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Genetic loss of immunoglobulin A does not influence development of alcoholic steatohepatitis in mice

Tatsuo Inamine, PhD^{1,2}, An-Ming Yang, MD¹, Lirui Wang, PhD^{1,3}, Kuei-Chuan Lee, MD, PhD¹, Cristina Llorente, PhD^{1,3}, and Bernd Schnabl, MD^{1,3}

¹Department of Medicine, University of California San Diego, La Jolla, CA

²Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

³Department of Medicine, VA San Diego Healthcare System, San Diego, CA

Abstract

Background—Chronic alcohol abuse is associated with intestinal dysbiosis and bacterial translocation. Translocated commensal bacteria contribute to alcoholic liver disease. Secretory immunoglobulin A (IgA) in the intestine binds bacteria and prevents bacterial translocation.

Aim—To investigate the functional role of IgA in ethanol-induced liver disease in mice.

Methods—We subjected wild-type (WT) and IgA-deficient littermate mice to Lieber DeCarli models of chronic ethanol administration and the model of chronic and binge ethanol feeding (the NIAAA model).

Results—Chronic ethanol feeding increased systemic levels of IgA, while fecal IgA was reduced in C57BL/6 WT mice. WT and *Iga*^{−/−} littermate mice showed similar liver injury, steatosis and inflammation following 4 weeks of ethanol feeding or chronic and binge ethanol feeding. IgA deficiency did not affect intestinal absorption or hepatic metabolism of ethanol. Pretreatment with ampicillin elevated intestinal IgA in WT littermate mice. Despite increased intestinal IgA, WT littermate mice exhibited a similar degree of liver disease compared with *Iga*^{−/−} mice after 7 weeks of ethanol feeding. Interestingly, bacterial translocation to mesenteric lymph nodes was increased in *Iga*^{−/−} mice fed an isocaloric diet, but was the same after ethanol feeding relative to WT littermate mice. The absence of intestinal IgA was associated with increased intestinal and plasma IgM in *Iga*^{−/−} mice after ethanol feeding.

Conclusions—Our findings indicate that absence of IgA does not affect development of alcoholic liver disease in mice. Loss of intestinal IgA is compensated by increased levels of intestinal IgM, which likely limits bacterial translocation after chronic ethanol administration.

Contact: Bernd Schnabl, MD, Department of Medicine, University of California San Diego, MC0063, 9500 Gilman Drive, La Jolla, CA 92093, Phone 858-822-5311, Fax 858-822-5370, beschnabl@ucsd.edu.

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Keywords

microbiome; alcoholic liver disease; microbiota; bacterial translocation

Introduction

Liver cirrhosis is the 12th leading cause of mortality worldwide (Lozano et al., 2012), and alcohol abuse is the etiology for 50% of cirrhosis-related deaths (Rehm et al., 2013). Chronic alcoholism can lead to intestinal dysbiosis, a dysfunctional gut barrier, and translocation of viable bacteria (Schnabl and Brenner, 2014, Wang et al., 2016). Translocation of viable bacteria from the intestinal lumen to extraintestinal organs results in progression of experimental alcoholic liver disease (Wang et al., 2016), and causes infections in patients with end-stage liver disease (cirrhosis) (Christou et al., 2007).

The intestinal immune system has developed several mechanisms to protect the human body from bacterial translocation and invasion. One adaptive immune response is the secretion of immunoglobulin A (IgA) from plasma cells. IgA is the major immunoglobulin isotype and several grams of IgA are released into the intestine each day in humans (Conley and Delacroix, 1987). Dendritic cells sample luminal bacteria and activate B cells to differentiate into plasma cells (Hooper and Macpherson, 2010). Plasma cells in the intestinal lamina propria secrete IgA specific for bacteria, which is transcytosed across intestinal epithelial cells (Hooper and Macpherson, 2010). In the gut lumen, secretory IgA serves as a first-line barrier that binds bacteria, limits the contact between bacteria and enterocytes, and prevents bacterial translocation (Hooper and Macpherson, 2010). Mice carrying a genetic defect in activation-induced cytidine deaminase (AID), which cannot undergo class switching to IgA and IgG, have bacterial translocation and intestinal bacterial overgrowth (Wei et al., 2011).

