TLR2 and TLR9 contribute to alcohol-mediated liver injury through induction of CXCL1 and neutrophil infiltration

Yoon Seok Roh¹,², Bi Zhang², Rohit Loomba², and Ekihiro Seki¹,²

¹Division of Gastroenterology, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA and ²Division of Gastroenterology, Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, California 92093, USA

Running Head: Role of TLR2 and TLR9 in early alcoholic hepatitis

Address for Correspondence:

Ekihiro Seki, M.D., Ph.D.
Division of Gastroenterology, Department of Medicine,
Cedars-Sinai Medical Center
8700 Beverly Blvd., DAVIS, Suite 2099
Los Angeles, CA 90048
Phone) 310-423-6605
Fax) 310-423-0157
E-mail) Ekihiro.Seki@cshs.org

Copyright © 2015 by the American Physiological Society.
List of Abbreviations:

AH, alcoholic hepatitis; TLR, Toll-like receptors; WT, wild-type; KC, Kupffer cell; ALD, alcoholic liver disease; HCC, hepatocellular carcinoma; BMDM, bone marrow-derived macrophages; NASH, non-alcoholic steatohepatitis; ALT, alanine aminotransferase; EtOH, ethanol; H&E, hematoxylin and eosin; HSC, hepatic stellate cell; ox-LDL, oxidized low-density lipoprotein; TG, triglycerides.
Abstract

Although previous studies reported the involvement of the TLR4-TRIF pathway in alcohol-induced liver injury, the role of TLR2 and TLR9 signaling in alcohol-mediated neutrophil infiltration and liver injury has not been elucidated. Since an alcohol-binge drinking is recognized to induce more severe form of alcohol liver disease, we used a chronic-binge ethanol-feeding model as a mouse model for early stage of alcoholic hepatitis. While a chronic-binge ethanol-feeding induced alcohol-mediated liver injury in wild-type mice, TLR2 and TLR9-deficient mice showed reduced liver injury. Induction of neutrophil-recruiting chemokines, including Cxcl1, Cxcl2 and Cxcl5, and hepatic neutrophil infiltration were increased in wild-type mice, but not in TLR2 and TLR9-deficient mice. In vivo depletion of Kupffer cells (KCs) by liposomal clodronate reduced liver injury and the expression of Il1b, but not Cxcl1, Cxcl2 and Cxcl5, suggesting KCs are partly associated with liver injury, but not neutrophil recruitment, in a chronic-binge ethanol-feeding model. Notably, hepatocytes and hepatic stellate cells (HSCs) produce high amounts of CXCL1 in ethanol-treated mice. The treatment with TLR2 and TLR9 ligands synergistically upregulated CXCL1 expression in hepatocytes. Moreover, the inhibitors for CXCR2, a receptor for CXCL1, and MyD88 suppressed neutrophil infiltration and liver injury induced by chronic-binge ethanol-treatment. Consistent with the above findings, hepatic CXCL1 expression was highly upregulated in patients with alcoholic hepatitis. In a chronic-binge ethanol-feeding model, the TLR2 and TLR9-dependent MyD88-dependent pathway mediates CXCL1 production in hepatocytes and HSCs; the CXCL1 then promotes neutrophil infiltration into the liver via CXCR2, resulting in the development of alcohol-mediated liver injury.

Keywords: ALD; MyD88; chemokine; AH; binge ethanol feeding
**Introduction**

Alcoholic liver disease (ALD) is a result of chronic consumption of excessive alcohol. The clinical spectrum of ALD includes alcoholic fatty liver, alcoholic hepatitis (AH), alcoholic cirrhosis and hepatocellular carcinoma (12). Alcoholic fatty liver is considered a reversible and non-progressive entity. AH, the most severe form of ALD, is associated with high mortality; up to 40 % of severe AH patients die within 6 months (22). Although AH shows such a high mortality, treatment of AH is still largely dependent on corticosteroid for controlling inflammation, which has not been improved in the past 40 years (14). Survived AH patients may either have co-existing cirrhosis or it may progress to alcoholic liver cirrhosis and 3-10% of cirrhotic patients ultimately develop hepatocellular carcinoma (HCC).

It has been shown that ethanol itself and its metabolite-induced cytotoxicity are directly related to ethanol-induced liver injury and additional immune responses can further augment hepatocyte injury and can lead to cirrhosis as well as HCC (21). Accumulating evidences demonstrate that TLR-mediated innate immune response is associated with liver damage, inflammation, fibrosis and tissue remodeling. Chronic alcohol intake is known to increase LPS levels in systemic circulation due to the increased intestinal permeability by the disruption of epithelial tight junction (41). Increased LPS in the liver subsequently activates Kupffer cells (KCs) and bone marrow-derived macrophages (BMDM) through the binding to TLR4, contributing to the pathogenesis of ALD (15, 17). Indeed, mice deficient in TLR4, CD14 and LBP are protective against alcohol-induced liver injury (15, 17, 39, 43). Consistently, gut sterilization with oral administration of non-absorbable antibiotics reduces plasma LPS levels and alcohol-induced liver inflammation and injury in mice (1). Although TLR4 activates both MyD88 and TRIF-dependent intracellular signaling pathways, the TRIF-IRF3 axis appears to be
more critical than the MyD88-dependent pathway for the development of ALD (15, 29).

