ORIGINAL RESEARCH

Intestinal Osteopontin Protects From Alcohol-induced Liver Injury by Preserving the Gut Microbiome and the Intestinal Barrier Function

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SUMMARY

Osteopontin protects from alcohol-associated liver disease by regulating the intestinal microbiome, which restores tryptophan metabolites and short-chain fatty acids signaling through aryl hydrocarbon receptor in intestinal epithelial cells. This preserves the integrity of the intestinal epithelial barrier and prevents bacterial translocation from the gut to the liver.

BACKGROUND & AIMS: The gut-liver axis plays a key role in the pathogenesis of alcohol-associated liver disease (ALD). We demonstrated that Opn-/- mice develop worse ALD than wild-type (WT) mice; however, the role of intestinal osteopontin (OPN) in ALD remains unknown. We hypothesized that overexpression of OPN in intestinal epithelial cells (IECs) could ameliorate ALD by preserving the gut microbiome and the intestinal barrier function.

METHODS: OpnKI IEC, OpnΔIEC, and WT mice were fed the control or ethanol Lieber-DeCarli diet for 6 weeks.

RESULTS: OpnKI IEC but not OpnΔIEC mice showed improved intestinal barrier function and protection from ALD. There were less pathogenic and more beneficial bacteria in ethanol-fed OpnKI IEC than in WT mice. Fecal microbiome transplant (FMT) from OpnKI IEC to WT mice protected from ALD. FMT from ethanol-fed WT to OpnKI IEC mice failed to induce ALD. Antimicrobial peptides, IL33, pSTAT3, aryl hydrocarbon receptor (Ahr), and tight-junction protein expression were higher in IECs from jejunum of ethanol-fed OpnKI IEC than of WT mice. Ethanol-fed OpnKI IEC showed more tryptophan metabolites and short-chain fatty acids in portal serum than WT mice. FMT from OpnKI IEC to WT mice enhanced IECs Ahr and tight-junction protein expression. Oral administration of milk OPN replicated the protective effect of OpnKI IEC mice in ALD.

CONCLUSION: Overexpression of OPN in IECs or administration of milk OPN maintain the intestinal microbiome by intestinal antimicrobial peptides. The increase in tryptophan

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metabolites and short-chain fatty acids signaling through the
Ahr in IECs, preserve the intestinal barrier function and protect
from ALD. (Cell Mol Gastroenterol Hepatol 2022; No. No. ; https://
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Gut-liver Axis; Short-chain Fatty Acids; Tryptophan.

C hronic alcohol consumption is a major cause of
liver-related morbidity and mortality worldwide.1,2
The gut-liver axis is a key player in alcohol-associated
liver disease (ALD).3 Alcohol and its metabolite acetale-
hyde, disrupt the intestinal epithelial barrier.4 Moreover,
ethanol lowers the production of antimicrobial peptides
(AMPs),5,6 causes intestinal dysbiosis – or changes in the
composition and abundance of bacterial species – and bac-
terial overgrowth.5 Intestinal dysbiosis leads to global
changes in intestinal metabolites, including branched-chain
amino acids, such as tryptophan (Trp), short-chain fatty
acids (SCFAs), and bile acids.7-9 As a result, there is
disruption of the intestinal barrier, leaky gut, and trans-
location of bacteria and bacterial products from the intes-
tinal lumen to the portal vein, which upon reaching the liver,
activate Kupffer cells (KCs) and infiltrating macrophages
(MFs) to trigger liver injury.7,10

Osteopontin (OPN) (encoded by the Spp1 gene) is a
soluble cytokine and a matrix-associated protein present in
most tissues and body fluids.11,12 Serum and hepatic OPN
increase in patients with alcoholic hepatitis and in animal
models of ALD.13,14 Importantly, OPN is expressed in in-
testinal epithelial cells (IECs)15 and maintains tight-junction
(TJ) protein complexes, enabling occludin to localize to the
TJs to be phosphorylated.15 Furthermore, OPN maintains
the homeostasis of intestinal commensal bacteria by pre-
serving TCRγδ+ intraepithelial lymphocytes,16 and acts as
an opsonin to induce phagocytosis of bacteria.17

Previous work from our laboratory demonstrated that
overexpression of OPN in hepatocytes or treatment with
milk osteopontin (mOPN) protected from ALD by blocking
the gut-derived lipopolysaccharide (LPS) effects in the
liver.2 However, the role of IEC-derived OPN in ALD remains
to be determined. We hypothesized that increasing the
expression of OPN in IECs could preserve the gut-liver axis
and protect from ALD. Thus, the aims of this work were,
first, to compare the hepatoprotective effects of natural in-
duction (due to alcohol) versus natural induction plus
overexpression OPN in IECs; second, to analyze if enhanced
expression of OPN in IECs could preserve the gut micro-
bioe and the intestinal epithelial barrier function to
protect from ALD; and third, to evaluate the therapeutic
potential of oral administration of mOPN to maintain the gut
microbial homeostasis under alcohol consumption.