IgA has been studied in the field of alcoholic liver disease for a long time (Lee, 1965). Increased systemic IgA levels are found in patients with alcoholic liver disease (van de Wiel et al., 1987). In addition, alcoholic liver disease is associated with IgA deposits in hepatic sinusoids. Liver IgA deposits appear specific for alcoholic liver disease, correlate with the amount and duration of alcohol consumption, but not with serum IgA concentrations (van de Wiel et al., 1987). Although intestinal or fecal IgA levels have not been reported after chronic alcohol consumption, the number of IgA secreting plasma cells in the intestine is reduced in an experimental model of ethanol-induced liver disease (Moro-Sibilot et al., 2016). There is currently no study investigating the functional role of IgA for the development of alcoholic liver disease. We investigated whether absence of IgA affects the progression of ethanol-induced liver disease via increased bacterial translocation.

Materials and Methods

Mice

Iga^{-/-} mice on a C57BL/6 genetic background have been described (Harriman et al., 1999). Heterozygous (*Iga*^{+/-}) mice were bred to generate littermate mice without disruption of *Iga* (WT) and mice with homozygous disruption (*Iga*^{-/-}). Female mice (age 8–10 weeks) were

fed with liquid diet containing ethanol for 4 weeks (the Lieber DeCarli diet model of chronic alcohol consumption), as previously described (Chen et al., 2015a). The Lieber DeCarli diet comprises Micro Stabilized Rod Liq AC IRR (LD101A; TestDiet, St. Louis, MO) and Maltodextrin IRR (9598; TestDiet, St. Louis, MO) and 200-proof ethanol (Gold Shield, Hayward, CA). The caloric intake from ethanol was 0 on day 1, 10% of total calories on days 2 to 3, 20% on days 4 to 5, 30% on day 6 to 7, and 36% from day 8 until the end. Control mice received an isocaloric amount of iso-maltose instead of ethanol. For chronic-plus-single-binge ethanol feeding (the NIAAA model) (Bertola et al., 2013), male and female mice (age 8–12 weeks) were fed with the Lieber DeCarli diet for 15 days followed by ethanol binge. The caloric intake from ethanol was 0 on day 1 to 5 and 36% from day 6 until the end. At day 16, mice were gavaged with a single dose of ethanol (5 g/kg body weight) in the early morning, and mice were sacrificed 9 hours after the gavage.

To eliminate IgA-reducing bacteria and increase intestinal IgA amount, female mice (age 10–12 weeks) were treated with ampicillin (1 mg/ml; Sigma, St. Louis, MO) in drinking water for one week, and then fed with the Lieber DeCarli diet for 7 weeks. The caloric intake from ethanol was 0 on day 1, 10% of total calories on days 2 to 4, 20% on days 5 to 7, 30% from day 8 until the end of 6 weeks, and 36% for the last week.

A subset of experiments used female C57BL/6 mice (age 8 weeks) and termed non-littermate WT mice. Non-littermate WT mice were fed with the Lieber DeCarli diet. The protocol described above for 7 weeks of feeding was used. Mice were fed one additional week with a 36% caloric intake from ethanol for a total of 8 weeks.

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Real-time qPCR

RNA was extracted from mouse liver using the TRIzol Reagent (Invitrogen, Carlsbad, CA). After DNase treatment with the RQ1 RNase-Free DNase (Promega, Madison, WI), RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ABI, Carlsbad, CA). Primer sequences were obtained from the NIH qPrimerDepot. The qPCR value was normalized to mouse 18S (Hartmann et al., 2012).

DNA was isolated from mesenteric lymph nodes of mice as previously described (Wang et al., 2016). Primer sequence for microbial 16S rRNA gene was previously published (Maeda et al., 2003). To quantify the total bacterial load present in mesenteric lymph nodes, the qPCR value for each sample was multiplied by the total amount of DNA (μg)/mg of mesenteric lymph nodes. Real-time qPCR was performed with the iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA) using the StepOnePlus Real-Time PCR System (ABI, Carlsbad, CA). PCR results are expressed relative to the levels of control-fed WT mice.

Fecal sample preparation

Mouse feces was homogenized in phosphate-buffered saline (PBS) containing cOmplete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN) with 1 mm glass beads, and centrifuged. Supernatant was used for ELISA.

Protein analysis

Interleukin (IL)1B, Tumor necrosis factor (TNF) (all eBioscience, San Diego, CA), IgA, IgM and albumin (all Bethyl Labs, Montgomery, TX) were measured by ELISA. Immunoblot analysis was performed using antibodies against cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1; Millipore, Billerica, MA) and beta-actin (Abcam, Cambridge, MA). Immunoblots were analyzed by densitometry using NIH Image J.