TLR2 and TLR9 recognize the surface structures of gram-positive bacteria (38) and the CpG containing DNA derived from virus and bacteria (2), respectively. We have previously reported that TLR2 and TLR9 signaling contribute to the development of non-alcoholic steatohepatitis (NASH) through activation of inflammasome and IL-1β signaling (27, 28). Since the common signaling pathways may be activated in both ALD and NASH, it is suggested that chronic alcohol consumption increases the hepatic and systemic levels of ligands for TLR2 and TLR9, which activate hepatic TLR2 and TLR9 signaling, resulting in induction of ALD. Indeed, serum levels of bacterial DNA are increased in patients with AH (5).

Neutrophil recruitment is strongly associated with the hepatocyte death in human AH (18, 45). Gao and colleagues recently established a new mouse model of ALD as chronic-binge ethanol feeding model that closely mimics drinking patterns of human alcoholics and exhibits the characteristics of the early stage of AH (6). In contrast to chronic ethanol-containing Lieber-De Carli diet model where liver macrophages play a role, the chronic-binge ethanol-feeding model induces hepatic neutrophil infiltration that recapitulates the feature of human AH. Depletion of neutrophils with anti Ly-6G blocking antibody suppressed liver injury in a chronic-binge ethanol feeding model, suggesting the crucial role of neutrophils in this model (6). Neutrophil infiltration promotes liver damage directly and/or indirectly through the generation of potent oxidants (hydrogen peroxide and chloramine) and serine proteases including proteinase-3 and elastase (30). Although the previous study has demonstrated that TLR2, 4 and 9 are involved in dysfunction of neutrophils in AH patients (35), the cellular and molecular mechanisms by which TLR2 and TLR9 signaling mediates the progression of AH and neutrophil infiltration have so far remained unclear.
In the present study, we investigated the functions of TLR2 and TLR9 signaling in hepatic neutrophil infiltration in the chronic-binge ethanol-feeding model, and examined the responsible cell types for the production of neutrophil chemoattractant, CXCL1. We also tested the potential of MyD88 blockade for the treatment of AH. Our results demonstrate that TLR2 and TLR9 signaling is required for CXCL1 production, neutrophil infiltration, and liver damage in the mouse model of early stage of AH.
Materials and Methods

Study Design

C57BL/6 wild type (WT) and TLR2-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME) and TLR9-deficient mice were originally generated by Dr. Akira (Osaka University, Suita, Japan). All mice including WT mice were bred in the University of California San Diego vivarium. TLR2-deficient and TLR9-deficient mice were back-crossed at least 10 generations onto the C57BL/6 background and displayed a similar hepatic phenotype as WT mice under standard laboratory chow. Female mice of each genotype were divided into two groups at 8-10 weeks of age: control diet and Lieber-DeCarli diet (Bio-Serv, Frenchtown, NJ) as followed by previous study (6). Each experiment included 6-8 mice/group and the data were the averages from at least two independent experiments.

For induction of mouse ALD, we followed the protocol of chronic plus binge ethanol feeding model as previously described (6) with slight modifications. Briefly, mice were fed with a control liquid diet \textit{ad libitum} for the first 5 days as an acclimatization step and subsequently fed with a Lieber-DeCarli diet (Bio-Serv, Frenchtown, NJ) containing 6.3% ethanol (vol/vol) for 10 days. In the morning on the 11th day, mice were subjected to receive single dose of binge ethanol (5g/kg of BW) and sacrificed 9 hours after the ethanol binge.

The mice received humane care according to National Institutes of Health recommendations outlined in the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

In vivo treatment
A MyD88 inhibitory peptide and a control peptide (100µg/mouse, Novus, Littleton, CO) were injected intraperitoneally twice (12 hours and 1 hour prior to binge). A selective CXCR2 antagonist (SB225002, 25mg/kg, Tocris, Minneapolis, MN) was administered orally 1 hour before binge. To deplete Kupffer cells, ethanol-fed mice were given clodronate liposome injections (200µl/mouse) intraperitoneally for two consecutive days (48 hours and 24 hours prior to binge).

In vitro and ex vivo study

Hepatocytes, KCs and hepatic stellate cell (HSCs) were isolated from mice fed a control and an ethanol-containing diet as previously described (27). In some experiments using hepatocytes, 200 µl of liposomal clodronate was injected intravenously 1 day before isolation to exclude the KC contamination. Hepatocytes, KCs and HSCs were cultured in serum-free M199, DMEM and RPMI 1640 (Gibco, Life Technologies, Grand Island, NY), respectively, for 16 hours before treatment with the specific ligands. Pam3CSK4 (200 ng/ml, Invivogen, San Diego, CA) and ODN1668 (5 µg/ml, Invivogen, San Diego, CA) were used to stimulate liver cells.

