltrate the livers. Con A injection-induced elevation of hepatic EPO activity was much higher in ethanol-fed mice than that in pair-fed mice ( $P < 0.05$ ; Fig. 4C).

**Effects of chronic ethanol consumption on multiple signals in T cell-mediated hepatitis.** The activation of multiple signals has been implicated in the development and progression of T cell-mediated hepatitis (21, 22, 48, 50). Therefore, Con A-mediated activation of these signals between pair- and ethanol-fed mice was compared. As shown in Fig. 5A, injection of Con A induces activation of STAT1 and STAT3 in the livers of pair-fed mice, as evidenced by the detection of phosphorylated STAT1 and STAT3. Such activation was markedly suppressed in ethanol-fed mice. STAT1 protein expression was also induced after injection of Con A, which was less evident in the ethanol-fed group relative to the pair-fed group. Similar STAT3 protein expression was detected in both Con A-treated pair- and ethanol-fed groups. Furthermore, Con A injection also caused activation of ERK1/2 in the liver; such activation was significantly enhanced in the ethanol-fed group compared with the pair-fed group (Fig. 5A).

Next, NF- $\kappa$ B activation was determined by using gel mobility shift assay. As shown in Fig. 5B, Con A injection was shown to induce NF- $\kappa$ B binding in pair-fed mice, with peak effect occurring at 1 h, whereas significantly higher and prolonged NF- $\kappa$ B binding was observed in ethanol-fed mice. Consistent with the results of the gel mobility shift assay, Western blot analysis showed that I $\kappa$ B was rapidly phosphorylated after injection of Con A in pair-fed mice, and this phosphorylation was much higher and prolonged in ethanol-fed mice (Fig. 5C). The I $\kappa$ B protein was rapidly degraded 1 h after Con A injection and recovered to normal levels at 3 h in pair-fed mice (Fig. 5C). In ethanol-fed mice, injection of Con A also caused rapid degradation of I- $\kappa$ B protein at 1 h, but the I- $\kappa$ B protein was not completely recovered until 12 h later (Fig. 5C).

**Effects of chronic ethanol consumption on expression of chemokines and adhesion molecules in T cell-mediated hepatitis.** The above data demonstrate that ethanol consumption regulates activation of various signaling pathways during T cell-mediated hepatitis. Next, we examined whether Con