Results

Ethanol Increases the Expression of OPN in IECs,
Which Protects From ALD

IECs express OPN,15 and we previously demonstrated
that ethanol drinking enhances the expression in mouse
IECs.1 To further confirm this, we analyzed the mRNA
expression of Opn in IECs of jejunum from wild-type (WT)
mice fed control or ethanol Lieber-DeCarli (LDC) diet.
Ethanol induced the expression of Opn mRNA in IECs
(Figure 1, A). To evaluate the role of the increased expres-

Abbreviations used in this paper: Ahr, aryl hydrocarbon receptor; ALD, alcohol-associated liver disease; ALT, alanine aminotransferase;
AMPs, antimicrobial peptides; FITC-dextran, fluorescein isothiocya-
trate-dextran; FM, fecal microbiome; FMT, fecal microbiome trans-
plant; H&E, hematoxylin and eosin; IECs, intestinal epithelial cells; IL1β, interleukin-1β; IM, intestinal microbiome; Jam4, junctional adhesion
molecule 4; JamA, junctional adhesion molecule A; KCs, Kupffer cells;
LC/MS, liquid chromatography–mass spectrometry; LDC, Lieber-
DeCarli; LPS, lipopolysaccharide; MFs, macrophages; mOPN, milk
osteopontin; OPN, osteopontin; Reg, Regenerating islet-derived
protein; SCFAs, short-chain fatty acids; TGs, triglycerides; TJ, tight-
junction; Tnfa, tumor necrosis factor-α; Trp, tryptophan; WT, wild-
type.

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mRNA expression of pro-inflammatory cytokines in the liver. Ethanol-fed OpnTKIIEC showed lower tumor necrosis factor-α (Tnfa), Il1b, Ccl2, and Ccl3 mRNAs than WT mice (Figure 2, F) but remained comparably high in ethanol-fed WT and OpnTKIIEC mice (Figure 2, G). Overall, these results suggest that overexpression of OPN in IECs preserves the intestinal epithelial barrier function, prevents translocation of LPS, and reduces KC activation and the production of pro-inflammatory cytokines, all of which protect from ALD.

The Increased Expression of OPN in IECs Prevents Alcohol-induced Dysbiosis

Alcohol drinking causes intestinal dysbiosis, which disrupts the gut barrier and contributes to ALD.37 Thus, we examined if ethanol-fed OpnTKIIEC mice were protected from alcohol-induced intestinal dysbiosis by analyzing the fecal microbiome (FM). The abundance of total bacteria was similar among groups of mice (Figure 3, A). The Shannon diversity index (alpha diversity) was higher in ethanol-fed
**Figure 1.** Continued.
Figure 2. The increased expression of OPN in IECs preserves gut permeability. WT, Opn<sup>KI</sup>IEC and Opn<sup>MEC</sup> mice were fed control or ethanol diet for 6 weeks to provoke ALD. Portal serum FITC-dextran (A–B), Portal serum LPS (C). Hepatic bacterial 16S rRNA. Data were normalized with 18S as housekeeping gene and fold change (FC) was calculated against WT control (D–E). mRNA expression of pro-inflammatory cytokines in the liver. Data were normalized with β-actin as housekeeping gene and FC was calculated against WT control (F–G). n = 6/group; data are presented as mean ± standard error of the mean. *P < .05 and **P < .01 vs control; †P < .05 and ††P < .01 vs WT ethanol. C, Control diet; E, ethanol diet.
than in control-fed Opn\textsuperscript{KI IEC} mice, indicating a diverse and equally distributed microbiome in ethanol-fed Opn\textsuperscript{KI IEC} mice (Figure 3, B). Weighted UniFrac analysis (beta diversity) revealed that the fecal bacteria in ethanol-fed Opn\textsuperscript{KI IEC} was different from WT but was comparable to control-fed Opn\textsuperscript{KI IEC} and WT mice (Figure 3, C). Eight bacterial phyla were most dominant in all groups of mice (Figure 3, D). The abundance of these phyla (Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Acidobacteria, Tenericutes, Deferribacteres) was similar in ethanol-fed and control-fed Opn\textsuperscript{KI IEC} but increased in ethanol-fed compared with control-fed WT mice, except for Deferribacteres, which decreased (Figure 3, E). Differential analysis of bacterial genera (Figure 3, F), showed that 36 genera, including \textit{Bacteroides}, \textit{Escherichia}, \textit{Enterococcus}, and \textit{Aerococcus}, all pathogenic and increased in ALD, were higher in ethanol-fed WT than in Opn\textsuperscript{KI IEC} mice (Figure 3, G). Twenty-two genera, including \textit{Bifidobacterium}, \textit{Eubacterium}, \textit{Prevotella}, and \textit{Alloprevotella}, linked to intestinal Trp and SCFAs metabolism and known to be beneficial, were higher in ethanol-fed Opn\textsuperscript{KI IEC} than in WT mice (Figure 3, H). Overall, these findings suggest that Opn\textsuperscript{KI IEC} mice are protected from alcohol-induced intestinal dysbiosis.