Histological Analysis

Mouse liver tissues were collected, fixed in 10% neutral buffered formalin solution for 48 h, routinely processed, and then embedded in paraffin. Tissue sections (4 µm) were prepared using a microtome (HM-340E, Thermo Fisher Scientific Inc., Waltham, MA) and placed on glass slides. Hematoxylin and eosin (H&E), TUNEL and immunohistochemical staining for Ly-6G (eBiosciences, San Diego, CA), F4/80 (eBiosciences, San Diego, CA), and CXCL1 (LifeSpan Bioscience, Seattle, WA) were performed. F4/80 and CXCL1 positive area were measured on at
The number of TUNEL or Ly-6G positive cells were counted on at least 8 random fields/slide and expressed as cells/HPF.

**RNA Isolation and quantitative RT-PCR Analysis**

RNA was extracted from mouse liver tissues and cells using TRIzol (Life Technologies, Grand Island, NY) plus column kit (NucleoSpin®, Clontech, Mountain View, CA), and treated with DNase I (Promega, Madison, WI). Extracted RNA was converted to complementary DNA using reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was then performed using a CFX96 Real Time System (Bio-Rad Laboratories, Irvine, CA) using SYBR Green I as a double-strand DNA-specific binding dye. After the reaction was completed, specificity was verified by melting curve analysis. Quantification was performed by comparing Ct values of each sample with normalization to 18S RNA. Sequences of primers were summarized in Table 1.

**Measurement for ALT and CXCL1**

Serum alanine aminotransferase (ALT) levels were measured by Infinity ALT Reagent (Thermo, Waltham, MA). The levels of CXCL1 in serum and cell supernatant were analyzed by ELISA kits (R&D, Minneapolis, MN).

**TG extraction and Measurement**

Hepatic triglycerides (TG) were extracted as described (27). TG contents were measured
using Triglyceride Measurement Kit (Pionte Scientific, Canton, MI) according to the manufacturer's instructions.

**Human Samples**

Unstained slides were obtained using the paraffin-embedded human liver tissue blocks of patients with AH and control patients with normal liver histology who had a clinical indication of a liver biopsy, and immunohistochemical staining was performed for CXCL1, and Ly-6G was used to assess neutrophil infiltration.

**Statistical Analysis**

All data were expressed as the mean ± standard error. Differences between two groups were compared using a two-tailed Student’s t-test. Correlation was analyzed by Spearman correlation test. A p-value < 0.05 was considered statistically significant.
**Results**

**TLR2 and TLR9 are required for liver injury and expression of neutrophil-recruiting chemokines in mice after chronic-binge alcohol treatment**

First, we investigated the role of TLR2 and TLR9 in the chronic-binge ethanol-feeding model using WT, TLR2 and TLR9-deficient mice. In WT mice, the early stage of AH was demonstrated by elevated serum ALT levels, hepatocytes death, neutrophil infiltration and steatosis. In contrast, TLR2 and TLR9-deficient mice showed reduced serum ALT levels and decreased number of TUNEL positive hepatocytes. However, degree of steatosis, as assessed by hepatic TG levels, was similar between WT and TLR2 or TLR9-deficient mice (Fig. 1A-B). The levels of proinflammatory cytokines, such as \( \text{Il1b} \), \( \text{Il6} \) and \( \text{Tnf} \) were elevated (1.5-2 fold) in WT livers while the hepatic levels of \( \text{Il1b} \) and \( \text{Il6} \) were significantly reduced in TLR2-deficient mice and only \( \text{Il1b} \) was suppressed in TLR9-deficient mice after the chronic-binge ethanol feeding (Fig. 1C). Since previous study showed the crucial role of neutrophils in the development of liver injury induced by chronic-binge ethanol feeding (6), we assessed neutrophil recruitment and neutrophil-recruiting chemokines. The expression of neutrophil-attracting chemokines, such as \( \text{Cxcl1} \), \( \text{Cxcl2} \), \( \text{Cxcl5} \) and \( \text{Ccl2} \), was markedly increased 6-15 fold in ethanol-treated WT livers whereas the expression of these chemokines were reduced in ethanol-treated TLR2 and TLR9-deficient mice (Fig. 1D). Increased hepatic protein expression of CXCL1 and serum level of CXCL1 in WT mice were significantly suppressed in TLR2 and TLR9-deficient mice (Fig. 1E). Accordingly, neutrophil infiltration was also suppressed in TLR2 and TLR9-deficient mice as assessed by the expression of Ly-6G (Fig. 1F). These results indicate that TLR2 and TLR9 signaling contribute to liver damage, production of neutrophil-recruiting chemokines and neutrophil infiltration in the chronic-binge ethanol-feeding model.
Hepatic expression of neutrophil-recruiting chemokines is independent of Kupffer cells in chronic-binge ethanol-feeding model

