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Myeloid-MyD88 contributes to ethanol-induced liver injury in mice linking hepatocellular death to inflammation

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Abstract

Background—Toll-like receptor 4 (TLR4) is critical for ethanol-induced liver injury. TLR4 signaling is mediated by two proximal adaptor molecules: myeloid differentiation primary-response protein (MyD88) and TLR-domain-containing adapter-inducing interferon- β (TRIF). Studies utilizing global knock-outs of MyD88 and TRIF identified a predominant role for TRIF signaling in the progression of ethanol-induced liver injury. In contrast, IL1 receptor, which signals solely via the MyD88 pathway, is also known to mediate ethanol-induced liver injury. We postulated that a cell specific role for MyD88 in myeloid cells might explain these apparently discrepant roles of MyD88. Here we made use of myeloid-specific MyD88-deficient (MyD88^{LysM-KO}) mice generated by crossing LysM-CRE mice with MyD88^{fl/fl} mice to test this hypothesis.

Methods—MyD88^{LysM-KO} and littermate controls were fed a Lieber-DeCarli ethanol containing diet or pair-fed control diets for 25 days.

Results—Littermate control, but not MyD88^{LysM-KO}, mice developed early stages of ethanol-induced liver injury including elevated plasma ALT and increased hepatic triglycerides. Lobular

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inflammation and expression of pro-inflammatory cytokines/chemokines was increased in control but not MyD88^{LysM-KO}. Further, ethanol-induced inflammasome activation, indicated by the presence of cleaved caspase-1 and mature IL-1 β protein, were also ameliorated in livers of MyD88^{LysM-KO} mice. In contrast, chronic ethanol-induced apoptosis, assessed via TUNEL staining, was independent of myeloid-MyD88 expression.

Conclusions—Collectively, these data demonstrate a cell specific role for MyD88 in the development of chronic ethanol-induced liver injury. While MyD88^{LysM-KO} still exhibited hepatocellular apoptosis in response to chronic ethanol, the absence of MyD88 on myeloid cells prevented the development of hepatic steatosis and inflammation.

Keywords

alcoholic liver disease; TLR4; MyD88; hepatic macrophages

Introduction

Alcohol causes a dysbiosis of the gut microflora associated with an increased intestinal permeability, resulting in increased translocation of bacterial products into the circulation (Rao et al., 2004; Nagy et al., 2016). The interaction of alcohol with bacterial products (peptidoglycan, LPS, flagellin, CpG DNA) and cytokines (such as TNF α , IL1 and IL6) alters the metabolism and immune response in the liver, causes liver injury and chronic inflammation, leading to alcoholic liver disease (ALD) (Wang et al., 2012; Nagy et al., 2016). Among the different toll-like receptors (TLR), evidence shows that TLR4 signaling is required for the development and pathogenesis of ALD (Roh and Seki, 2013). TLR4 has ability to activate two distinct pathways: MyD88-dependent and MyD88-independent/TRIF-dependent pathways (Takeda and Akira, 2015). The MyD88-dependent pathway is mediated by the adaptors MyD88 and TIRAP, leading to activation of NF κ B and MAPK family members and induction of inflammatory cytokines (Yamamoto et al., 2003; Takeda and Akira, 2015). The MyD88-independent pathway involves the adaptors TRIF and TRAM, leading to activation of IRF3 and induction of Type I interferons (IFNs), as well as NF κ B activation (Fitzgerald et al., 2003; Zhao et al., 2008). Making use of global knock-outs for TLR4, MyD88 and TRIF, studies have shown that chronic ethanol-induced liver injury is predominantly mediated via TLR4 and the MyD88-independent TRIF/IRF3-dependent cascade, but not the MyD88 pathway (Uesugi et al., 2001a; Zhao et al., 2008). However, it is important to note that the IL1 receptor (IL1R), which exclusively signals through the MyD88 pathway, is also required for the pathogenesis of ALD (Petrasek et al., 2012). Recent evidence indicates that activation of TLR4 signaling by low concentrations of LPS, similar to those observed both in patients with ALD and murine models of chronic ethanol exposure, results in the formation of a MyD88-IRAKM myddosome, leading to the activation of NF κ B and expression of inflammatory genes (Zhou et al., 2016). Importantly, IRAKM is only expressed in myeloid cells and IRAKM-deficient mice are protected from chronic ethanol-induced liver disease (Zhou et al., 2016). Thus, from the data available, it remains unclear how the MyD88-dependent pathway participates and contributes to the development and pathogenesis of ALD.