Biochemical analysis

Plasma levels of ALT and hepatic levels of triglyceride were measured using the Infinity ALT Kit (Thermo Fisher Scientific, Waltham, MA) and Triglyceride Liquid Reagents Kit (Pointe Scientific, Canton, MI), respectively. Plasma levels of ethanol were measured using the Ethanol Assay Kit (BioVision, Milpitas, CA).

Staining procedures

For histologic analysis of liver tissues, formalin-fixed liver sections were stained with hematoxylin and eosin. Frozen liver sections were analyzed by Oil Red O staining.

Statistical analysis

Results are represented as mean $\pm$ SEM. Significance was evaluated using the Mann-Whitney U test. To compare fecal IgA levels before and after ampicillin treatment, the Wilcoxon signed-rank test was used. A *P* value less than 0.05 was considered to be statistically significant.

Results

Chronic alcohol administration results in lower intestinal IgA levels

Using a chronic model of ethanol administration in mice, we confirmed (Moro-Sibilot et al., 2016) that plasma IgA levels increase as compared with mice fed an isocaloric diet (Fig. 1A). Unlike systemic IgA levels, fecal IgA concentrations were significantly lower following chronic ethanol feeding for 8 weeks (Fig. 1B).

Loss of IgA does not affect chronic ethanol-induced liver disease in mice

Intestinal IgA is important to bind bacteria and prevent their translocation across the mucosal barrier (Macpherson et al., 2015). Translocation of viable bacteria to the mesenteric lymph nodes and the liver contributes to progression of ethanol-induced liver disease (Wang et al., 2016). To investigate whether suppressed intestinal IgA will promote development of alcoholic liver disease, we compared the effects of feeding ethanol for 4 weeks to *Iga*^{-/-} mice (Harriman et al., 1999) (C57BL/6 genetic background) versus wild-type (WT) littermates. Ethanol-induced liver disease was not different in *Iga*^{-/-} and WT mice as

evidenced by similar levels of liver injury (Fig. 2A and B) and steatosis (Fig. 2C and D). Although 4 weeks of feeding an oral Lieber DeCarli diet did not cause a significant increase of hepatic IL1B and TNF protein in WT mice, *Iga*^{-/-} mice showed slightly elevated levels of IL1B and TNF (Fig. 2E). To determine whether IgA deficiency alters intestinal absorption or hepatic metabolism of ethanol, blood alcohol levels were measured, which were not different between WT and *Iga*^{-/-} mice (Fig. 3A). Hepatic ADH and CYP2E1 are the two major enzymes metabolizing ethanol (Chen et al., 2015b). Hepatic *Adh1* gene expression was not significantly different between genotypes before and after ethanol feeding (Fig. 3B). Ethanol induced hepatic CYP2E1 protein in WT mice to a similar degree as compared with IgA deficient mice (Fig. 3C). In addition, intestinal permeability increased to a similar extent in alcohol-fed WT and *Iga*^{-/-} mice as assessed by measuring fecal albumin (Hartmann et al., 2013, Wang et al., 2015) (Fig. 3D). Together, our results suggest that IgA deficiency does not affect development of alcoholic liver disease in mice.

Increasing intestinal IgA level does not protect WT mice from chronic ethanol-induced liver disease

Fecal IgA concentrations vary between mouse cages even within the same vivarium. Vertically transmissible bacteria in mice with low concentrations of fecal IgA dominantly lower fecal IgA levels in mice with high concentrations of fecal IgA after co-housing or fecal transplantation (Moon et al., 2015). Despite the profound difference in fecal IgA, serum IgA levels were similar between mice with low and high fecal IgA concentrations (Moon et al., 2015). This suggests that gut specific bacteria contribute to degradation of intestinal IgA. We therefore assessed plasma and fecal IgA in WT littermate mice. We confirmed that plasma IgA levels increased after ethanol feeding in WT littermate mice (Fig. 4A); these levels were comparable to non-littermate WT mice (all on C7BL/6 genetic background) (Fig. 1A). However, absolute fecal IgA concentrations were lower in WT littermate (Fig. 4B) than non-littermate mice (Fig. 1B), and a trend toward lower fecal IgA was observed after ethanol feeding for 4 weeks (Fig. 4B). This raises the possibility that fecal IgA in WT littermate mice is below the threshold that confers protection against experimental liver disease.