KC is the primary cell type to produce inflammatory cytokines involved in the pathogenesis of ALD (17, 34). We examined the role of KCs in neutrophil recruitment and liver injury using chronic-binge ethanol-feeding model. KCs were depleted by injection of liposomal chlodronate and the mice were subjected to the chronic-binge ethanol feeding. The depletion of KCs was confirmed by blunted hepatic expression of F4/80 (Fig. 2A and 2E). We observed the reduction of serum ALT levels accompanied by the decreased expression of proinflammatory cytokines, including \textit{Il1b}, \textit{Il6} and \textit{Tnf} in KC-depleted mice compared with vehicle-treated ones (Fig. 2B and 2C). Intriguingly, the expression of neutrophil-recruiting chemokines was not decreased by KC depletion, suggesting that the source of neutrophil chemoattractants is the cells other than KCs in this model (Fig. 2D). Consistently, KC depletion did not alter the infiltration of Ly-6G-positive neutrophils (Fig. 2E). These results indicate that, in addition to neutrophils previously described, KCs partly contribute to the induction of liver injury, but are not associated with production of neutrophil-recruiting chemokines in chronic-binge ethanol-feeding model.

Hepatocytes and hepatic stellate cells are responsible for producing CXCL1 in mouse ALD

To identify the cellular sources of neutrophil-attracting chemokines, other than KCs, in chronic-binge ethanol feeding model, we isolated different liver cell populations including hepatocytes, HSCs, and KCs. Hepatocytes and HSCs isolated from ethanol-fed mice had increased mRNA expression of \textit{Cxcl1} compared with cells from pair-fed animals. Interesting,
KCs from ethanol-fed mice showed reduced expression of Cxcl1 compared with KCs from pair-fed mice (Fig. 3A), which supports our data showing that KCs have minor roles in neutrophil infiltration induced by chronic-binge ethanol treatment (Fig. 2). To further investigate the mechanism underlying TLR2 and TLR9-dependent CXCL1 production, we treated primary cultured hepatocytes with a synthetic TLR2 ligand (Pam3CSK4) and TLR9 ligand (ODN1668). The ODN1668 alone could not induce Cxcl1 expression, but pretreatment with Pam3CSK4 enabled primary hepatocytes to produce CXCL1 in response to ODN1668 (Fig. 3B). Notably, the synergistic effect induced by TLR2 and TLR9 ligands was observed only in hepatocytes, but not in HSCs (Fig. 3C). These results indicate that TLR2 and TLR9 signaling has capacity to induce CXCL1 production in hepatocytes and HSCs.

A selective CXCR2 blockade inhibits neutrophil infiltration and alcohol-mediated liver injury

Since CXCL1 is a potent neutrophil-recruiting chemokine, we examined the correlation between liver injury and CXCL1 levels in mice with or without chronic-binge ethanol feeding. As illustrated in Fig. 4A, serum ALT levels positively correlate with serum CXCL1 levels in mice. These results prompted us to test whether systemic blockade of CXCR2, a receptor for CXCL1, has a therapeutic effect on liver injury through inhibiting neutrophil recruitment in chronic-binge ethanol-feeding model. The treatment with SB225002, a selective CXCR2 antagonist, suppressed liver injury as demonstrated by reduced serum ALT levels (Fig. 4B). The expression of neutrophil markers (Ly6g and Elane) and the infiltration of Ly-6G-positive neutrophils were also reduced in mice treated with SB225002 compared with vehicle-treated mice (Fig. 4C and 4D). These results suggested that inhibition of the CXCL1-CXCR2 axis
suppresses liver injury through suppressing neutrophil infiltration in chronic-binge ethanol-feeding model.

MyD88 inhibition attenuates CXCL1 production and liver injury in chronic-binge ethanol-feeding model.

Given that both TLR2 and TLR9-deficient mice were protected against alcohol insults (Fig. 1-4), MyD88, a common adaptor molecule for IL-1 receptor and all TLRs, except for TLR3, may play a crucial role in liver injury induced by chronic-binge ethanol-treatment. To test the therapeutic potential of MyD88 inhibition, we treated mice with a MyD88 inhibitory peptide. Inhibiting MyD88 significantly suppressed liver injury (Fig. 5A) and serum CXCL1 levels in the chronic-binge ethanol-treated mice (Fig. 5B). These results were further supported by the reduction of CXCL1 and neutrophil markers in the liver (Fig. 5C and 5D). The MyD88 inhibition also reduced the hepatic $\text{Il1b}$ expression, but not $\text{Il6}$ and $\text{Tnf}$, which resembles the results from TLR2 and 9-deficient mice after the chronic-binge ethanol-treatment (Fig.1C).

These results demonstrated that the MyD88 blockade could prevent liver damage through the suppression of CXCL1, IL-1β production and neutrophil recruitment in acute-on-chronic ethanol feeding model.

Hepatic CXCL1 levels are upregulated in patients with alcoholic hepatitis

To understand the clinical relevance of CXCL1 in human disease, we analyzed hepatic CXCL1 expression in patients with AH. Consistent with the animal study, CXCL1 expression
was strongly upregulated and neutrophil infiltration was increased in AH patients (Fig. 6A and 6B). Our data suggests that the upregulation of hepatic CXCL1 and neutrophil recruitment are associated with the development of AH.

