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Reduced Early Alcohol-Induced Liver Injury in CD14-Deficient Mice

Ming Yin,* Blair U. Bradford,2* Michael D. Wheeler,* Takehiko Uesugi,* Matthias Froh,* Sanna M. Goyert,† and Ronald G. Thurman*

Activation of Kupffer cells by gut-derived endotoxin is associated with alcohol-induced liver injury. Recently, it was shown that CD14-deficient mice are more resistant to endotoxin-induced shock than wild-type controls. Therefore, this study was designed to investigate the role of CD14 receptors in early alcohol-induced liver injury using CD14 knockout and wild-type BALB/c mice in a model of enteral ethanol delivery. Animals were given a high-fat liquid diet continuously with ethanol or isocaloric maltodextrin as control for 4 wk. The liver to body weight ratio in wild-type mice (5.8 ± 0.3%) was increased significantly by ethanol (7.3 ± 0.2%) but was not altered by ethanol in CD14-deficient mice. Ethanol elevated serum alanine aminotransferase levels nearly 3-fold in wild-type mice, but not in CD14-deficient mice. Wild-type and knockout mice given the control high-fat diet had normal liver histology, whereas ethanol caused severe liver injury (steatosis, inflammation, and necrosis; pathology score = 3.8 ± 0.4). In contrast, CD14-deficient mice given ethanol showed minimal hepatic changes (score = 1.6 ± 0.3, p < 0.05). Additionally, NF-κB, TGF-β, and TNF-α were increased significantly in wild-type mice fed ethanol but not in the CD14 knockout. Thus, chronic ethanol feeding caused more severe liver injury in wild-type than CD14 knockout, supporting the hypothesis that endotoxin acting via CD14 plays a major role in the development of early alcohol-induced liver injury. The Journal of Immunology, 2001, 166: 4737–4742.

Endotoxins (LPS) represent the major component of the outer membrane of Gram-negative bacteria and have been implicated in sepsis, organ failure, and shock (1). Accumulating evidence suggests that endotoxins and proinflammatory cytokines also participate in early alcohol-induced liver injury (2). Elevated circulating endotoxin most likely activates Kupffer cells to release many potent effectors and cytokines, thus leading to tissue injury. The following evidence supports this hypothesis: 1) Ethanol increases permeability of the isolated small bowel to endotoxin in a dose-dependent manner (3) and elevates circulating endotoxin (4). 2) Reduction of Gram-negative bacteria in the intestines (i.e., sources of endotoxin) with antibiotics (5) or lactobacillus administration (6) minimized early alcoholic liver injury. 3) Early ethanol-induced liver injury can be prevented by gadoxilinium chloride, a selective Kupffer cell toxicant (7). 4) Early alcohol-induced liver injury was attenuated with TNF-α Abs and was prevented in TNF receptor-1 knockout mice (8, 9).

CD14, a GPI-anchored glycoprotein that is highly expressed on the surface of human monocytes and most macrophages (10), has been demonstrated to be part of a specific cellular LPS binding site (11) and is a key mediator of septic shock induced by endotoxin (10, 12, 13). Circulating LPS binds to LPS-binding protein (LBP)3 and activates monocytes/macrophages via binding to the CD14 receptor (14, 15). Kupffer cells, the resident liver macrophages, are the major population of the monocyte-macrophage lineage (16) and contain CD14 receptors (4, 17). Recent studies show that alcohol exposure increases the expression of CD14 in Kupffer cells (18) and estrogen sensitizes Kupffer cells to LPS via increases in CD14. The interaction of LPS with CD14 triggers a signaling cascade and results in induction of cytokines (IL-6, IL-1, and TNF-α) that are known to participate in liver injury (2). It has been shown previously that CD14-deficient mice do not produce significant levels of these cytokines even when exposed to high levels of LPS (12). To test the hypothesis that CD14 is involved in early alcohol-induced liver injury, the response of CD14-deficient mice to ethanol was compared with that wild-type mice by evaluating parameters of hepatic injury using a murine enteral ethanol feeding model (9). After 4 wk of continuous enteral ethanol feeding, not only was steatosis observed, but inflammation and necrosis also occurred in livers of wild-type mice. However, in mice lacking CD14 receptors, hepatic pathology due to alcohol was largely blocked. These studies show that CD14, presumably on Kupffer cells, plays a critical role in early alcohol-induced liver injury and further support a role for endotoxin in this disease. Preliminary accounts of this study have been reported elsewhere (19).