Antibiotic treatment with ampicillin eliminates IgA-suppressive bacteria and reverses the IgA-low phenotype by increasing fecal IgA (Moon et al., 2015). We therefore pre-treated our WT and *Iga*^{-/-} littermate mice with ampicillin for 7 days, which significantly increased fecal IgA levels (Fig. 4C). Pretreatment with ampicillin maintained fecal IgA at high levels in WT littermate mice fed an isocaloric diet for 7 weeks (Fig. 4D). Chronic ethanol feeding lowered fecal IgA levels (Fig. 4D) as we have observed in WT non-littermate mice (Fig. 1B).

To induce a more robust inflammatory response in the liver after ampicillin treatment, we chose to extend our model to 7 weeks of Lieber DeCarli diet feeding. Despite increasing intestinal IgA in WT littermate mice with ampicillin pretreatment, there was no significant difference in liver injury (Fig. 5A and B), steatosis (Fig. 5C and D) and inflammation (Fig. 5E) between WT and *Iga*^{-/-} mice. Plasma levels and hepatic metabolism of ethanol did not differ between WT and IgA deficient mice, except for a slight increase of hepatic *Adh1*

mRNA in *Iga*^{-/-} mice fed with ethanol (Fig. 6A–D). These results confirm our initial findings that loss of IgA does not modulate ethanol-induced liver disease in mice.

IgA deficiency causes a compensatory increase in fecal and plasma IgM levels after chronic ethanol feeding

Translocation of viable bacteria to mesenteric lymph nodes contributes to ethanol-induced liver disease in mice (Wang et al., 2016). IgA is secreted from plasma cells residing in the lamina into the intestinal lumen to bind bacteria and to prevent translocation of bacteria across the intestinal epithelial barrier (Macpherson et al., 2015). As reported previously (Wang et al., 2016), chronic ethanol feeding in WT led to an increase of bacteria in mesenteric lymph nodes (MLN) (Fig. 7A). *Iga*^{-/-} mice fed an isocaloric diet showed more translocated bacteria in the mesenteric lymph nodes than WT mice; however, there was no significant difference between WT and *Iga*^{-/-} mice following chronic ethanol feeding (Fig. 7A). These results suggest that chronic ethanol feeding lowers fecal IgA (Fig. 4D), which is associated with increased bacterial translocation in WT mice, while *Iga*^{-/-} mice do not show more bacteria in mesenteric lymph nodes after chronic alcohol administration.

IgA deficiency is accompanied by compensatory increases in fecal and plasma IgM (Harriman et al., 1999). To assess this possibility, we detected IgM in the feces and plasma. Fecal and plasma IgM did not change in WT mice after alcohol administration (Fig. 7B). *Iga*^{-/-} mice fed an isocaloric diet showed a trend toward higher fecal IgM. In contrast, *Iga*^{-/-} mice fed ethanol for 7 weeks showed a significant increase in fecal IgM as compared with WT littermate mice (Fig. 7B). Plasma IgM was increased in isocaloric diet and ethanol-fed *Iga*^{-/-} mice as compared with WT mice (Fig. 7C). It is possible that this (likely compensatory) increase in fecal IgM limits bacterial translocation in *Iga*^{-/-} mice when compared with WT littermate mice after ethanol feeding. Secretory IgM is known to substitute for IgA (Macpherson et al., 2015) and to bind bacteria in the intestinal lumen (van der Waaij et al., 2004).

Genetic IgA deficiency does not alter liver injury in a model of chronic and binge ethanol feeding

The NIAAA model, comprised of chronic Lieber DeCarli feeding and a single dose of ethanol gavage, is known to induce more robust liver injury (Bertola et al., 2013). We also subjected WT and IgA-deficient littermate mice to the NIAAA model feeding. Although the model induced more severe liver injury, WT and *Iga*^{-/-} mice showed similar levels of liver injury (Fig. 8A) and steatosis (Fig. 8B and C). In addition, male and female mice did not show significant differences in liver injury and steatosis (Fig. 8A–C).

