Original Article

MOLECULAR METABOLISM

Hepatocyte toll-like receptor 4 deficiency protects against alcohol-induced fatty liver disease

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ABSTRACT

Objective: Recent studies have suggested a critical role for toll-like receptor 4 (TLR4) in the development of alcoholic liver disease. As TLR4 is widely expressed throughout the body, it is unclear which TLR4-expressing cell types contribute to alcohol-induced liver damage.

Methods: We selectively ablated TLR4 in hepatocytes and myeloid cells. Male mice were fed a liquid diet containing either 5% alcohol or pair-fed a control diet for 4 weeks to examine chronic alcohol intake-induced liver damage and inflammation. In addition, mice were administered a single oral gavage of alcohol to investigate acute alcohol drinking-associated liver injury.

Results: We found that selective hepatocyte TLR4 deletion protected mice from chronic alcohol-induced liver injury and fatty liver. This result was in part due to decreased expression of endogenous lipogenic genes and enhanced expression of genes involved in fatty acid oxidation. In addition, mice lacking hepatocyte TLR4 exhibited reduced mRNA expression of inflammatory genes in white adipose tissue. Furthermore, in an acute alcohol binge model, hepatocyte TLR4 deficient mice had significantly decreased plasma alanine transaminase (ALT) levels and attenuated hepatic triglyceride content compared to their alcohol-gavaged control mice. In contrast, deleting TLR4 in myeloid cells did not affect the development of chronic-alcohol induced fatty liver, despite the finding that mice lacking myeloid cell TLR4 had significantly reduced circulating ALT concentrations.

Conclusions: These findings suggest that hepatocyte TLR4 plays an important role in regulating alcohol-induced liver damage and fatty liver disease.

Keywords Alcoholic fatty liver disease; Toll-like receptor 4; Hepatocyte; Myeloid cell; Liver injury; Inflammation

1. INTRODUCTION

Overconsumption of alcohol is a serious, worldwide health problem. Evidence suggests that inflammation is an essential factor contributing to the initiation and progression of alcoholic liver disease (ALD) [1]. Ethanol metabolites can directly induce inflammation via the activation of NF-κB and production of tumor necrosis factor alpha (Tnfα) [2]. In addition, alcohol impairs gut permeability, leading to translocation of endotoxin/lipopolysaccharide (LPS) to the portal vein and circulation [3–6]. Circulating LPS interacts with its receptor, toll-like receptor 4 (TLR4), to activate signal transduction and generate inflammatory cytokines, including Tnfα and interleukin 1β (IL-1β) [7]. Previous studies have shown that mutant TLR4 mice [8], or mice deficient in TLR4 [9,10], display attenuated alcohol-induced fatty liver disease, suggesting a direct role for TLR4 in the development of ALD.

The specific TLR4-expressing cells that contribute to alcohol-induced liver damage remain unclear. TLR4 is widely expressed in several cell types, including hepatocytes [11–17]. Despite mediating a relatively low inflammatory response [11,18,19], several recent studies, including our own, have demonstrated the critical role of hepatocyte TLR4 in disease development. Hepatocyte TLR4 regulates obesity-associated hepatic fat accumulation, inflammation, and insulin resistance [19–21]. Furthermore, hepatocyte TLR4 mediates the synergistic effects of alcohol and hepatitis C virus-induced liver damage and

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oncogenesis [12]. These observations led us to hypothesize that hepatocyte TLR4 may regulate alcohol-induced fatty liver disease.

Accumulating evidence also suggests a critical role for macrophage TLR4 in ALD. For instance, Inokuchi et al. have reported that bone marrow-derived TLR4-expressing cells contribute to alcohol-induced steatohepatitis [10]. While bone marrow-derived cells include a variety of TLR4-expressing cell types, we specifically focused on the role of myeloid cell TLR4 in alcohol-induced liver damage and fatty liver development.

In this study, we showed that selective hepatocyte TLR4 deletion protected mice from chronic alcohol-induced liver injury and fatty liver. We found that mice lacking hepatocyte TLR4 had decreased expression of endogenous lipogenic genes, enhanced expression of genes involved in fatty acid oxidation, and reduced expression of inflammatory genes in white adipose tissue. Furthermore, in an acute alcohol binge model, hepatocyte TLR4 deficient mice had significantly decreased plasma alanine transaminase (ALT) levels and attenuated hepatic triglyceride content compared to alcohol-gavaged control mice. In contrast, while mice lacking myeloid cell TLR4 had significantly reduced circulating ALT concentrations, the development of chronic alcohol-induced fatty liver was not affected. Taken together, these findings suggest that hepatocyte TLR4 plays an important role in regulating both chronic and acute alcohol-induced liver damage and fatty liver disease.