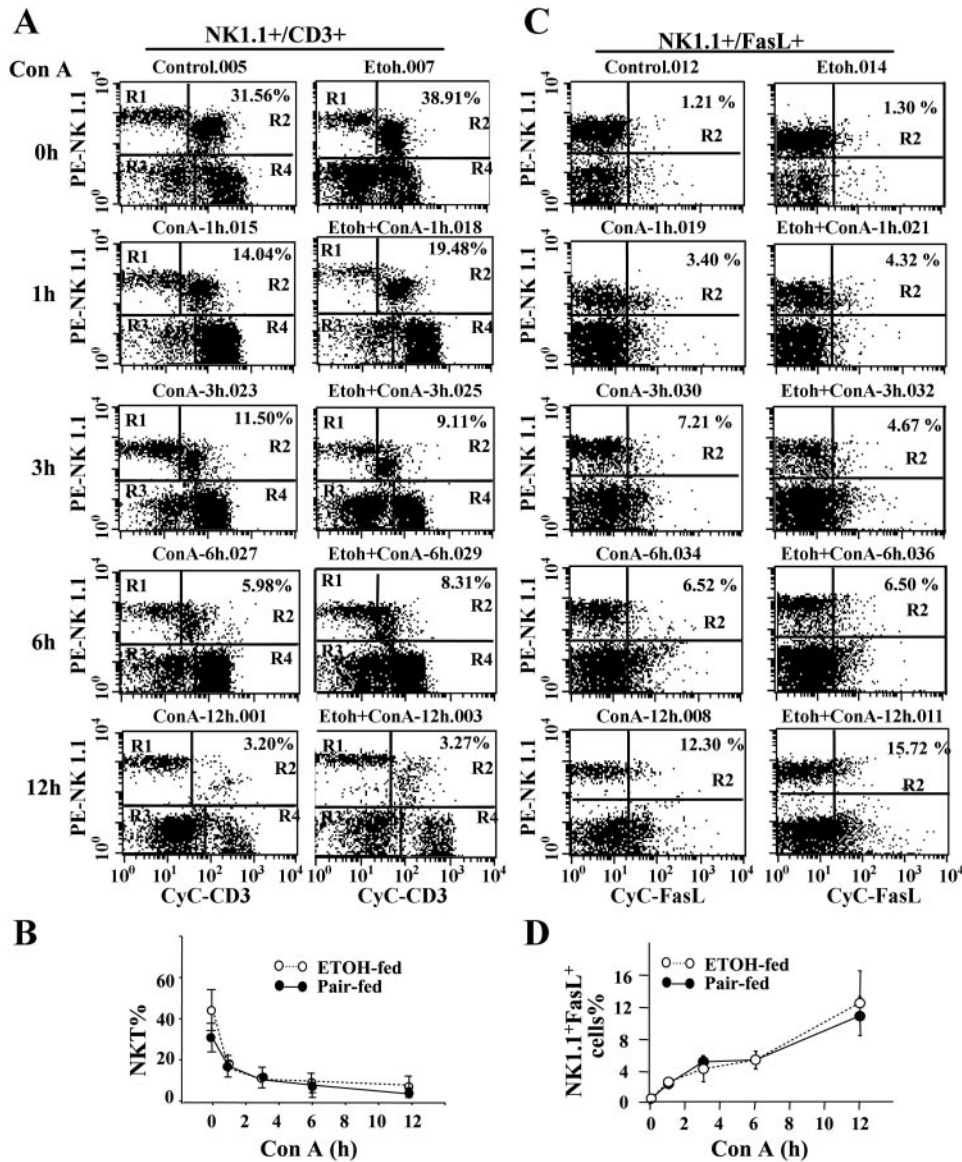


Fig. 3. Effects of chronic ETOH consumption on activation of natural killer T (NKT) cells and expression of Fas ligand (FasL) on NKT cells after administration of Con A. Pair- and ETOH-fed mice were injected with Con A (5  $\mu$ g/g). After 3, 6, or 9 h, hepatic lymphocytes were isolated. The surface expression of NK1.1<sup>+</sup>CD3<sup>+</sup> (A) and NK1.1<sup>+</sup>FasL<sup>+</sup> (C) was analyzed by flow cytometry. A representative example of 3 independent experiments is shown. The *top right* quadrant (gate R2) in A shows CD3<sup>+</sup>NK1.1<sup>+</sup> double-positive cells (% of the total hepatic MNCs), which was summarized in B. The *right panel* in C (gate R2) shows FasL<sup>+</sup> positive cells (% of the total hepatic MNCs), which was summarized in D. Values in B and D are shown as means  $\pm$  SE from 3 mice at each time point. \* $P < 0.01$  compared with corresponding pair-fed groups.

A-induced expression of various chemokines and adhesion molecules, which are controlled by these signaling pathways, is regulated in ethanol-fed mice compared with pair-fed mice. As shown in Fig. 6A, Con A injection induced expression of various chemokines and adhesion molecules in the liver, with peak effect between 1 and 6 h. Compared with pair-fed mice, Con A induction of VCAM-1, ICAM-1, Eotaxin-1, Eotaxin-2, macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, and MCP-1 in ethanol-fed mice was prolonged, whereas induction of MIG and IP-10 was decreased. To further confirm the prolonged induction of these chemokines and adhesive molecules, we compared and quantified their expression at the 12-h point after Con A injection. As shown in Fig. 6, B and C, induction of ICAM-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, and MCP-1 was significantly potentiated in ethanol-fed mice compared with that in pair-fed mice 12 h after injection of Con A, whereas induction of MIG was decreased.

## DISCUSSION

In this paper, we demonstrated that chronic ethanol consumption markedly potentiates T cell-mediated hepatitis induced by injection of Con A in mice. Furthermore, it was determined that ethanol potentiation of T cell-mediated hepatitis is due mainly to enhancing the NF- $\kappa$ B signaling pathway and the subsequent induction of NF- $\kappa$ B-controlled chemokines and adhesion molecule expression in the liver. As shown in Fig. 5, B and C, NF- $\kappa$ B binding, I $\kappa$ B phosphorylation, and I $\kappa$ B degradation after Con A injection were significantly enhanced in ethanol-fed mice compared with pair-fed mice. Consistent with this finding, ICAM-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, and MCP-1 expression, which are controlled by NF- $\kappa$ B (11, 18, 37), are significantly prolonged in ethanol-fed mice after Con A injection compared with pair-fed mice. It has been reported (31, 32) that ethanol consumption causes activation of NF- $\kappa$ B and subsequent induction of chemokines and adhesion mole-