Discussion

The human body has developed multiple layers of protection in the intestine to avoid invasion and translocation of viable bacteria. Intestinal barriers include the production of mucins by intestinal goblet cells and formation of a thick mucus layer covering intestinal epithelial cells (McGuckin et al., 2011). The mucus layer keeps luminal bacteria in distance to intestinal epithelial cells. A second protective adaptation is the secretion of antimicrobial

molecules by intestinal enterocytes and Paneth cells, in particular regenerating islet derived 3 (REG3)-B and G (Hooper and Macpherson, 2010). REG3 lectins are mostly retained in the mucus layer and minimize colonization of bacteria in the inner mucus layer (Vaishnava et al., 2011). A third mechanism to reduce the contact between bacteria and enterocytes is secretion of IgA into the intestinal lumen by plasma cells residing in the lamina propria. Binding of IgA to bacteria prevents their translocation and invasion into the host (Hooper and Macpherson, 2010).

Chronic alcohol consumption results in translocation of viable bacteria and can therefore be considered as failure of the body's protective barriers. We have started to systematically investigate the importance of each of the intestinal barriers. Our current study suggests that chronic ethanol feeding lowers intestinal IgA level. This could be due to lower IgA secreting plasmablasts in the lamina propria following ethanol treatment (Moro-Sibilot et al., 2016), or impaired transcellular transport of IgA via the polymeric immunoglobulin receptor (pIgR) (Diebel et al., 2002). Although, genetic deficiency in IgA increased bacterial translocation, it did not promote further increase of bacterial translocation and exacerbate liver disease in mice fed with alcohol. This is similar to our earlier findings demonstrating that genetic ablation of Mucin-2 (MUC2) - the major and most abundant secreted mucin in the small and large intestine (Velcich et al., 2002) - is not sufficient to worsen alcoholic liver disease (Hartmann et al., 2013). In both cases, one would expect a failing intestinal firewall to allow increased bacterial translocation to mesenteric lymph nodes and liver. As it turns out, failure of one circuit within the intestinal firewall (Balmer et al., 2014) is not sufficient to cause failure of the entire system. Adaptive mechanisms are compensating for these single failures. In the current study, IgA deficiency results in increased intestinal IgM after ethanol feeding. Intestinal IgM is able to bind bacteria (van der Waaaj et al., 2004). IgM protects intestinal epithelial cells from *Shigella flexneri* *in vitro*, and the protective effect was higher than that of IgA (Longet et al., 2014). A different compensatory and protective mechanism is induced in MUC2 deficient mice after chronic ethanol administration. REG3 lectins are induced causing a reduction of ethanol-associated intestinal bacterial overgrowth and less bacterial translocation (Hartmann et al., 2013). Interestingly, REG3B or REG3G deficiency appears to be sufficient to increase bacterial translocation and alcoholic liver disease in mice (Wang et al., 2016). It seems that REG3 lectins might be master regulators for bacterial translocation during alcoholic liver disease. Overall, this suggests that barrier protection is not a static process and several components contribute and compensate for each other. These compensatory mechanisms allow evaluation of their therapeutic potential to stabilize a dysfunctional gut barrier.

A recently published study demonstrated that IgA producing plasmablasts are primed in gut-associated lymphoid tissue, and are able to home to the liver (Moro-Sibilot et al., 2016). IgA secreting plasmablasts increase in the liver after chronic-binge ethanol feeding in mice, which might account for increased systemic IgA commonly seen after chronic alcohol consumption (Moro-Sibilot et al., 2016). Blocking the migration of plasmablasts from intestinal Peyer's patches to the liver using the chemical inhibitor FTY720, ameliorates ethanol-induced liver disease in mice (Moro-Sibilot et al., 2016). The main concern is that this chemical FTY720 inhibitor is modulating sphingosine-1 phosphate receptor and is not specific in blocking liver homing of plasmablasts. FTY720 is inhibiting the migration of

several other immune cells, and shows protective properties in a T-cell mediated liver injury model (Yin et al., 2012). Thus, more carefully designed experiments are required to investigate the unknown function of hepatic IgA secreting plasma cells and sinusoidal IgA deposits.

In summary, a picture of the intestinal defense system during alcoholic liver disease is starting to emerge. IgA deficiency is not sufficient to promote alcoholic liver disease in mice, because adaptations such as increased intestinal and plasma IgM, and possibly other yet unknown mechanisms compensate for the loss of IgA. Future studies with inducible gene manipulation are required to reduce the time for these adaption processes to develop. This will eventually lead to the identification of new targets for the treatment of bacterial translocation and alcoholic liver disease.