While the activity of MyD88 on immune cells is most well understood, MyD88 is expressed in multiple cell types that can contribute to ALD, including hepatocytes (Duparc et al., 2016), endothelial cells (Jagavelu et al., 2010), intestinal epithelial cells (Everard et al., 2014), as well as cells in the central nervous system (Kleinridders et al., 2009). There is accumulating evidence from other models of chronic inflammatory diseases that there are cell-specific functions for MyD88. For example, there is an interaction between MyD88 on endothelial cells and myeloid cells in the development of high-fat diet induced inflammation and insulin resistance (Yu et al., 2014). Further, hepatocyte MyD88 contributes to the regulation of bile acids and specific nuclear receptors that regulate glucose and lipid metabolism (Duparc et al., 2016). Therefore, it is possible that there is a cell-type specific interplay between the contributions of MyD88 in multiple cell types during progression of ALD.

Taken together, these data led us to hypothesize that myeloid MyD88 may specifically contribute to the progression of chronic ethanol-induced liver injury. Here we have used myeloid-specific MyD88-deficient (MyD88^{LysM-KO}) mice to study specific contributions of MyD88 in myeloid cells during progression of ethanol-inducing liver injury. Loss of MyD88 in myeloid cells reduced ethanol-induced increases in plasma ALT and hepatic steatosis. Ethanol-induced lobular inflammation and expression of pro-inflammatory mediators was also attenuated in livers of MyD88^{LysM-KO} mice. Loss of MyD88 in myeloid cells also blunted the accumulation of mature IL-1 β and cleaved caspase-1, indicating a reduction in activation of the inflammasome. Taken together, these results indicate that myeloid-MyD88 participates in multiple aspects of ethanol-induced liver injury.

Methods

All authors had access to the study data and reviewed and approved the final manuscript.

Materials

MyD88^{fl/fl} and LysM-CRE mice were purchased from Jackson Labs (Bar Harbor, Maine). A colony of LysM-CRE-MyD88^{fl/+} mice was established at the Cleveland Clinic by serial crossing the parental MyD88^{fl/fl} strain with the LysM-CRE strain. For experiments, the LysM-CRE-MyD88^{fl/+} mice were crossed with MyD88^{fl/fl} to generate MyD88^{LysM-KO}. Littermate LysM-CRE-MyD88^{fl/+} mice were used as controls. Lieber-DeCarli high-fat diet ethanol and control diets were purchased from Dyets (Bethlehem, PA).

Antibodies were from the following sources: CYP2E1 and CD45 (AbCAM, Cambridge, MA), 4-hydroxynonenal (Alpha Diagnostics, San Antonio, TX), TNF α (Fitzgerald Inc, North Acton, MA), F4/80 (AbD Serotec, Raleigh, NC), HSC70 and β -actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Anti-IL-1 β antibody (3ZD) was obtained from the Biological Resources Branch of the NIH. Anti-caspase-1 (p20) antibody was generated as previously described by L. Franchi and G. Nunez (Franchi and Nunez, 2008). Antibody against Mincle was from MBL Ltd (Japan). Alexa fluor-488 conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). TUNEL assay kit Apop Tag @ Plus in Situ apoptosis detection kit was purchased from Millipore (Billerica, MA, cat. No. S7111).

Mouse models

All procedures using animals were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Female mice were housed in shoe-box cages (2 animals/cage) with microisolator lids. Standard microisolator handling procedures were used throughout the study. Mice were randomized into ethanol-fed and pair-fed groups and then adapted to control liquid diet for 2 days. The ethanol-fed group was then allowed free access to an ethanol containing diet with increasing concentrations of ethanol: 1 and 2% (vol/vol) each for 2 days, then 4 and 5% ethanol each for 7 days, and finally 6% ethanol for an additional week. The 6% (vol/vol) diet provided ethanol as 32 percent of total calories in the diet. Control mice were pair-fed diets which iso-calorically substituted maltose dextrins for ethanol over the entire feeding period. At the end of the feeding protocol, mice were anesthetized, blood samples taken into non-heparinized syringes from the posterior vena cava, livers excised and mice euthanized by exsanguination. Portions of each liver were then either fixed in formalin or frozen in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc., Torrance CA) for histology, frozen in RNAlater (Qiagen, Valencia, CA) or flash frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was transferred to EDTA-containing tubes for the isolation of plasma. Plasma was then stored at -80°C .