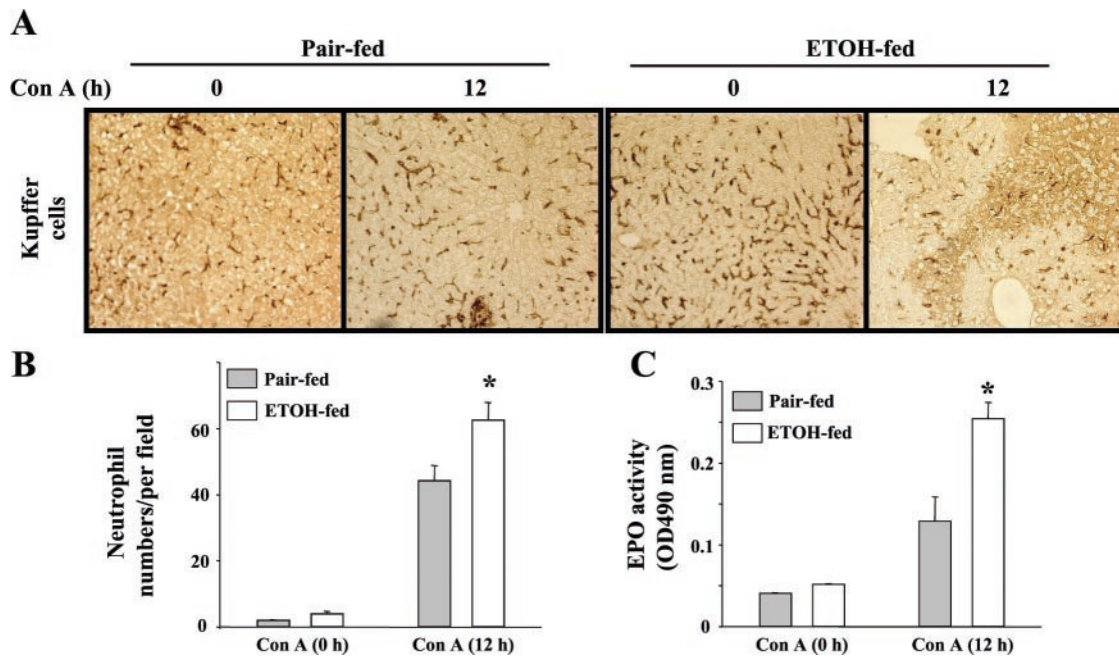
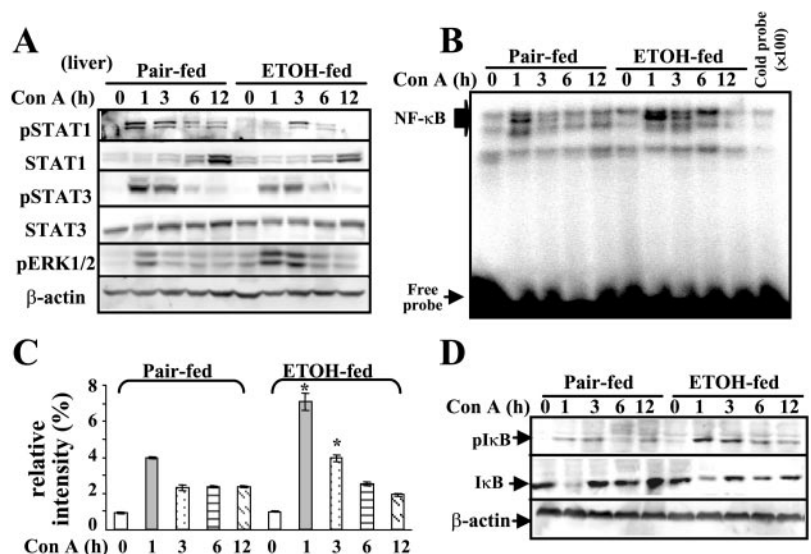