Materials and Methods

Animals

Female CD14-deficient mice generated as described elsewhere (12, 20) were backcrossed 10 times with BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Age- and sex-matched wild-type BALB/c mice, weighing 21–24 g, served as controls. Mice were screened for the presence of the CD14 receptor mRNA (+/+ or −/−) in wild-type mice and the absence of the CD14 receptor mRNA in knockout mice by RNase protection assay.

*Department of Pharmacology, Laboratory of Hepatology and Toxicology, University of North Carolina, Chapel Hill, NC 27599; and Division of Molecular Medicine, North Shore University Hospital, Cornell University Medical College, Manhasset, NY 11030

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2 Address correspondence and reprint requests to Blair U. Bradford, Department of Pharmacology, Laboratory of Hepatology and Toxicology, CB 7365, Mary Ellen Jones Building, University of North Carolina, Chapel Hill, NC 27599-7365. E-mail address: beub@med.unc.edu

3 Abbreviations used in this paper: LBP, LPS-binding protein; ALT, alanine aminotransferase; LT-β, lymphotoxin-β; MIF, migration inhibitory factor.
All animals received humane care in compliance with institutional guidelines. Body weight was measured before surgery and at necropsy after 4 wk of continuous delivery of control or ethanol-containing diet.

Surgery
The principle surgical procedures were similar to methods previously described by Tsukamoto et al. (21) in rats, with modifications based on the size of mice (9). Briefly, mice were anesthetized by injection of pentobarbital sodium (50 mg/kg; Abbott Laboratories, North Chicago, IL), and laparotomy was performed under sterile surgical conditions. A PE90 polyethylene tube (Becton Dickinson, Sparks, MD) was placed in the squamous part of the stomach. The tube was anchored to the stomach wall with Dacron and fixed to the abdominal wall. It was then tunneled s.c. to the dorsal aspect of the neck followed by closing of the abdominal wall with 7-0 prolene sutures. The tube was then pulled through a 250P polysulfone attachment mouse button (Instech Laboratories, Plymouth Meeting, PA) and spring coil. The button was fixed under the skin with its metal spring coil outside of the body to protect the tube. The feeding tube was attached to an infusion pump by means of a swivel, allowing complete mobility of the mouse within a metabolic cage. Animals were allowed to recover for 1 wk with free access to chow diet and water before starting alcohol-containing or control high-fat liquid diets.

Diet
A basic liquid diet was prepared according to Thompson and Reitz (22) as described previously and supplemented with lipotropes as described by Morimoto et al. (23). The control diet (1.3 kcal/ml) contained corn oil as fat (34% of total calories), protein (23%), carbohydrate (43%), plus minerals and vitamins. For the isocaloric high-fat diet, dextrose-maltose was replaced by ethanol (9). The liquid diet was fed continuously at the rate of 11 ml/day to achieve weight gain. Throughout the experimental period of liquid diet delivery, mice had free access to cellulose pellets as a source of fiber (Harlan Teklad, Madison, WI).

Experimental protocol
CD14-deficient and wild-type mice were randomly allocated into two experimental groups of five to seven mice each and were fed either ethanol-containing or an isocaloric high-fat control diet. Animals received diets by oral gavage (23, 24) using an adaptive feeding tube system (Instech Laboratories, Plymouth Meeting, PA). The liquid diet was fed to the mouse within a metabolic cage. Animals were allowed to recover for 1 wk before starting alcohol-containing or control high-fat liquid diets.