2. MATERIALS AND METHODS

2.1. Animal care and alcohol feeding protocol

$Tlr4^{LKO}$, $Tlr4^{\Delta m\Phi}$ mice and their corresponding littermate control $Tlr4^{fl/fl}$ mice have been previously described [19]. 10–12 week old male mice on a mixed (B6/129) genetic background were subjected to a modified alcohol feeding protocol to induce early-stage alcoholic liver injury [22]. Mice were acclimated to a control liquid diet (Bio-Serv, F1259SP) for the first day. From days 2–5, a subset of mice was given the liquid diet (Bio-Serv, F1258SP) supplemented with increasing concentrations of ethanol from 1% to 4%. For the subsequent 4 weeks, mice were fed a liquid diet containing either 5% (v/v) ethanol or pair-fed a control diet. Mice were then fasted for 4 h and euthanized for blood and tissue collection. Animals were housed in a temperature-controlled environment on a 12-hour light/12-hour dark cycle. Care of all animals and procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas (UT) Southwestern Medical Center at Dallas.

2.2. Single oral gavage of alcohol

Male mice (10–12 weeks old) were fasted for 2 h in the morning from 8am to 10am. After measuring body weight, mice were given an oral gavage of 5 g/kg BW of alcohol (31.5%, vol/vol) or maltose dextrin (45%, wt/vol). 6 h later, mice were anesthetized for blood and liver collection.

2.3. Determination of hepatic triglyceride contents

Mice were euthanized, and blood was collected from the inferior vena cava. Then 10 mL PBS was slowly delivered to the left ventricle of the heart to remove residual blood. Livers were removed and snap-frozen in liquid nitrogen and stored at −80 °C. Frozen liver tissues were used for lipid extraction by the UT Southwestern Metabolic Phenotyping Core Facility. After extraction, triglyceride contents were measured using Infinity Reagent assays (Thermo Fisher Scientific).

2.4. Plasma parameters

Mice were anesthetized and blood was collected in EDTA-coated tubes. Plasma was separated by centrifugation at 8,000 g for 12 min and stored at −80 °C. Plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured using the Vitros 250 Chemistry Analyzer (Ortho Clinical Diagnostics) by the UT Southwestern Metabolic Phenotyping Core.

2.5. Hepatic oxidative stress

Hepatic oxidative stress was measured by TBARS (TCA Method) Assay Kit (Cayman Chemical Co.) following the manufacturer's instructions.

2.6. Quantitative real-time PCR (qPCR)

Total RNA from liver and white adipose tissue was extracted using RNA Stat60 (Teltest). Complementary DNA was synthesized using the High Capacity cDNA Kit (Applied Biosystems). qPCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems). The relative amounts of all mRNAs were calculated using the ΔΔCT assay. Primers for Tlr4 (ID: Mm0445274_m1), Acc1 (ID: Mm01304277_m1), Fas (ID: Mm00662319_m1), Scd1 (ID: Mm00772290_m1), PPARα (ID: Mm00440939_m1), Cpt1α (ID: Mm01231183_m1), Acox1 (ID: Mm01246834_m1), Tnfα (ID: Mm00443258_m1), IL-6 (ID: Mm00446190_m1), IL-1β (ID: Mm00434228_m1), Mcp1 (ID: Mm00441242_m1), Cd11c (ID: Mm00498698_m1) were purchased from Applied Biosystems. mRNA contents were normalized to 18s (ID: Hs99999901_s1) for liver and GAPDH (ID: Mm99999915_g1) for white adipose tissue, respectively.

2.7. Immunoblot analysis

Liver tissues were homogenized in lysis buffer containing 2% SDS, 50 mM Tris-HCl (pH 6.8) and phosphatase inhibitor (PI78420, Thermo Scientific), followed by centrifugation at 12,000 r.p.m. for 15 min. Supernatants were stored at −20 °C until assays were performed. Tissue protein concentrations were determined by bicinchoninic acid (BCA) Kit (Pierce). 50 μg of protein were loaded onto 4%–12% Bis-Tris gel for electrophoresis (Novex™ NuPAGE™, Invitrogen). Proteins were then transferred onto nitrocellulose membranes and immunoblotted with specific antibodies that recognized ACC1 [23–25] (508D, rabbit polyclonal anti-mouse antibody), FAS [23,24] (320D, rabbit polyclonal anti-mouse antibody), SCD1 (Cell Signaling Technology), and β-actin (Cell Signaling Technology). After incubation with ECL (Pierce), chemiluminescence was imaged with a LI-COR Odyssey® Fc imager and quantified using LI-COR Image Studio™ software (LI-COR Biotechnology).

2.8. Circulating cytokines

Plasma concentrations of Tnfα, IL-1β, and Mcp1 were determined using the MILLIPLEX MAP Mouse Cytokine/Chemokine-Premixed 32 Plex assay (Millipore).

2.9. Isolation of peritoneal macrophage, SVF, and adipocytes from control and alcohol-fed mice

After four weeks of diet feeding, mice were anesthetized, and macrophages were collected by flushing the peritoneal cavity with cold PBS. After centrifugation at 1,000 rpm for 10 min at 4 °C, cell pellets were resuspended and plated in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Two hours later, floating cells were removed by washing with warm PBS. Adherent macrophages were cultured overnight and used for RNA isolation [19]. Epididymal fat pads were collected from the same mice. Tissues were minced and digested using collagenase (Gibco, #17703-034) at 37 °C for 2 h with shaking. The cell suspension was filtered through a 100 μm cell strainer, washed with DMEM containing 10%