Fig. 4. ETOH consumption potentiates Con A injection-mediated infiltration of neutrophils and eosinophils in the liver. *A*: pair- and ETOH-fed mice were injected with Con A ( $5 \mu\text{g/g}$ ) for 12 h. Liver sections were prepared and subjected to immunohistochemistry using anti-F4/80 antibodies to detect macrophages. *B*: liver sections in *A* were also immunostained with anti-MPO antibody to detect neutrophils. Ten fields were randomly selected, and positive cells were counted. Values are shown as means  $\pm$  SE from 5 mice at each time point. \* $P < 0.05$  compared with corresponding pair-fed groups. *C*: pair- and ETOH-fed mice were injected with Con A ( $5 \mu\text{g/g}$ ) for 12 h. Hepatic eosinophil peroxidase activities were measured. Values are shown as means  $\pm$  SE from 5 mice at each time point. \* $P < 0.01$  compared with pair-fed group.

cles in rodent livers as well as in monocytes from human patients with alcoholic hepatitis. For example, Nanji et al. (33) reported that intragastric feeding of an ethanol diet with fish oil or corn oil induced significant activation of NF- $\kappa$ B and induction of regulated on activation normal T-expressed and presumably secreted, MCP-1, MIP-1 $\alpha$ , MIP-2, IP-10, cytokine-induced neutrophil chemoattractant, and ENA-78 in the livers of rats compared with feeding rats with an ethanol diet containing saturated fatty acids. The activation of NF- $\kappa$ B and induction of chemokines and adhesion molecules strongly correlated with liver inflammation and injury in this intragas-

tric model (33). The activation of NF- $\kappa$ B in monocytes was also reported in human alcoholic liver disease (20). Additionally, the critical role of NF- $\kappa$ B activation in alcoholic liver injury has been clearly demonstrated through delivery of the adenoviral I $\kappa$ B super-repressor gene, which reduced early alcohol-induced liver injury in rats (49). However, the mechanisms underlying ethanol-mediated activation of NF- $\kappa$ B are not fully understood. Recent data (31, 44) show that alcohol exposure elevates TNF- $\alpha$  levels, which are mediated via multiple mechanisms including potentiation of LPS-activated ERK1/2-Egr-1 signal pathway, stabilization of TNF- $\alpha$  mRNA

Fig. 5. Chronic ETOH consumption dysregulates STATs and NF- $\kappa$ B signaling pathways in T cell-mediated hepatitis induced by Con A. *A*: pair- and ETOH-fed mice were injected with Con A ( $5 \mu\text{g/g}$ ) at various time points; total liver protein extracts were immunoblotted using various antibodies as indicated. *B* and *C*: activation of NF- $\kappa$ B was measured by using gel mobility shift analysis of NF- $\kappa$ B binding (*B*) and Western blot analysis of I $\kappa$ B phosphorylation (pI- $\kappa$ B) and I $\kappa$ B degradation (*C*). In *B*, 100-fold cold NF- $\kappa$  probe was included in the gel shift binding assay. Data in *A-C* are representatives of 3 independent experiments with similar results.