Discussion

The present study clearly demonstrated that activation of TLR2 and TLR9 signaling contributes to hepatic production of CXCL1, recruiting neutrophils into the liver in the chronic-binge ethanol feeding model, a mouse model of early stage AH. Our data also demonstrated that the biological relevant cellular source of CXCL1 is not KCs. Instead, we found that hepatocytes and HSCs are the major cellular sources of CXCL1. Notably, there is a synergistic effect of TLR2 and TLR9 signaling to induce CXCL1 production in hepatocytes, which induces the recruitment of neutrophils into the liver. Increased serum CXCL1 levels correlate with serum ALT in the current ethanol-feeding model. Furthermore, we demonstrated the therapeutic potential of CXCR2 blockade (a responsible receptor for CXCL1) and MyD88 inhibitor (a common adaptor molecule for all TLRs, except for TLR3, and IL-1 receptor) in the chronic-binge ethanol-feeding model. Finally, we demonstrated that CXCL1 expression was highly upregulated in patients with AH. Taken together, our data indicates that TLR2 and TLR9 signaling mediates CXCL1-mediated neutrophil infiltration and liver injury in acute-on-chronic ethanol-feeding model.

TLR2 and TLR9 are the sensors for the cell wall components derived from gram-positive bacteria (38) and the unmethylated CpG-containing DNA derived from virus and bacteria (2), respectively. TLR2 also recognizes a number of endogenous ligands, such as
saturated fatty acids, serum amyloid A, and oxidized low-density lipoprotein (ox-LDL) (13, 40, 42). On the other hand, denatured DNA produced from damaged cells and mitochondria can be recognized by TLR9 (16, 44). Of note, alcohol consumption has been reported to induce oxidization of LDL (3). These findings were further confirmed by the positive correlation of alcohol intake (10 g per day) with an increase of 2.4 U/L of circulating ox-LDL (31). Furthermore, ethanol treatment alters the permeability of mitochondrial membrane and promotes mitochondrial swelling through mitochondrial ROS production, thereby leading to mitochondrial damage (23). Thus, liver cells may be exposed to sufficient levels of TLR2 and TLR9 ligands during the excessive alcohol consumption.

The previous studies reported that chronic alcohol-induced liver damage and inflammation are prevented by TLR4 deficiency, but not MyD88 deficiency (15, 17). The TLR4-dependent TRIF-IRF3 axis mediates alcohol-induced liver injury through activation of pro-apoptotic Bax in hepatocyte (29). Thus, MyD88-dependent pathway has been underestimated in the pathogenesis of ALD. All TLRs, except for TLR3, and IL-1 receptor use MyD88 as an adaptor molecule, which activate NF-κB and MAPK pathways (37). These signaling cascades lead to the production of various cytokines and chemokines (7). In contrast to ALD, MyD88 has been highlighted as a critical regulator in various liver diseases including liver fibrosis, NASH and HCC (32, 33). Recently, concerns about the experiments using whole-body MyD88-knockout (KO) mice have been discussed. Since MyD88 KO mice are severely immunocompromised and their commensal bacteria are often altered, functional compensation could occur, thereby masking or distortion of the phenotypes of these KO mice (25, 36). More careful and intensive investigation on the function of MyD88 in the pathogenesis of ALD should be facilitated. We will discuss in more detail below.
Neutrophil infiltration is a prominent feature of AH in patients and significantly correlates with the severity and the mortality of AH patients (18, 45). However, the mechanism of neutrophil infiltration in AH has not been fully elucidated. Our data demonstrated that TLR2 and TLR9, two TLRs that activate MyD88-dependent pathway, are critical for neutrophil recruitment and its mediated pathogenesis in the chronic-binge ethanol-feeding model. These results were supported by the previous report showing that doxycycline-induced neutrophil infiltration is dependent on MyD88, TLR2, and TLR9, but independent of TRIF, TLR3, and TLR4 (20). Of note, CXCL1 production exclusively depends on the signaling through MyD88-dependent pathway, but not TRIF. This may be explained by the fact that the CXCL1 promoter contains only NF-κB site, but not IRF site (9). Therefore, CXCL1 production is predominantly regulated by MyD88-dependent pathway.