Histopathology and TUNEL staining

Formalin-fixed tissues were paraffin-embedded, sectioned, coded and stained with hematoxylin and eosin. Steatosis and inflammation were scored by our experienced pathologist (X. Liu) on a scale of 0 to 3 on the basis of inflammatory cells infiltrated. Apoptosis was detected using the TUNEL assay and TUNEL positive cells were co-localized with CD45, as described previously (Smathers et al., 2016). All images presented in the results are representative of at least 3 images per liver and 4 mice per experimental condition.

Biochemical assays

Plasma samples were assayed for alanine aminotransferase (ALT) using a commercially available enzymatic assay kit (Sekisui Diagnostics, Lexington, MA), following the manufacturer's instructions. Total hepatic triglycerides were assayed using the Triglyceride Reagent Kit from Pointe Scientific Inc. (Lincoln Park, Michigan).

Western blot analysis

Frozen liver tissue (0.5 –1.0 g) was homogenized in lysis buffer (10 ml/g tissue) and protein concentration were measured using the BCA assay (Pritchard et al., 2007). Liver lysates were then used for Western blot analysis of Cytochrome P450 2E1 (CYP2E1), interleukin 1β and caspase-1. HSC 70 or β -actin were used as the loading control.

Isolation of RNA and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated and reverse transcribed followed by amplification using qRT-PCR. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S.

Statistical Analysis

Values shown in all figures represent the mean $\pm$ SEM ($n = 4$ pair-fed, $n = 6$ ethanol-fed). Analysis of variance was performed using the general linear models procedure (SAS, Carey, IN). Data were log-transformed as necessary to obtain a normal distribution. Follow-up comparisons were made by least square means testing. P values of less than 0.05 were considered significant. Student's t-test was used for comparing values obtained from two groups (Figure 2B and 2F only).

Results

Myeloid MyD88 contributes to ethanol-induced steatosis and hepatocyte injury

MyD88^{LysM-KO} mice and their littermate controls were allowed free access to Lieber-DeCarli ethanol-diet for 25 days, with gradually increasing concentrations of ethanol to a final concentration of ethanol as 32% calories during the final week of feeding or pair-fed control diets. Neither body weight, ethanol consumption nor induction of CYP2E1 was affected by genotype (Table 1). Hepatic steatosis, visualized with Oil Red O staining (Figure 1A) or assessed biochemically (Figure 1B), was increased in control mice in response to chronic ethanol feeding, but this response was reduced in MyD88^{LysM-KO} mice. Similarly, chronic ethanol feeding increased plasma ALT, an indicator of hepatocyte injury, in control, but not in MyD88^{LysM-KO} mice (Figure 1C).

Deficiency of MyD88 in myeloid cells reduces ethanol-induced hepatic inflammation

Chronic ethanol feeding to control mice increased lobular inflammation and steatosis, based on histological scoring (Figure 1D/E). These responses were reduced in MyD88^{LysM-KO} mice (Figure 1D/E). Similarly, chronic ethanol feeding increased expression of MCP-1 and IL1 β mRNA in control, but not MyD88^{LysM-KO} mice (Figure 2A). While chronic ethanol did not increase TNF α mRNA expression, immunoreactive TNF α was higher in control mice after ethanol feeding, but not in MyD88^{LysM-KO} mice (Figure 2A/B). Although Mincle was expressed in livers of ethanol-fed control mice, co-localized with F4/80, Mincle-positive cells were not detected in livers of MyD88^{LysM-KO} mice (Figure 2C/D). In parallel, ethanol-induced phosphorylation of SYK, a downstream target of Mincle, was also attenuated in MyD88^{LysM-KO} mice (Figure 2E). Finally, ethanol-induced accumulation of 4-hydroxynonenal-adducts, a dosimeter of oxidative stress, was also reduced in livers of MyD88^{LysM-KO} mice (Figure 2F).