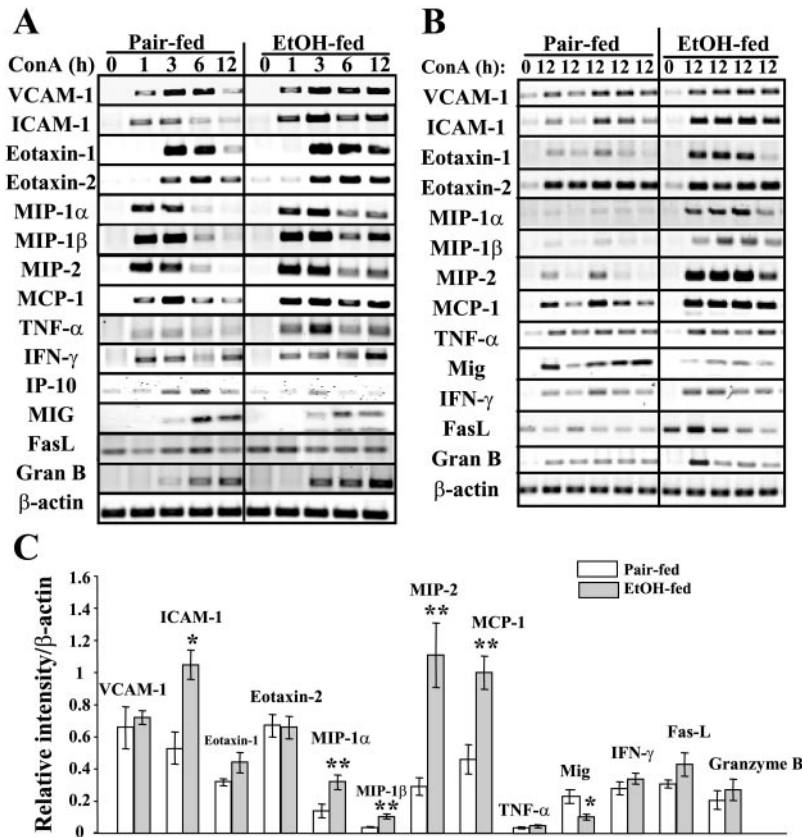


Fig. 6. Effects of ETOH feeding on expression of chemokines, cytokines, and adhesion molecules in T cell-mediated hepatitis induced by Con A. **A**: pair- and ETOH-fed mice were injected with Con A (5  $\mu$ g/g) for various time points. Total RNA samples were prepared and subjected to RT-PCR using various primers as indicated. **B**: pair-fed mice ( $n = 5$ ) and ETOH-fed mice ( $n = 4$ ) were injected with Con A (5  $\mu$ g/g) for 12 h. Total RNA samples were prepared and subjected to RT-PCR using various primers as indicated. **C**: the band densities in **B** blots were quantified, and the relative intensity to  $\beta$ -actin is shown. Values are shown as means  $\pm$  SE from 5 mice in the pair-fed group and 4 mice in ETOH-fed group. \* $P < 0.05$ , \*\* $P < 0.01$  compared with pair-fed groups. MIP, macrophage inflammatory protein.

(23, 24, 42), and induction of oxidative stress (1, 51). Hence, elevated levels of TNF- $\alpha$  could be an important mechanism contributing to NF- $\kappa$ B activation. Here, we showed that expression of TNF- $\alpha$  mRNA was significantly higher 3 h after injection of Con A in ethanol-fed mice compared with pair-fed mice (Fig. 6A), suggesting that ethanol potentiated NF- $\kappa$ B activation in T cell-mediated hepatitis, partly mediated via an increase in TNF- $\alpha$  production.

Activation of hepatic NKT cytotoxicity through expression of FasL against hepatocytes has also been shown to play an important role in Con A-induced liver injury (45). Our results in this paper showed that the cytotoxicity of hepatic NKT cells from ethanol-fed mice against hepatocytes from the same mice was similar to the cytotoxicity of hepatic NKT cells from pair-fed mice against hepatocytes from pair-fed mice (Fig. 2C), suggesting that ethanol potentiation of T cell-mediated hepatitis is not through enhancing NKT cell cytotoxicity. Further investigation showed that the cytotoxicity of ethanol-fed mouse liver NKT cells was lower than that of pair-fed mouse liver NKT cells, because the ability of ethanol-fed mouse liver NKT cells to kill the same target cells significantly decreased compared with NKT cells from pair-fed mouse liver. This is likely due to a downregulation of FasL expression in NKT cells after injection of Con A from ethanol-fed mice compared with pair-fed mice (Fig. 3, C and D). Moreover, hepatocytes from ethanol-fed mice were more susceptible to NKT cell killing compared with pair-fed mouse hepatocytes. Although the cytotoxicity of liver MNCs is not enhanced in ethanol-fed mice (Fig. 2), adoptive transfer of liver MNCs from ethanol-fed mice, compared with transfer of pair-fed liver MNCs, en-

hanced Con A-induced liver injury (Fig. 2D). Similarly, Cao et al. (7) reported that transfer of liver-associated T cells from ethanol-fed rats potentiated Con A-induced liver injury compared with cell transfer from pair-fed rats, which is likely mediated by enhanced TNF- $\alpha$  production. Taken together, these findings suggest that ethanol consumption may dysregulate liver MNCs, such as enhancing TNF- $\alpha$  production, and consequently T cell-mediated hepatitis is enhanced.