### The Intestinal Microbiome (IM) From Opn\textsuperscript{KI IEC} Mice Protects From ALD

To determine if the IM from Opn\textsuperscript{KI IEC} protects from ALD, we transplanted FM from Opn\textsuperscript{KI IEC} to WT mice and fed them with control or ethanol diet for 6 weeks. H&E staining (Figure 4, A), the steatosis and inflammation scores, liver TGs, and serum ALT activity (Figure 4, B) showed less alcohol-induced liver injury in WT after fecal microbiome transplant (FMT) from Opn\textsuperscript{KI IEC} mice compared with mice without FMT. The increase in portal serum FITC-dextran in ethanol-fed WT mice was prevented by FMT from Opn\textsuperscript{KI IEC} mice (Figure 4, B). Ethanol-fed WT mice with FMT from Opn\textsuperscript{KI IEC} mice showed lower bacterial 16S rRNA (Figure 4, C), and decreased the mRNA expression of pro-inflammatory cytokines (\textit{Tnfa}, \textit{Il1b}, \textit{Ccl2}, \textit{Ccl3}) in liver compared with mice without FMT (Figure 4, D). The duodenum, jejunum, ileum, and colon from these mice did not

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**Figure 2.** Continued.
show structural changes after FMT and ethanol feeding (not shown). These data indicate that the IM from Opn<sup>KI</sup> IEC mice improves intestinal epithelial barrier function, lowers hepatic pro-inflammatory cytokines, and protects from ALD.

**Figure 3.** The increased expression of OPN in IECs prevents alcohol-induced dysbiosis. WT and Opn<sup>KI</sup> IEC mice were fed control or ethanol diet for 6 weeks to provoke ALD; then, 16S rRNA sequencing was performed in stools to analyze the FM. Total bacterial abundance (A). Shannon index for alpha diversity (B). Principal component analysis plot of weighted UniFrac distances for beta diversity (C). Operational taxonomic unit (%) of bacterial phyla (D). Differential analysis of bacterial phyla (E). Volcano plot of bacterial genera (F). Highly abundant bacterial genera in ethanol-fed WT (G) and in ethanol-fed Opn<sup>KI</sup> IEC (H) mice. N = 8 (4 males + 4 females)/group. *P < .05 vs control; **false discovery rate (FDR) < 0.05; ***FDR < 0.01; and ****FDR < 0.001 vs WT ethanol. C, Control diet; E, ethanol diet.

**Overexpression of OPN in IECs Preserves the IM and Protects From ALD**

To examine if overexpression of OPN in IECs changes the IM in Opn<sup>KI</sup> IEC mice, we performed FMT from ethanol-fed
WT mice to Opn<sup>KI</sup> IEC mice and fed them with control or ethanol diet for 6 weeks to provoke ALD. FM analysis showed that 8 bacterial phyla were the most prevalent in these mice (Figure 5, A). Ethanol-fed Opn<sup>KI</sup> IEC mice with FMT from ethanol-fed WT mice showed higher total bacteria and alpha diversity compared with ethanol-fed WT mice with FMT from ethanol-fed WT mice (Figure 5, B–C). The beta diversity analysis showed that the IM in ethanol-fed Opn<sup>KI</sup> IEC mice with FMT from ethanol-fed WT mice was different from that of ethanol-fed WT mice with FMT from ethanol-fed WT mice (Figure 5, D). These results suggest that overexpression of OPN in IECs regulates the IM. To assess if the OPN-mediated regulation of the IM protects from ALD, we evaluated liver injury. H&E staining (Figure 5, E), the steatosis and inflammation scores, liver TGs, and serum ALT activity (Figure 5, F) demonstrated less liver injury in ethanol-fed Opn<sup>KI</sup> IEC mice with FMT from ethanol-fed WT mice compared with ethanol-fed WT mice with FMT from ethanol-fed WT mice. The former showed decreased portal serum FITC-dextran, less bacterial 16S rRNA in liver, and lower hepatic mRNA expression of Tnfa, Il1b, Ccl2, and Ccl3, compared with the latter (Figure 5, F–H), indicating improved gut barrier function in Opn<sup>KI</sup> IEC mice. The duodenum, jejunum, ileum, and colon from these mice did not