KC is a major producer of pro-inflammatory cytokines to play crucial roles in the development of ALD and NASH (17, 34). In the chronic-binge ethanol-feeding model, in vivo depletion of KCs did not affect the production of neutrophil-recruiting chemokines and neutrophil infiltration in the liver, indicating that neutrophil-mediated alcohol-mediated liver injury is independent of KCs. The KC-mediated pathogenesis of liver injury may be mediated through production of inflammatory cytokines in the chronic-binge ethanol-feeding model. Other than KCs, our study highlighted hepatocytes and HSCs as the responsible cell types to produce neutrophil attracting chemokines in ALD. During neutrophil migration and adhesion into the endothelial cell, they encounter immobilized chemokines from extravascular cells, which would give them further directional cue for migration and infiltration (11, 26). These extravascular cells could be hepatocytes and HSCs in current model. Importantly, hepatocytes showed the synergistic effect induced by TLR2 and TLR9 ligands. Given that endocytosis is a prerequisite
for proper endosomal TLR responses, hepatocytes generally do not respond to TLR9. Interestingly, pretreatment with a TLR2 ligand enables hepatocytes to synergistically respond to the TLR9 ligand to produce CXCL1. In same line with our present study, it has been reported that the co-treatment with TLR2 and TLR9 ligands induces the synergistic effect on immune responses upon bacterial infection (10). Moreover, accumulating evidences have suggested that TLR2 activation increases endocytotic activity through activation of Rab5 (4, 19). Thus, the synergistic effects of TLR2 and TLR9 may be mediated by TLR2-induced enhancement of endocytosis of TLR9 ligand to hepatocytes.

The previous study reported by Hritz et al demonstrated the redundancy of TLR2 and MyD88 in chronic feeding model of Lieber-DeCarli diet containing ethanol (15). In our study using the chronic-binge ethanol-feeding model, TLR2 and TLR9 signaling, and MyD88 are required for the production of IL-1β and neutrophil-attracting chemokines and neutrophil recruitment, thereby inducing liver injury. In contrast, TLR2 and TLR9 signaling, and MyD88 are dispensable for production of KC-derived inflammatory cytokines, such as IL-6 and TNFα, which, in part, corroborates with the findings observed by Hritz et al (15). KC, a main producer of IL-6 and TNFα, is a crucial cell type in the conventional chronic Lieber-DeCarli diet model (15), but KC does not play a major role in the recruitment of neutrophils in the chronic-binge ethanol-feeding model. Compared with the conventional chronic Lieber-DeCarli diet model, the chronic-binge ethanol-feeding model shows high degrees of liver injury and inflammation, and neutrophil infiltration (6). The previous study showed that depletion of neutrophil with neutralizing antibody (Anti-Ly-6G Ab) inhibits liver injury (6). Thus, the chronic-binge ethanol-feeding model recapitulates neutrophil-mediated early stage of AH. In contrast, in the conventional Lieber-DeCarli diet model, neutrophil may not play a major role, and the
pathogenesis may be mainly mediated by KCs.

Mortality and morbidity of AH is still very high (8). However, corticosteroids and pentoxifylline are the only drugs currently available for the treatment of AH (14). Therefore, we have to seek new and effective interventions for AH. Since AH is thought to be neutrophil-dependent (18, 45) and the chronic-binge ethanol-feeding model causes liver injury driven by neutrophils (6), we took advantages of the chronic-binge ethanol-feeding model to test the new therapeutic agents. Our present study demonstrated that TLR2, TLR9 and CXCL1 are crucial factors for the development of acute-on-chronic mouse ALD and suggests that these molecules can be targets for the treatment of ALD. MyD88 is a common denominator for signaling of IL-1 signaling and all TLRs (except for TLR3) including TLR2 and TLR9 (37). We postulated that modulation of MyD88 could regulate the detrimental response by alcohol in the liver. To examine the protective effect of MyD88 inhibition on alcohol-induced liver injury, we administered a MyD88 inhibitory peptide to the chronic-binge ethanol-treated mice. The MyD88 inhibition successfully suppressed the production of neutrophil-recruiting chemokines and IL-1β, but not of IL-6 and TNFα. MyD88 inhibitor may be insufficient for complete suppression of IL-6 and TNFα while this inhibitor sufficiently reduced neutrophil recruitment and liver injury in the chronic-binge ethanol-feeding model. We also tested the preventive effect of CXCR2 blockade on the chronic-binge alcohol-feeding model. CXCR2, a responsible receptor for CXCL1, is highly expressed on neutrophils (24). CXCR2 blockade with a selective antagonist successfully inhibited neutrophil chemotaxis to the ethanol-treated livers. Collectively, the TLR-MyD88 signaling and the CXCL1-CXCR2 interaction can be attractive targets for the treatment of neutrophil-mediated early stage AH.

In conclusion, TLR2 and TLR9 promote CXCL1 production in hepatocytes and HSCs,
which in turn induces neutrophil recruitment into the liver via CXCR2. This pathway plays a central role for the pathogenesis of alcohol-induced neutrophil-mediated liver injury. We also demonstrated that MyD88 inhibition and CXCR2 blockage attenuate alcohol-induced liver injury. Thus, targeting TLR2, TLR9, MyD88 and CXCL1-CXCR2 to restrict neutrophil infiltration may become a novel, effective intervention for the early stage of AH.

Grants:

This study was supported by NIH grant R01AA02172 (E. Seki), R01DK085252 (E. Seki); and by the 2014 Congressman John Joseph Moakley Postdoctoral Research Fellowship from American Liver Foundation (Y.S. Roh). RL is supported in part by the American Gastroenterological Association (AGA) Foundation – Sucampo – ASP Designated Research Award in Geriatric Gastroenterology and by a T. Franklin Williams Scholarship Award; Funding provided by: Atlantic Philanthropies, Inc, the John A. Hartford Foundation, the Association of Specialty Professors, and the American Gastroenterological Association and grant K23-DK090303.