Ethanol-induced activation of caspase-1 and IL1 β are myeloid-MyD88-dependent

Chronic ethanol feeding activates the inflammasome pathway, characterized by the presence of mature IL1 β , active caspase 1 and increased expression of NLRP3 and ASC in the liver (Petrasek et al., 2012). Because of the close association between the TLR4/MyD88 pathway and inflammasome activation in macrophages, here we hypothesized that Myeloid-MyD88-mediated hepatocyte injury, in response to ethanol feeding, is mediated through activation of the inflammasome pathway. Indeed, we found that while chronic ethanol feeding increased the cleavage of both IL1 β and caspase-1 in livers of control mice, MyD88^{LysM-KO} mice were protected from ethanol-induced activation of the inflammasome (Figure 3).

Loss of MyD88 in myeloid cells did not affect ethanol-induced apoptosis in mouse liver

Chronic ethanol feeding induces programmed cell death pathways including both apoptosis and necroptosis (Wu and Cederbaum, 2005; Roychowdhury et al., 2013). Hepatocyte apoptosis is associated with the pro-fibrotic effects of ethanol, as phagocytosis of apoptotic hepatocytes activates hepatic stellate cells (Roychowdhury et al., 2012). In contrast, apoptosis of macrophages is associated with the resolution of inflammatory responses (Tabas, 2010). Therefore, the consequences of apoptosis to liver homeostasis likely depend on the cell type undergoing apoptotic cell death. Chronic ethanol feeding increased TUNEL⁺ cells in both control and MyD88^{LysM-KO} mice (Figure 4A). Morphologically, the TUNEL⁺ cells resembled both hepatocytes and non-parenchymal cells, consistent with previous reports (Smathers et al., 2016). TUNEL positive non-parenchymal cells were co-localized with CD45 (Figure 4B). The number of TUNEL⁺/CD45⁺-double positive cells in mouse liver following ethanol feeding was not affected by genotype.

Discussion

Chronic ethanol feeding enhances TLR4-dependent signaling in hepatic macrophages via activation of both the MyD88- and TRIF-depending signaling pathways (Mandal et al., 2010) and deletion of TLR4 protects mice from ethanol-induced hepatic injury (Hritz et al., 2008). Therefore, it was surprising when studies utilizing global knock-outs of MyD88 found that MyD88^{-/-} mice were not protected from ethanol-induced liver injury (Hritz et al., 2008). Interestingly, MyD88 expression in myeloid cells is critical for the development of chronic inflammation in a high fat diet-induced obesity model (Yu et al., 2014). This important role for myeloid-MyD88 in hepatic injury in response to high-fat diet (Yu et al., 2014), coupled with the fact that mice with global MyD88-deficiency are susceptible to bacterial infection, suggested that myeloid-specific deletion of MyD88 would be a useful tool to study the contribution of MyD88 to ethanol-induced liver injury. Therefore, by targeted deletion of MyD88 in myeloid cells using the LysM-CRE system, here we tested the hypothesis that myeloid-MyD88 contributes to chronic ethanol-induced liver injury. Indeed, ethanol-induced steatosis and plasma ALT/AST, markers of hepatocyte injury, were reduced in MyD88^{LysM-KO} mice compared to controls. In parallel, ethanol-induced hepatic lobular inflammation along with the mRNA expression of the pro-inflammatory mediators including TNF α , MCP1 and IL-1 β were also ameliorated in MyD88^{LysM-KO} mice.

Increased lobular inflammation and expression of the pro-inflammatory cytokines/chemokines in mouse liver is characteristic of the progression of chronic ethanol-induced liver injury (Wang et al., 2012; Nagy et al., 2016). The TLR4-MyD88 pathway is responsible for sensing pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) (Jin et al., 2013). Mice deficient in TLR4 are resistant to ethanol-induced liver injury (Uesugi et al., 2001b). In response to activation by PAMP/DAMPs, hepatic macrophages increase expression of inflammatory mediators. We have also recently discovered that low concentrations of endotoxin also increase the expression of Mincle, a C-type lectin receptor that acts as a sensor for cell death, on hepatic macrophages (Zhou et al., 2016). Importantly, chronic ethanol feeding to mice also increased expression of Mincle on hepatic macrophages. Thus, increased expression of

Mincle likely contributes to the exacerbation of inflammatory responses during ethanol exposure, providing a link between hepatocyte cell death and inflammatory responses (Zhou et al., 2016).

Chronic ethanol feeding induces multiple programmed cell death pathways in the liver which includes apoptosis, necroptosis and pyroptosis. Apoptosis of hepatocytes has been implicated to liver injury while macrophage apoptosis is crucial for resolution of hepatic inflammation.

