Research Article

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Activation of toll-like receptor 3 attenuates alcoholic liver injury by stimulating Kupffer cells and stellate cells to produce interleukin-10 in mice

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Background & Aims: The important function of toll-like receptor (TLR) 4 in Kupffer cells and hepatic stellate cells (HSCs) has been well documented in alcoholic liver injury. However, little is known about the role of TLR3. Thus, we tested whether TLR3 activation in HSCs and Kupffer cells could attenuate alcoholic liver injury *in vivo*, and investigated its possible mechanism *in vitro*.

Methods: Alcoholic liver injury was achieved by feeding wild type (WT), TLR3 knockout ($TLR3^{-/-}$) and interleukin ($IL\text{-}10^{-/-}$) mice with high-fat diet plus binge ethanol drinking for 2 weeks. To activate TLR3, polyinosinic–polycytidylic acid (poly I:C) was injected into mice. For *in vitro* studies, HSCs and Kupffer cells were isolated and treated with poly I:C.

Results: In WT mice, poly I:C treatment reduced alcoholic liver injury and fat accumulation by suppressing nuclear factor-κB activation and sterol response element-binding protein 1c expression in the liver. In addition, freshly isolated HSCs and Kupffer cells from poly I:C-treated mice showed enhanced expression of IL-10 compared to controls. Infiltrated macrophage numbers and the expression of tumor necrosis factor-α, monocyte chemoattractant protein-1 and IL-6 on these cells were decreased after poly I:C treatment. *In vitro*, poly I:C treatment enhanced the expression of IL-10 via a TLR3-dependent mechanism in HSCs and Kupffer cells. Finally, the protective effects of poly I:C on alcoholic liver injury were diminished in $TLR3^{-/-}$ and $IL\text{-}10^{-/-}$ mice.

Conclusions: TLR3 activation ameliorates alcoholic liver injury via the stimulation of IL-10 production in HSCs and Kupffer cells. TLR3 could be a novel therapeutic target for the treatment of alcoholic liver injury.

Keywords: Toll-like receptor 4; Poly I:C; Endocannabinoid; Steatohepatitis.
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Abbreviations: LPS, lipopolysaccharide; TLR, toll-like receptor; CB1R, cannabinoid receptor 1; HSC, hepatic stellate cell; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; 2-AG, 2-arachidonoylglycerol; SREBP1c, sterol response element-binding protein 1c; FAS, fatty acid synthase; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; Poly I:C, polyinosinic-polycytidylic acid; IRF, interferon regulatory factor; NK cell, natural killer cell; WT, wild type; ALT, alanine transaminase; AST, aspartate transaminase; TG, triglyceride; RT-PCR, reverse-transcription polymerase chain reaction; MNC, mononuclear cell; FACS, fluorescence-activated cell sorting; MIP, macrophage inflammatory protein; α-SMA, α-smooth muscle actin; COL1A2, type 1 collagen alpha 2.

Introduction

Alcoholic liver disease is one of the major causes of morbidity and mortality worldwide [1]. Accumulating data indicate that alcoholic steatohepatitis is caused not only by hepatocyte injury and reactive oxygen stress, but also by the enhanced binding of specific ligands to their receptors, including lipopolysaccharide (LPS) binding to toll-like receptor (TLR) 4 and endocannabinoid binding to cannabinoid receptor 1 (CB1R) in hepatic cells [2–4]. The LPS molecule and its receptor TLR4 are considered to be critical to the activation of both Kupffer cells and hepatic stellate cells (HSCs) [3–6]. The binding of LPS to TLR4 generally induces the activation of nuclear factor-κB (NF-κB), resulting in the production of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) [4,7]. In addition, alcohol consumption activates hepatic CB1R via the HSC-derived 2-arachidonoylglycerol (2-AG), an endocannabinoid that enhances hepatic lipogenesis by upregulating sterol response element-binding protein 1c (SREBP1c) and fatty acid synthase (FAS), but suppresses AMP-activated protein kinase (AMPK) and carnitine palmitoyltransferase 1 (CPT1) in hepatocytes, subsequently leading to the accumulation of fat in the liver [2]. In most cases, 2-AG is converted from diacylglycerols by diacylglycerol lipases (DAGL) and degraded by fatty acid amide hydrolase (FAAH) [8]. Thus, TLR4 and CB1R-mediated hepatic inflammation and steatosis likely play an important role in the pathogenesis of alcoholic liver injury.