Disclosures: There is no conflict of interest to disclose for all authors.

Author’s Contributions:

Y. S. R.: study design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript; B. Z.: acquisition and analysis of data; R. L.: analysis and
interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding; E. S.: study supervision, study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis, obtained funding.

References


11. Feng D, Nagy JA, Pyne K, Dvorak HF, and Dvorak AM. Neutrophils emigrate from venules...


Figure Captions

**Figure 1.** TLR2 and TLR9-deficient mice exhibit reduced liver injury and neutrophil-recruiting chemokine expression in chronic plus binge ethanol-induced ALD

(A-E) WT, TLR2 and TLR9-deficient female mice were subjected to control and Lieber-DeCarli diet (n=10 per group for Pair-fed, n=14-16 per group for EtOH-fed; each experiment was performed with 5 to 8 mice per group and repeated two times). (A) Liver injury was assessed by measuring serum ALT levels. (B) Hepatocyte death was analyzed by TUNEL staining and TUNEL-positive cells were counted. Hepatic steatosis was determined by measuring hepatic TG levels. The results are expressed as mg of TG per g of liver. (C) Expression of proinflammatory cytokines (*Il1b, Il6* and *Tnf*) was determined by qRT-PCR and shown as fold change compared with pair-fed WT mice. (D) Expression of neutrophil-recruiting chemokines (*Cxcl1, Cxcl2, Cxcl5* and *Cxcl5*)...
and Ccl2) was determined by qRT-PCR and shown as fold change compared with pair-fed WT mice. (E) Hepatic expression of CXCL1 was assessed by immunohistochemistry and quantified by measuring CXCL1-positive area. Serum CXCL1 levels were measured by ELISA. (F) The hepatic neutrophil infiltration was determined by immunohistochemistry for Ly-6G and the number of Ly-6G-positive cells was counted. Data are presented as means ± SEM per group. *p < 0.05; n.s., not significant; n.d., not detected. Original magnification, ×100 (H&E), ×200 (TUNEL, CXCL1 and Ly-6G).

Figure 2. Hepatic expression of neutrophil-recruiting chemokines induced by alcohol is independent of Kupffer cells

(A-E) In vivo depletion of Kupffer cells (KCs) was achieved by injection of clodronate liposome (200 µl/mouse) for 2 consecutive days (n=6 per group). (A) KC depletion was confirmed by hepatic expression of F4/80 using qRT-PCR. (B) Liver injury was assessed by measuring serum ALT levels. (C) Expression of proinflammatory cytokines (Il1b, Il6 and Tnf) was determined by qRT-PCR and shown as fold change compared with vehicle-treated pair-fed mice. (D) Expression of neutrophil-recruiting chemokines (Cxcl1, Cxcl2 and Cxcl5) was determined by qRT-PCR and shown as fold change compared with vehicle-treated pair-fed mice. (E) KCs and neutrophils were stained by immunohistochemistry for F4/80 and Ly-6G, respectively. (F) The quantification was done by measuring F4/80-positive area or counting Ly-6G-positive cells. Data are presented as means ± SEM per group. **p < 0.01, ***p < 0.001; n.s., not significant. Original magnification, ×200 (F4/80 and Ly-6G).
Figure 3. Hepatocytes and hepatic stellate cells are the major cell types for the production of CXCL1

(A-C) Each liver fraction (hepatocytes, HSCs and KCs) was isolated from livers of EtOH and Pair-fed mice. Representative results are presented from two independent experiments (each isolation was assayed in triplicate). (A) The Cxcl1 expression in each fraction was determined by qRT-PCR and shown as fold change compared with those of pair-fed mice. (B-C) Hepatocytes and HSCs were pretreated with Pam3CSK4 (200 ng/ml), and then ODN1668 (5µg/ml) was added for 30 minutes (qRT-PCR) and 6 hours (ELISA). The Cxcl1 mRNA and CXCL1 protein levels in hepatocytes (B) and HSCs (C) were determined by qRT-PCR and ELISA, respectively. Data are presented as means ± SEM per group. *p < 0.05, **p < 0.01; n.s., not significant.

Figure 4. Treatment with a CXCR2 antagonist attenuates alcohol-induced neutrophil-mediated liver injury

(A) Correlation between serum CXCL1 and ALT levels in mice with or without ALD was analyzed by Spearman correlation test. (B-D) The CXCR2 blockade was accomplished by an oral administration of a selective antagonist, SB225002 (n=16 per group; each experiment was performed with 8 mice per group and repeated two times). (B) Liver injury was assessed by measuring serum ALT levels. (C) Expression of neutrophil markers (Ly6g and Elane) was determined by qRT-PCR and shown as fold change compared with vehicle-treated mice. (D) The neutrophil infiltration was determined by immunohistochemistry for Ly-6G and its quantification was done by counting Ly-6G-positive cells. Data are presented as means ± SEM per group. *p < 0.05. Original magnification, ×200 (Ly-6G).
Figure 5. Blocking MyD88 protects against alcohol-mediated CXCL1 production and liver injury