Since loss of myeloid-MyD88 reduced ethanol-induced TNF α in mouse liver, an inducer of both apoptosis and necroptosis, we hypothesized that myeloid-MyD88 induced hepatocyte injury through activation of TNF α -induced apoptosis and/or necroptosis in mouse liver. In contrast, we found that ethanol-induced apoptosis and necroptosis remained unaffected in livers of MyD88^{LysM-KO} mice, suggesting that myeloid-MyD88 contributes to a different mode of cell death in response to chronic ethanol feeding. Apoptosis can be triggered by multiple pathways in response to ethanol feeding, which include excess reactive oxygen species formation, metabolism-induced hypoxia, Fas ligand activation or up-regulation of the pro-inflammatory mediators such as TNF α (Barnes et al., 2014). Although, loss of MyD88 in myeloid cells was able to reduce ethanol-induced oxidative stress and TNF α , there are still a variety of other pathways that can regulate apoptosis, which are not directly dependent on MyD88. It is therefore not possible to pinpoint one specific pathway contributing to apoptosis in absence of MyD88.

Caspase-1-mediated inflammasome activation is recently implicated to ethanol-induced liver injury. Wild-type mice treated with IL1R antagonist or mice lacking IL1R or caspase 1, a central molecule of inflammasome activation, are protected from ethanol-induced liver injury (Petrasek et al., 2012). Upon activation, Caspase-1 cleaves IL-1 β , a pro-inflammatory molecule as well as GSDMD, a key step during the execution phase of pyroptosis (Wallach et al., 2016). Since both DAMPS and PAMPS are linked to induction of pyroptosis and MyD88 is critical for sensing DAMPS/PAMPS, loss of MyD88 in myeloid cells is expected to reduce ethanol-induced pro-pyrototic signals in mouse liver. In line of this hypothesis, here we found that ethanol-induced activation of Caspase-1, as well as cleavage of IL-1 β were attenuated in livers of MyD88^{LysM-KO} mice, suggesting that Myeloid-MyD88 contributes to ethanol-induced liver injury through induction of pyroptosis.

Taken together, these data indicate that myeloid-MyD88 has no influence on hepatocellular apoptosis or necroptosis. These modes of programmed cell death still occur, likely in response to the direct effects of ethanol metabolism on hepatocytes. However, despite ongoing apoptosis or necroptosis, our data indicate that the absence of MyD88 signaling in myeloid cells interrupts the cycle of ethanol-induced PAMP and DAMP signaling in the liver that eventually leads to exacerbated inflammatory responses during the progression of ethanol-induced liver injury.

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Abbreviations

TLR4	Toll-like receptor 4
MyD88	molecules: myeloid differentiation primary-response protein
TRIF	TLR-domain-containing adapter-inducing interferon- β
MyD88^{LysM-KO}	myeloid-specific MyD88-deficient mice
ALD	alcoholic liver disease
IFNs	Interferons
TNFα	Tumor necrosis factor α
IL1	Interleukin 1
IL6	Interleukin 6
NLRP3	NLR family, pyrin domain containing 3
TUNEL	Terminal deoxynucleotidyl transferase-dUTP nick end labeling
PAMP	Pathogen associated molecular patterns
DAMP	Damage-associated molecular patterns
SEM	Standard error of mean
CYP2E1	Cytochrome P450 2E1

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Figure 1. Ethanol-induced steatosis and hepatocyte injury were attenuated in livers of MyD88^{LysM-KO} mice

MyD88^{LysM-KO} mice and littermate controls were allowed free access to diets with increasing concentrations of ethanol or pair-fed a control diet. (A) Frozen liver sections were stained with Oil-Red O to visualize neutral lipids. Images were acquired using 20X objective. (B) Hepatic triglyceride content was measured in whole liver homogenate. (C) ALT activity was measured in plasma. (D) Paraffin-embedded liver sections were stained with hematoxylin and eosin. All images were acquired using a 10X objective. (E) Histological scoring for lobular inflammation and steatosis. Values represent means $\pm$ SEM. Values with different alphabetical superscripts were significantly different from each other, $p < 0.05$. $n = 4-6$.