During the past decade, several interesting studies suggested that TLR3 and IL-10 participate in the suppression or killing of activated HSCs and Kupffer cells in a variety of models of liver injury [9–13]. Polyinosinic–polycytidylic acid (poly I:C), a double-stranded RNA mimic ligand to TLR3, attenuates LPS-induced liver injury via the downregulation of TLR4 expression on macrophages and it ameliorates carbon tetrachloride-induced liver

fibrosis via the activation of natural killer (NK) cell cytotoxicity against activated HSCs in a TLR3-dependent manner [9,10]. In addition, IL-10 acts as a negative regulator for Kupffer cells and HSCs in liver inflammation and fibrosis [11–13]. Interestingly, several recent studies reported that a certain double-stranded RNA virus infection triggered the expression of IL-10 through interferon regulatory factor (IRF) 3 signaling [14] and that activation of TLR3 signaling induced IRF3 activation [15], suggesting the potential production of IL-10 via a poly I:C-mediated TLR3 pathway. However, little is known about the effects of TLR3-mediated IL-10 production on Kupffer cells and HSCs in alcoholic liver injury.

Here, we explored whether TLR3 activation by poly I:C regulates alcoholic liver injury. Our results suggest that TLR3 activation in HSCs and Kupffer cells plays an antagonistic role against the TLR4-mediated signal pathway via the production of IL-10, which may be a crucial mechanism for ameliorating alcoholic liver injury in mice.

Materials and methods

Animals and experimental design

Male C57BL/6 wild type (WT), *TLR3* knockout ($TLR3^{-/-}$) and $IL\text{-}10^{-/-}$ mice (20–24 g), purchased from The Jackson Laboratory (Bar Harbor, ME), were backcrossed to the B6 strain for more than 9 generations. For the study of acute alcoholic liver injury, mice were fed a standard chow diet (STD), or high-fat diet + isocaloric sugar water drinking without or with poly I:C (Pair or Pair + PIC), or high-fat diet + binge ethanol drinking without or with poly I:C (EtOH or EtOH + PIC) for 2 weeks. For binge drinking, 4 g/kg ethanol (3 g/kg for $IL\text{-}10^{-/-}$ mice) was administered once per day by gavage. Because of hepatotoxicity at high doses of poly I:C, a low dose of poly I:C (0.5 μg/g) was injected to mice i.p. every other day. All experimental procedures were approved by the Institutional Animal Care and Use Committee of KAIST.

Serum chemistry, CBA assay, and hepatic levels of triglyceride

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and cholesterol were measured using the VetTest Chemistry analyzer (IDEXX laboratory Inc, Westbrook, ME), and levels of serum cytokines were measured with the cytometry bead array (CBA) mouse inflammation kit (BD bioscience, San Jose, CA). Total contents of hepatic triglycerides were measured as described previously [2].

Staining

Liver sections were stained with hematoxylin and eosin (H&E) or Oil-Red O solution (Sigma) as described previously [2]. Immunostaining was performed with anti-F4/80 (Novous biologicals, Littleton, CO), and IL-10 (R&D systems), then visualized with Alexa Fluor®594 conjugated anti-mouse IgG or anti-rat IgG secondary antibody (Invitrogen) and VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. F4/80 positive Kupffer cells were alternatively visualized with avidin–biotin peroxidase complex solution using an ABC kit (Vector), 3,3-diamino benzidine (DAB; Invitrogen). All visual inspections were done using Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a CCD camera and computer-assisted image analysis with DP2-BSW at various magnifications.

Reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR

Total RNAs from isolated cells or liver tissues were extracted using Trizol (Invitrogen). One to 2 μg of extracted RNA was reverse-transcribed using the SuperScript® II reverse transcriptase (Invitrogen) to make complementary DNA. Gene specific primers (Supplementary Table 1) were used for PCR analysis. Details of the method and calculations of fold increases over control were previously described [2].

Immunoblot analysis

As previously described [2], proteins from cells and tissues were extracted with RIPA buffer. Immunoblot analyses were performed with 50–80 μg proteins from liver homogenates using antibodies to anti-β-actin, CYP2E1 (Sigma), anti-phospho-NF-κBp65 (S536), NF-κBp65, FAS, AMPKα, phospho-AMPKα (Thr172) (all are from Cell signaling), SERBP1c (BioVision, Mountain View, CA) and CPT1 (Alpha diagnostic). Proteins were separated on 10% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and then visualized using ECL system (GE healthcare).

Isolation of liver mononuclear cells (MNCs), Kupffer cells and hepatic stellate cells

Total liver MNCs were isolated from the whole liver tissue as previously described [16]. Briefly, after sacrificing the mice, liver tissues were immediately homogenized and filtered through a 70-μm cell strainer (BD bioscience). After the elimination of hepatocytes, the supernatant was collected, washed in sterile PBS, and resuspended in 40% Percoll (GE healthcare). The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 25 min at 2400 rpm. Liver MNCs were collected from the interface and then washed twice in sterile PBS. Isolated Kupffer cells and hepatic stellate cells (HSCs) were collected by *in situ* collagenase perfusion and differential centrifugation on OptiPrep (Sigma) density gradient as described previously [2,17]. The purity of Kupffer cells and HSCs was assessed by immunohistochemistry with anti-F4/80 antibody and flow cytometric analysis or by typical light microscopic appearance and vitamin A autofluorescence (purity, over 90%). See Supplementary data for a more detailed description.

Fluorescence-activated cell sorting (FACS)

Isolated liver MNCs were stained with anti-CD45 (30-F11), anti-F4/80 (BM8), (eBioscience, San Diego, CA), anti-CD11b (M1/70), anti-CD3e (145-2C11), anti-NK1.1 (PK138), anti-CD4 (RM4-5), and anti-Gr1 (RB6-8C5) (BD bioscience). The stained cells were analyzed using BD™ LSR II Flow Cytometer (BD bioscience) and FlowJo software (Tree Star, Ashland, OR).

In vitro experiments

Isolated cells were treated with poly I:C (10 μg/ml). Collected cells and supernatants were subjected to PCR analysis or IL-10 measurement.

Statistical analysis

Data are expressed as means ± SEM. To compare values, Student's *t* test was performed for paired data and one-way ANOVA with a Turkey *post hoc* test was performed for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Poly I:C treatment ameliorates alcoholic liver injury and steatosis in mice

In contrast to pair-fed mice (Pair, Pair + PIC), body weights of ethanol-fed mice (EtOH, EtOH + PIC) gradually decreased compared with those of the control (STD). However, there were no significant effects of poly I:C on body weight and diet intake (Fig. 1A, Supplementary Fig. 1A). Serum levels of ALT, AST and TG were significantly increased in EtOH-fed mice compared to the pair-fed group, while liver injuries were attenuated by poly I:C treatment in the EtOH + PIC compared with those of the EtOH (Fig. 1B). In addition, poly I:C treatment decreased inflammatory cytokines such as TNF-α, MCP-1 and IL-6, whereas it significantly increased twofold the anti-inflammatory IL-10 levels in the sera of the EtOH + PIC compared with those of the EtOH (Fig. 1C). NK cell activation and interferon-γ production by poly I:C were