In contrast to potentiation of NF- $\kappa$ B signaling pathway, ethanol consumption decreased Con A-mediated activation of multiple STATs in the liver compared with pair-fed mice. As shown in Fig. 5A, injection of Con A caused activation of STAT1 and STAT3 in the livers of pair-fed mice, which was significantly decreased in ethanol-fed mice. Previously, it was shown that acute and chronic ethanol exposure inhibited IL-6 activation of STAT3 and IFN- $\gamma$  activation of STAT1, which is partly mediated via activation of ERK1/2 and PKC (9, 10, 34). Here, we also showed that activation of ERK1/2 is significantly higher in ethanol-fed mice after Con A injection compared with pair-fed mice, suggesting that enhanced ERK1/2 activation could be involved in the downregulation of STAT activation in ethanol-fed mice. Activation of IL-6/STAT3 has been shown to protect against liver injury in Con A-induced T cell hepatitis (21); thus downregulation of STAT3 signaling may also contribute to ethanol potentiation of Con A-induced liver injury.

T cell-mediated immune responses have been implicated in the pathogenesis of a variety of human liver disorders (3, 19, 25) including autoimmune liver disease (29) and viral hepatitis (8, 38). Our findings here show that chronic ethanol consump-

tion potentiates Con A-induced T cell-mediated hepatitis in mice via dysregulation of cytokine signals such as stimulation of NF- $\kappa$ B signaling but inhibition of STAT signaling. Chronic alcohol consumption likely also dysregulates these cytokine signals and accelerates the development and progression of T cell-mediated injury in human liver diseases.