Figure 2. MyD88-deficiency in myeloid cells prevented ethanol-induced lobular inflammation and increased expression of pro-inflammatory cytokines/chemokines

MyD88^{LysM-KO} mice and littermate controls were allowed free access to diets with increasing concentrations of ethanol or pair-fed a control diet. **(A)** Expression of mRNA for IL1 β , MCP-1 and TNF α was detected in mouse livers using qRT-PCR measurement and normalized to 18S RNA. **(B/C)** Paraffin-embedded liver sections of ethanol-fed groups were immuno-stained for **(B)** TNF α (Control: 51.4 $\pm$ 19.6 and MyD88^{LysM-KO}: 2.04 $\pm$ 0.7) or **(C)** Mincle (Control: 12.0 $\pm$ 2.02 and MyD88^{LysM-KO}: 1.67 $\pm$ 0.67). Images were acquired using 20X objectives. **(D)** Immuno-reactive Mincle (green) were co-stained with F4/80 (red). Images were acquired using a 40X objective. **(E)** Lysates were prepared from whole liver and analyzed by Western blot for the expression of SYK and phosphor-SYK. β -actin was used as a loading control. Band densities were analyzed using ImageJ software and normalized to β -actin. **(F)** Paraffin-embedded liver sections of ethanol-fed groups were immuno-stained for 4-hydroxynonenal (Control: 278.4 $\pm$ 108.8 and MyD88^{LysM-KO}: 35.17 $\pm$ 10.2). Images were acquired using 20X objective. Images are representative of 6 ethanol-fed mice. Values represent mean $\pm$ SEM. Values with different alphabetical superscripts were significantly different from each other, $p < 0.05$. $n = 4-6$.

Figure 3. Absence of MyD88 in myeloid cells attenuated ethanol-induced increase of cleaved caspase 1 and IL-1 β in mouse liver

MyD88^{LysM-KO} mice and littermate controls were allowed free access to diets with increasing concentrations of ethanol or pair-fed a control diet. Lysates were prepared from whole liver and analyzed by Western blot analysis for the presence of cleaved IL-1 β and cleaved caspase-1 (p20). β -actin was used as a loading control. Band densities were analyzed using ImageJ software and normalized to β -actin. Data represent mean $\pm$ SEM. n=4–6. Values with different alphabetical superscripts were significantly different from each other, $p < 0.05$. n=4–6.

Figure 4. Myeloid MyD88 deficiency did not attenuate hepatocellular apoptosis
 MyD88^{LysM-KO} mice and littermate controls were allowed free access to diets with increasing concentrations of ethanol or pair-fed a control diet. Frozen liver sections were subjected to (A) TUNEL and (B) TUNEL/CD45-co-staining. Images were acquired using a 40X objective. TUNEL⁺ and TUNEL⁺/CD45⁺ cells were enumerated per 40X frame. Blue arrows: TUNEL⁺/CD45⁺ cells, white arrows: TUNEL⁺/CD45⁻ cells. Values represent mean $\pm$ SEM. Values with different alphabetical superscripts were significantly different from each other, $p < 0.05$. $n = 4-6$.

Table 1
Body weight and food intake in control and MyD88^{LysM-KO} mice

MyD88^{LysM-KO} mice and littermate controls were allowed free access to diets with increasing concentrations of ethanol or pair-fed a control diet. Pair-fed control groups received same amount of food ingested by the ethanol-fed group on the previous night (designated Pair-fed in table). CYP2E1 expression was measured by Western blot using the whole liver homogenate and normalized to HSC70. Values represent means $\pm$ SEM. Values with different alphabetical superscripts were significantly different from each other, $p < 0.05$. $n=4-6$.

	Control		MyD88 ^{LysM-KO}	
	Pair-fed	EtOH-fed	Pair-fed	EtOH-fed
Initial Body weight (g)	18.2 $\pm$ 1.6	21.7 $\pm$ 2.0	19.4 $\pm$ 1.6	19.6 $\pm$ 0.9
Final Body weight (g)	18.1 $\pm$ 1.1	20.8 $\pm$ 2.5	22.1 $\pm$ 1.8	21.6 $\pm$ 2.1
Average daily food intake (ml/cage)	Pair-fed	24.5 $\pm$ 5.3	Pair-fed	23.3 $\pm$ 5.6
CYP2E1/HSC70 (arbitrary units)	0.30 $\pm$ 0.02 ^a	0.87 $\pm$ 0.12 ^b	0.32 $\pm$ 0.05 ^a	0.85 $\pm$ 0.11 ^b