Pharmacology and assay for ethanol
Concentrations of ethanol in urine are representative of blood alcohol levels (25). Mice were housed in metabolic cages that separated urine from feces and urine samples were collected over 24 h for each mouse. Ethanol levels in urine were determined daily by measuring absorbance at 366 nm against a 180-bp fragment were as follows: forward, 5'-TGCACCTGCATGCGAAGCC-3'; reverse, 5'-CAACAGTAAACCGCTTTAAGAC-3'.

RT-PCR amplification of CD14 cDNA
Mouse Kupffer cell RNA was extracted by standard phenol:chloroform extraction and ethanol precipitation. The digested products were separated on a 6% acrylamide gel. The RNA probe used to detect CD14-specific RNA was generated by in vitro transcription of linearized pCR-CD14 after cleavage with HindIII. The pCR-CD14 plasmid was generated by insertion of the RT-PCR amplification fragment (180 bp) of mouse Kupffer cell cDNA into the pCR-TOPO cloning plasmid (Invitrogen, San Diego, CA). The RNA probes (PharMingen) were used to detect GAPDH-specific RNA (as control), L32 as a ribosomal housekeeping gene, TNF-α, lymphotoxin-β (LT-β), macrophage migration inhibitory factor (MIF), and TGF-β were generated by in vitro transcription of mouse GAPDH template (PharMingen). The protected RNA was visualized by autoradiography.

EMSA
A gel mobility shift assay was used in this study to assess the amount of active protein involved in protein–DNA interactions. The limitations of this method are the amount of nuclear protein needed for assay as well as the number of cells isolated from the rat liver. Binding conditions for NF-kB were characterized and EMSAs were performed as described in detail elsewhere (27). Briefly, extracts (40 μg) from liver tissues were preincubated for 10 min on ice with 1 μg of poly(dI-dC) and 2 μg of BSA (both from Pharmacia Biotech, Piscataway, NJ) in a buffer that contained 1 mM HEPES (pH 7.6), 40 mM MgCl₂, 0.1 M NaCl, 8% glycerol, 0.1 mM DTT, 0.05 mM EDTA, and 2 μl of a 3²P-labeled DNA probe (10,000 cpm/μl) that contained 0.4 ng of double-stranded oligonucleotide. Mixtures were incubated for 20 min on ice and resolved on 5% polyacrylamide (29:1 cross-linking) and 0.4X Tris-borate-EDTA gels. After electrophoresis, gels were dried and exposed to Kodak film (Kodak, Rochester, NY). Data were quantitated by scanning autoradiograms with GelScan XL (Pharmacia).

Statistics
ANOVA was used for the determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney U rank sum test was used. Data are presented as mean ± SEM. A p < 0.05 was selected before the study as the level of significance.

Results
Body and liver weights
Liver and body weight were determined to assess the general health of the animals. All animals survived surgery and gained weight during 4 wk of continuous delivery of high-fat liquid diets with or without ethanol. There were no significant differences in body weight among the groups studied. At necropsy, liver to body weight ratios in wild-type mice receiving ethanol were significantly higher than in wild-type mice fed control diet (Fig. 1). In contrast, the ratio in CD14-deficient mice given ethanol was significantly lower than in wild-type mice fed ethanol. Thus, ethanol caused significantly greater enlargement of livers in wild-type than in CD14 knockout mice.

Urine ethanol
Urine ethanol concentrations were monitored to the degree of intoxication. As reported previously in studies with rats (28) or C57BL mice (7, 9), urine ethanol levels also fluctuated in the CD14-deficient or wild-type BALB/c mice in a cyclic pattern from 18 to >500 mg/dl likely due to thyroid hormones and stress as was recently reported in rats (29). Average urine ethanol concentrations...
during 4 wk of ethanol exposure were 158 ± 25 mg/dl in wild-type mice and 162 ± 26 mg/dl in the knockouts, values which were not statistically different.

**Serum transaminases**

As a marker of liver injury, serum ALT was monitored throughout the study. Before continuous administration of liquid diets, the serum level of ALT in both wild-type and CD14 knockout mice was 44 ± 6 U/L. In wild-type mice, 4 wk of ethanol exposure significantly increased the serum ALT 3-fold (126 ± 27 U/L; Fig. 2); however, ethanol exposure did not increase ALT in CD14-deficient mice.