(A-D) A MyD88 inhibitory peptide and a control peptide (100µg/mouse) were administered with i.p. injection to EtOH-fed WT mice (n=16 per group; each experiment was performed with 8 mice per group and repeated two times). (A) Liver injury was assessed by measuring serum ALT levels. (B) Serum CXCL1 levels were measured by ELISA. (C) Expression of Cxcl1, neutrophil markers (Ly6g and Elane) and proinflammatory cytokines (Il1b, Il6 and Tnf) was determined by qRT-PCR and shown as fold change compared with vehicle-treated mice. (D) Hepatic expression of CXCL1 and neutrophil infiltration were determined by immunohistochemistry for CXCL1 and Ly-6G, respectively. Their quantification was performed. Data are presented as means ± SEM per group. *p < 0.05, **p < 0.01; n.s., not significant. Original magnification, ×200 (CXCL1 and Ly-6G).

Figure 6. Hepatic CXCL1 expression is upregulated in patients with alcoholic hepatitis

(A) Hepatic CXCL1 expression was determined by immunohistochemistry on liver biopsy specimens from control individuals or patients with AH (n=5 for control, n=10 for patient with AH). The quantification was done by measuring CXCL1-positive area. (B) Neutrophil infiltration was analyzed by immunohistochemistry for Ly-6G and its quantification on liver biopsy specimens of patients with AH. Data are presented as means ± SEM per group. **p < 0.01. Original magnification, ×200 (CXCL1), ×400 (Ly-6G).
Figure 2

A

**

F4/80 mRNA

Vehicle Clod.

EtOH-fed

B

*

ALT (U/L)

Vehicle Clod.

EtOH-fed

C

**

Il1b mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

**

I6 mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

***

Trm mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

D

n.s.

Cxc1 mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

n.s.

Cxc2 mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

n.s.

Cxc5 mRNA

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

E

F4/80 Ly-6G

Pair-fed Vehicle Vehicle Clod.

EtOH-fed

F

F4/80

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

Ly-6G

n.s.

Vehicle Vehicle Clod.

Pair-fed EtOH-fed

Positive area (%) Positive (cells/HPF)
Figure 3

A

Hepatocytes

Stellate cells

Kupffer cells

B

Hepatocytes

ODN 1668

Pam3CSK4

-  

+  

-  

+  

C

Stellate cells

ODN 1668

Pam3CSK4

-  

+  

-  

+  

n.s.

n.d.
Figure 4

A

Spearman correlation coefficient
$r=0.5561, p<0.0001$
$n=54$

B

C

D

Ly-6g mRNA

Elane mRNA

Ly-6G positive (cells/HPF)
Figure 5

A

B

C

D

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i

Ctrl Peptide  MyD88 i
Figure 6

A

CXCL1

No-EtOH

EtOH

CXCL1-kC positive area (%)

No-EtOH

EtOH

B

Ly-6G

No-EtOH

EtOH

Ly-6G positive (cells/hpf)

No EtOH

EtOH

**

*

*
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>AGTCCCTGCCCTTTGTACACA</td>
<td>CGATCCGAGGGCCTCCTA</td>
</tr>
<tr>
<td>Il1b</td>
<td>GGTCAAGGTTGGGAAGCAG</td>
<td>TGTGAAATGCCACCTTTTGA</td>
</tr>
<tr>
<td>Il6</td>
<td>ACCAGAGGAAATTTCAATAGGC</td>
<td>TGATGCACTTGCAAGAAAACA</td>
</tr>
<tr>
<td>Tnf</td>
<td>AGGGTCTGGGCCATAGAAC</td>
<td>CCACCACGCTCTTCTGTCTAC</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>TGCAACCAAAACGGGAAGTC</td>
<td>GTCAGAAGCCACGGTCCTAC</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>AAAGTTTGCTTGGACCCCTGAA</td>
<td>CTCAGACAGCGAGCACTC</td>
</tr>
<tr>
<td>Cxcl5</td>
<td>TGATCCCTGCAGGTCACA</td>
<td>CTGCGAGTGCATCCCCCTTA</td>
</tr>
<tr>
<td>Ly6g</td>
<td>TGCGTTGGCTCTGGAGATAGA</td>
<td>CAGAGTAGGGGCGAGATGG</td>
</tr>
<tr>
<td>F4/80</td>
<td>ATCTCCCTGGATGTTGCTTGGCTTG</td>
<td>AGCCGTCTGGTTGTCACTTGG</td>
</tr>
<tr>
<td>Elane</td>
<td>ACAACTGCTGAACGACATTGTGA</td>
<td>TGCACGTGGGCTTTAATGGTA</td>
</tr>
<tr>
<td>Ccl2</td>
<td>ATTTGGGATCATCTTGGTGTG</td>
<td>CCTGCTTTCACAGTTGCC</td>
</tr>
</tbody>
</table>