## REFERENCES

- Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology* 124: 778–790, 2003.
- Bhattacharya R and Shuhart MC. Hepatitis C and alcohol: interactions, outcomes, and implications. *J Clin Gastroenterol* 36: 242–252, 2003.
- Bogdanos DP, Mieli-Vergani G, and Vergani D. Virus, liver and autoimmunity. *Dig Liver Dis* 32: 440–446, 2000.
- Bone-Larson CL, Simpson KJ, Colletti LM, Lukacs NW, Chen SC, Lira S, Kunkel SL, and Hogaboam CM. The role of chemokines in the immunopathology of the liver. *Immunol Rev* 177: 8–20, 2000.
- Bosron WF, Lumeng L, and Li TK. Genetic polymorphism of enzymes of alcohol metabolism and susceptibility to alcoholic liver disease. *Mol Aspects Med* 10: 147–158, 1988.
- Cao Q, Batey R, Pang G, and Clancy R. Altered T-lymphocyte responsiveness to polyclonal cell activators is responsible for liver cell necrosis in alcohol-fed rats. *Alcohol Clin Exp Res* 22: 723–729, 1998.
- Cao Q, Batey R, Pang G, and Clancy R. Ethanol-altered liver-associated T cells mediate liver injury in rats administered Concanavalin A (Con A) or lipopolysaccharide (LPS). *Alcohol Clin Exp Res* 23: 1660–1667, 1999.
- Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, McHutchison JG, Alter HJ, and Chisari FV. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology* 33: 267–276, 2001.
- Chen J, Bao H, Sawyer S, Kunos G, and Gao B. Effects of short and long term ethanol on the activation of signal transducer and activator transcription factor 3 in normal and regenerating liver. *Biochem Biophys Res Commun* 239: 666–669, 1997.
- Chen J, Kunos G, and Gao B. Ethanol rapidly inhibits IL-6-activated STAT3 and C/EBP mRNA expression in freshly isolated rat hepatocytes. *FEBS Lett* 457: 162–168, 1999.
- Ciesielski CJ, Andreakos E, Foxwell BM, and Feldmann M. TNF $\alpha$ -induced macrophage chemokine secretion is more dependent on NF- $\kappa$ B expression than lipopolysaccharides-induced macrophage chemokine secretion. *Eur J Immunol* 32: 2037–2045, 2002.
- Crabb DW. Ethanol oxidizing enzymes: roles in alcohol metabolism and alcoholic liver disease. *Prog Liver Dis* 13: 151–172, 1995.
- Crabb DW, Dipple KM, and Thomasson HR. Alcohol sensitivity, alcohol metabolism, risk of alcoholism, and the role of alcohol and aldehyde dehydrogenase genotypes. *J Lab Clin Med* 122: 234–240, 1993.
- Day CP. Who gets alcoholic liver disease: nature or nurture? *J R Coll Physicians Lond* 34: 557–562, 2000.
- De la M Hall P, Lieber CS, DeCarli LM, French SW, Lindros KO, Jarvelainen H, Bode C, Parlesak A, and Bode JC. Models of alcoholic liver disease in rodents: a critical evaluation. *Alcohol Clin Exp Res* 25 Suppl 5: 254S–261S, 2001.
- French SW and Tsukamoto H. Animal models for alcoholic liver disease. *Hepatology* 10: 898–899, 1989.
- Grant BF, Dufour MC, and Harford TC. Epidemiology of alcoholic liver disease. *Semin Liver Dis* 8: 12–25, 1988.
- Grove M and Plumb M. C/EBP, NF- $\kappa$ B, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 $\alpha$  immediate-early gene. *Mol Cell Biol* 13: 5276–5289, 1993.
- Heneghan MA and McFarlane IG. Current and novel immunosuppressive therapy for autoimmune hepatitis. *Hepatology* 35: 7–13, 2002.
- Hill DB, Barve S, Joshi-Barve S, and McClain C. Increased monocyte nuclear factor- $\kappa$ B activation and tumor necrosis factor production in alcoholic hepatitis. *J Lab Clin Med* 135: 387–395, 2000.
- Hong F, Jaruga B, Kim WH, Radaeva S, El-Assal ON, Tian Z, Nguyen VA, and Gao B. Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J Clin Invest* 110: 1503–1513, 2002.
- Jaruga B, Hong F, Sun R, Radaeva S, and Gao B. Crucial Role of IL-4/STAT6 in T cell-mediated hepatitis: up-regulating eotaxins and IL-5 and recruiting leukocytes. *J Immunol* 171: 3233–3244, 2003.
- Kishore R, Hill JR, McMullen MR, Frenkel J, and Nagy LE. ERK1/2 and Egr-1 contribute to increased TNF- $\alpha$  production in rat Kupffer cells after chronic ethanol feeding. *Am J Physiol Gastrointest Liver Physiol* 282: G6–G15, 2002.
- Kishore R, McMullen MR, and Nagy LE. Stabilization of tumor necrosis factor  $\alpha$  mRNA by chronic ethanol: role of A + U-rich elements and p38 mitogen-activated protein kinase signaling pathway. *J Biol Chem* 276: 41930–41937, 2001.
- Kita H, Mackay IR, Van De Water J, and Gershwin ME. The lymphoid liver: considerations on pathways to autoimmune injury. *Gastroenterology* 120: 1485–1501, 2001.
- Koteish A, Yang S, Lin H, Huang X, and Diehl AM. Chronic ethanol exposure potentiates lipopolysaccharide liver injury despite inhibiting Jun N-terminal kinase and caspase 3 activation. *J Biol Chem* 277: 13037–13044, 2002.
- Lieber CS. Susceptibility to alcohol-related liver injury. *Alcohol Alcohol Suppl* 2: 315–326, 1994.
- Lieber CS and DeCarli LM. Alcoholic liver injury: experimental models in rats and baboons. *Adv Exp Med Biol* 59: 379–393, 1975.
- Lohr HF, Schlaak JF, Lohse AW, Bocher WO, Arenz M, Gerken G, and Meyer Zum Buschenfelde KH. Autoreactive CD4<sup>+</sup> LKM-specific and anticonotypic T-cell responses in LKM-1 antibody-positive autoimmune hepatitis. *Hepatology* 24: 1416–1421, 1996.
- Lumeng L and Crabb DW. Genetic aspects and risk factors in alcoholism and alcoholic liver disease. *Gastroenterology* 107: 572–578, 1994.
- McClain CJ, Barve S, Deaciuc I, and Hill DB. Tumor necrosis factor and alcoholic liver disease. *Alcohol Clin Exp Res* 22: 248S–252S, 1998.
- Nagy LE. Recent insights into the role of the innate immune system in the development of alcoholic liver disease. *Exp Biol Med (Maywood)* 228: 882–890, 2003.
- Nanji AA, Jokelainen K, Rahemtulla A, Miao L, Fogt F, Matsumoto H, Tahan SR, and Su GL. Activation of nuclear factor  $\kappa$ B and cytokine imbalance in experimental alcoholic liver disease in the rat. *Hepatology* 30: 934–943, 1999.
- Nguyen VA, Chen J, Hong F, Ishac EJ, and Gao B. Interferons activate the p42/44 mitogen-activated protein kinase and JAK-STAT (Janus kinase-signal transducer and activator transcription factor) signaling pathways in hepatocytes: differential regulation by acute ethanol via a protein kinase C-dependent mechanism. *Biochem J* 349: 427–434, 2000.
- Peters MG and Terrault NA. Alcohol use and hepatitis C. *Hepatology* 36, Suppl 1: S220–S225, 2002.
- Radaeva S, Jaruga B, Hong F, Kim WH, Fan S, Cai H, Strom S, Liu Y, El-Assal O, and Gao B. Interferon- $\alpha$  activates multiple STAT signals and down-regulates c-Met in primary human hepatocytes. *Gastroenterology* 122: 1020–1034, 2002.
- Roebuck KA and Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J Leukoc Biol* 66: 876–888, 1999.
- Rosen HR, Miner C, Sasaki AW, Lewinsohn DM, Conrad AJ, Bakke A, Bouwer HG, and Hinrichs DJ. Frequencies of HCV-specific effector CD4<sup>+</sup> T cells by flow cytometry: correlation with clinical disease stages. *Hepatology* 35: 190–198, 2002.
- Schiff ER. Hepatitis C and alcohol. *Hepatology* 26, Suppl 1: 39S–42S, 1997.
- Schneider T and Issekutz AC. Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase. Application in a Brown Norway rat model of allergic pulmonary inflammation. *J Immunol Methods* 198: 1–14, 1996.
- Sermon F, Le Moine O, Gustot T, Quertinmont E, Louis H, Nagy N, Degraef C, and Deviere J. Chronic alcohol exposure sensitizes mice to galactosamine-induced liver injury through enhanced keratinocyte chemoattractant and defective IL-10 production. *J Hepatol* 39: 68–76, 2003.
- Shi L, Kishore R, McMullen MR, and Nagy LE. Chronic ethanol increases lipopolysaccharide-stimulated Egr-1 expression in RAW 264.7 macrophages: contribution to enhanced tumor necrosis factor  $\alpha$  production. *J Biol Chem* 277: 14777–14785, 2002.
- Simpson KJ, Henderson NC, Bone-Larson CL, Lukacs NW, Hogaboam CM, and Kunkel SL. Chemokines in the pathogenesis of liver disease: so many players with poorly defined roles. *Clin Sci (Lond)* 104: 47–63, 2003.
- Szabo G. New insights into the the molecular mechanisms of alcoholic hepatitis: a potential role for NF- $\kappa$ B activation? *J Lab Clin Med* 135: 367–369, 2000.