**Pathological evaluation**

Fig. 3 shows representative photomicrographs of livers from wild-type and CD14 knockout mice after 4 wk of exposure to control or ethanol diets. There were no pathological changes seen in wild-type or CD14 knockout mice receiving high-fat control diet (Fig. 3, A and B). Accordingly, the pathology score of livers from wild-type and knockout mice fed control high-fat diet was minimal (Fig. 4). However, marked fatty accumulation and mild to moderate inflammation and necrosis were observed in wild-type mice given ethanol (Fig. 3C), with an average pathology score of 3.8 ± 0.6 (Fig. 4D). This value was significantly greater than values observed in wild-type mice given control diet (Fig. 4D). Fatty accumulation in wild-type mice receiving ethanol was panlobular, with massive large droplets of fat in pericentral areas and midzonal regions near central veins. In livers from CD14-deficient mice, however, only mild fatty accumulation was detected (Fig. 3D), with mild inflammation but no necrosis. As a result, the pathology scores of livers from CD14 knockout mice receiving ethanol diet were only 1.7 ± 0.4 (Fig. 4D), values which were significantly lower than ethanol-treated, wild-type mice (p < 0.05).

**Inflammatory markers in liver**

An RNase protection assay was used to determine the tissue levels of message for TNF-α, LT-β, MIF, and TGF-β. Ethanol caused

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**FIGURE 2.** Effect of continuous diet delivery with or without ethanol on serum ALT levels. Blood samples were collected at necropsy, i.e., 4 wk after high-fat liquid diet feeding in the presence or absence of ethanol. ALT levels were determined as described in Materials and Methods. Data presented are mean ± SEM, n = 5–7 mice/group. Two-way ANOVA was used for determination of statistical differences. *p < 0.05 compared with the other three groups of mice by one-way ANOVA with Tukey’s post hoc test. KO, Knockout.

**FIGURE 3.** Representative photomicrographs of livers from wild-type and CD14 knockout mice after 4 wk of continuous diet delivery with or without ethanol. Animals were treated as described in Materials and Methods. Livers from wild-type mice receiving high-fat control diet (A), CD14 knockouts fed high-fat control diet (B), wild-type mice given ethanol diet (C), and CD14 knockouts given ethanol diet (D) are shown. Original magnification, ×100. With higher magnification, E and F show inflammation and necrosis in wild-type animals fed ethanol; G and H depict histology without inflammation and necrosis in CD14-deficient mice receiving ethanol. H-F represents high-fat; EtOH represents animals given the ethanol diet. Representative photomicrographs.
about a 2.5-fold increase in tissue levels of TNF-α mRNA in livers from wild-type mice (Fig. 5) as well as the inflammatory cytokine LT-β. This phenomenon was not observed in CD14 knockout mice. TGF-β and LT-β were doubled after ethanol in the wild-type but not in CD14 knockout mice (Fig. 5). MIF was detected in all groups. To assess the involvement of NF-κB after alcohol treatment in both strains of mice, activation was measured by the EMSA in liver (Fig. 6). A doubling of NF-κB activation occurred after ethanol treatment in wild-type mice. In CD14 knockout mice, however, activation of NF-κB did not occur after ethanol treatment.

Discussion
The role of LBP and CD14 in early alcohol-induced liver injury
Activation of monocytes and macrophages by endotoxin (LPS) from Gram-negative bacteria has been extensively studied to define the mechanisms that underlie innate immune responses against bacterial pathogens (30). Numerous studies have revealed that LPS, instead of being directly toxic to cells or organs, exerts its biological effects indirectly through stimulation of host cells to produce endogenous mediators including cytokines and free radicals (15, 30–33), leading to inflammation and requires additional molecules for signal transduction, including Toll-like receptors (34, 35). It was very recently reported that alcohol-induced liver injury, using the same model used in this study, is blunted in the Toll-like receptor 4 mutant C3H/HeJ mouse (36). Taken together, this study and the work presented here support the hypothesis that a signaling pathway comprised of CD14 and Toll-like receptor 4 is required for development of pathology due to ethanol.