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Abbreviations

Adh	alcohol dehydrogenase
ALT	alanine aminotransferase
Cyp	cytochrome p450 enzyme
EtOH	ethanol
IL	interleukin
Ig	immunoglobulin
MLN	mesenteric lymph nodes
Reg3	regenerating islet derived-3
TNF	tumor necrosis factor
WT	wild-type

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Figure 1. Fecal IgA levels decrease after chronic ethanol administration in mice
C57BL/6 non-littermate mice were fed an oral control diet (n=6) or ethanol diet (n=8–14) for 8 weeks. (A) Plasma IgA levels. (B) Fecal IgA levels. * $P < .05$.

Figure 2. Alcohol-induced liver disease in *Iga*^{-/-} mice

WT mice and their *Iga*^{-/-} littermates were fed an oral control diet (n=4–5) or ethanol diet (n=10–12) for 4 weeks. (A) Plasma levels of ALT. (B) Representative liver sections after hematoxylin and eosin staining. (C) Hepatic triglyceride content. (D) Representative Oil Red O-stained liver sections. (E) Hepatic expression of IL1B and TNF protein. Scale bars, 100μm. **P*<.05.

Figure 3. Absorption and hepatic metabolism of ethanol in *Iga*^{-/-} mice

WT mice and their *Iga*^{-/-} littermates were fed an oral control diet (n=4–5) or ethanol diet (n=8–12) for 4 weeks. (A) Plasma levels of ethanol. (B) Hepatic expression of *Adh1* mRNA. (C) Immunoblot analysis of hepatic CYP2E1. (D) Fecal albumin content. **P*<.05.

Figure 4. Plasma and fecal IgA level in WT littermate mice

(A) Plasma IgA levels of WT littermate mice fed an oral control diet (n=4) or ethanol diet (n=11) for 4 weeks. (B) Fecal IgA levels in WT littermate mice fed an oral control diet (n=4) or ethanol diet (n=13) for 4 weeks. (C) Fecal IgA levels of non-treated (day 0; n=10) or ampicillin treated WT littermate mice for 7 days (day 7; n=10). (D) Fecal IgA level of WT littermate mice fed an oral control diet (n=5) or ethanol diet (n=10) for 7 weeks. Mice were pretreated with ampicillin for 7 days prior to Lieber DeCarli feeding. * $P < .05$.

Figure 5. Alcohol-induced liver disease in *Iga*^{-/-} mice after pretreatment with ampicillin WT mice and their *Iga*^{-/-} littermates were treated with ampicillin in the drinking water for 7 days followed by feeding an oral control diet (n=4-5) or ethanol diet (n=10) for 7 weeks. (A) Plasma levels of ALT. (B) Representative liver sections after hematoxylin and eosin staining. (C) Hepatic triglyceride content. (D) Representative Oil Red O-stained liver sections. (E) Hepatic expression of IL1B and TNF protein. Scale bars, 100µm. **P*<.05.

Figure 6. Absorption and hepatic metabolism of ethanol in *Iga*^{-/-} mice after pretreatment with ampicillin

WT mice and their *Iga*^{-/-} littermates were treated with ampicillin for 7 days followed by feeding an oral control diet (n=4–5) or ethanol diet (n=8–10) for 7 weeks. (A) Plasma levels of ethanol. (B) Hepatic expression of *Adh1* mRNA. (C) Immunoblot analysis of hepatic CYP2E1. **P* < .05.

Figure 7. Bacterial translocation and fecal IgM levels in WT littermate and *Iga*^{-/-} mice after pretreatment with ampicillin

WT mice and their *Iga*^{-/-} littermates were treated with ampicillin for 7 days followed by feeding an oral control diet (n=4–5) or ethanol diet (n=9–10) for 7 weeks. (A) Total bacteria in mesenteric lymph nodes assessed by qPCR. (B) Fecal IgM levels. **P*<.05.

Figure 8. Liver disease in *Iga*^{-/-} mice following chronic-plus-single-binge ethanol feeding WT mice (male n = 7; female n = 4) and their *Iga*^{-/-} littermates (male n = 4; female n = 5) were subjected to chronic ethanol feeding followed by a single dose of ethanol gavage. (A) Plasma levels of ALT. (B) Representative liver sections after hematoxylin and eosin staining. (C) Hepatic triglyceride content. Scale bars, 100μm.