45. **Takeda K, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, and Okumura K.** Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci USA* 97: 5498–5503, 2000.
46. **Tiegs G.** Experimental hepatitis and role of cytokines. *Acta Gastroenterol Belg* 60: 176–179, 1997.
47. **Tiegs G, Hentschel J, and Wendel A.** A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest* 90: 196–203, 1992.
48. **Trautwein C, Rakemann T, Brenner DA, Streetz K, Licato L, Manns MP, and Tiegs G.** Concanavalin A-induced liver cell damage: activation of intracellular pathways triggered by tumor necrosis factor in mice. *Gastroenterology* 114: 1035–1045, 1998.
49. **Uesugi T, Froh M, Arteel GE, Bradford BU, Gabele E, Wheeler MD, and Thurman RG.** Delivery of I $\kappa$ B superrepressor gene with adenovirus reduces early alcohol-induced liver injury in rats. *Hepatology* 34: 1149–1157, 2001.
50. **Wolf D, Schumann J, Koerber K, Kiemer AK, Vollmar AM, Sass G, Papadopoulos T, Bang R, Klein SD, Brune B, and Tiegs G.** Low-molecular-weight hyaluronic acid induces nuclear factor- $\kappa$ B-dependent resistance against tumor necrosis factor  $\alpha$ -mediated liver injury in mice. *Hepatology* 34: 535–547, 2001.
51. **Zhou Z, Wang L, Song Z, Lambert JC, McClain CJ, and Kang YJ.** A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF- $\alpha$  production. *Am J Pathol* 163: 1137–1146, 2003.