Ethanol and endotoxin
Studies demonstrating the strong resistance of CD14-deficient mice to endotoxin suggest that CD14 plays a predominant role in endotoxin shock (12, 37). The development of the CD14 knockout mice provides a powerful tool for studies of the role of endotoxin in various diseases. It is well known that ethanol exposure increases circulating endotoxin (4). However, ethanol-induced hepatic injury was minimized significantly here in CD14-deficient mice (Fig. 4). This indicates that cells of the immune system respond to LPS through a pathway involving the CD14 receptor (13, 38) and supports the hypothesis that...
elevated circulating endotoxin by excessive alcohol intake activates resident hepatic macrophages (Kupffer cells) to release endogenous inflammatory mediators via activation of NF-κB (Figs. 6 and 7). The activation of NF-κB by enteral ethanol treatment in the rat is known (39). It has been shown that NF-κB activation is not increased after 1 wk of ethanol treatment (40). This suggests that longer exposure to ethanol is critical as was observed here (Fig. 6). The increase in NF-κB activation is consistent with the hypothesis that endotoxin binds to CD14, activates the Kupffer cells, and elicits the production of reactive oxygen species which increases NF-κB (Fig. 7). The absence of activation of NF-κB in the CD14 knockout mouse after enteral ethanol strengthens this hypothesis.

Recently, it was reported that chronic ethanol increases mRNA for TGF-β expression in rats ~2 fold after 2 wk of enteral ethanol (41). In these experiments, mRNA for LBPD and CD14 were not increased by chronic endotoxin but were increased after acute endotoxin. The authors conclude that down-regulation of mRNA for CD14 and LBPD may occur as an adaptation in chronic alcohol abuse. The results in mice presented here using a chronic model of ethanol delivery provide an alternative explanation. TGF-β protein is doubled after ethanol treatment (see Fig. 5 and Results) in wild-type mice but is not elevated in the CD14 knockout mouse fed control or ethanol-containing diet, suggesting that CD14 is necessary for TGF-β activation and not for down-regulation.

**Cytokines**

TNF-α is a central proinflammatory cytokine (42). TNF-α acting through its receptor-1 pathway plays a predominant role in LPS-induced inflammatory diseases (43) and mediates the lethal effects of endotoxin (44). TNF-α is increased in alcoholics with hepatitis and levels correlate with survival (35) and appears to be the principle mediator of early alcohol-induced liver injury since injury was blocked in mice lacking the TNF receptor-1 (9). In the present study, increased levels of TNF-α (Fig. 5) along with the most severe hepatic injury among the groups studied were observed in the livers from wild-type mice fed ethanol, whereas these effects were blocked in CD14-deficient mice. Therefore, these results are consistent with the hypothesis that excessive intake of alcohol increases circulating endotoxin which activates Kupffer cells via the CD14 receptor to release TNF-α, leading to liver injury (Fig. 7).

In this study, macrophage MIF was detected in all treatment groups studied (Fig. 5) and is commonly expressed in liver and other tissues (45) as an inflammatory cytokine that can also induce TNF-α (46). Studies with anti-MIF Ab demonstrate a protective effect in models of endotoxin-induced injury (47). After 4 wk of dietary treatment with or without ethanol, a mild inflammatory infiltration was observed in mice receiving a high-fat control diet. This is different from the results of a previous study using mice on a C57BL/6J background (9), where no inflammation was detected in animals receiving a high-fat control diet. This phenomenon is possibly due to the fact that BALB/c mice are more susceptible to inflammation (48) involving, in part, the inflammatory cytokine MIF.

In summary, the results of the present study are consistent with the hypothesis that CD14 receptors play a major role in vivo in the pathogenesis of alcohol-induced liver injury; thus, drugs or genes that target CD14 signaling pathways may prove beneficial in treating alcoholic hepatitis.

**References**