**Figure 3.** Continued.
Figure 3. Continued.
Figure 4. The IM from Opn<sup>KI</sup> IEC mice protects from ALD. WT mice were transplanted with FM from Opn<sup>KI</sup> IEC and were fed control or ethanol diet for 6 weeks to provoke ALD. Liver H&E staining (green arrows, macrovesicular steatosis; yellow arrows, microvesicular steatosis; red arrows, inflammatory foci; CV, central vein; PV, portal vein) (A). Body weight, liver weight, liver to body weight ratio, pathology scores, liver TGs, serum ALT activity, serum alcohol levels, and portal serum FITC-dextran (B). Hepatic bacterial 16S rRNA. Data were normalized with 18S as housekeeping gene and fold change (FC) was calculated against no-FMT control (C). mRNA expression of hepatic pro-inflammatory cytokines. Data were normalized with β-actin as housekeeping gene and FC was calculated against no-FMT control (D). n = 4/group; data are expressed as mean ± standard error of the mean. *P < .05 and ***P < .001 vs control; P < .05 vs no-FMT ethanol. C, Control diet; E, ethanol diet.
show structural changes (not shown). Thus, these results indicate that overexpression of OPN in IECs preserves the gut microbiome, the intestinal epithelial barrier function, and protects from ALD.

**OPN Preserves the Gut Microbiome by Inducing AMPs Expression in IECs, Which Maintain the Intestinal Barrier Function and Protect From ALD**

To understand how IEC-derived OPN preserves the gut microbiome upon alcohol exposure, we examined the expression of AMPs in IECs from jejunum. Regenerating islet-derived protein (Reg) 3β and 3γ mRNA and protein were higher in IECs from ethanol-fed OpnK\textsuperscript{KI} IEC mice than in WT mice (Figure 6, A–B). Interleukin-33 (IL33) and phosphorylation of STAT3, known to enhance AMPs in IECs,\textsuperscript{19} were higher in jejunal IECs from control and ethanol-fed OpnK\textsuperscript{KI} IEC mice, whereas IL33 was higher only in jejunal IECs from ethanol-fed OpnK\textsuperscript{KI} IEC mice (Figure 6, A and C). To determine if the effect of OPN in the gut microbiome preserved the intestinal barrier function, first we measured portal serum Trp metabolites and SCFAs, produced by the gut microbiome, and known to improve gut barrier function.\textsuperscript{20} Second, we evaluated the mRNA expression of aryl hydrocarbon receptor (Ahr), which regulates gut barrier function via Trp metabolites\textsuperscript{21} and SCFAs.\textsuperscript{22} Third, we analyzed mRNA expression of TJ proteins in IECs from jejunum. Trp, indole metabolites, and SCFAs increased in portal serum from ethanol-fed OpnK\textsuperscript{KI} IEC mice compared with WT mice (Figure 6, D–G). Ethanol-fed OpnK\textsuperscript{KI} IEC mice showed higher Ahr, Occludin, junctional adhesion molecule A (JamA), and junctional adhesion molecule 4 (Jam4) mRNA expression in IECs from jejenum than in WT mice (Figure 6, H). AHR protein expression was similar in IECs from jejunum in control and ethanol-fed OpnK\textsuperscript{KI} IEC mice (Figure 6, B).

Next, to establish the link among OPN, Ahr, and TJ proteins, the mRNA expression of AMPs (Reg3γ and Reg3γ), IL33, Ahr, and TJ proteins (Occludin, Claudin3, JamA, Jam4) were analyzed in IECs from jejunum of Opn\textsuperscript{SILEC} and WT mice fed control or ethanol diet for 6 weeks. Opn\textsuperscript{SILEC} mice did not show meaningful changes in Reg3γ, Reg3γ, IL33, Ahr, Occludin, Claudin3, JamA, and Jam4 after ethanol feeding (Figure 6, I). To confirm that the FM from OpnK\textsuperscript{KI} IEC mice preserved the intestinal barrier function, the expression of Ahr, Occludin, Claudin3, JamA, and Jam4 was measured in IECs from jejunum of WT mice with FMT from OpnK\textsuperscript{KI} IEC mice. Ethanol-fed WT mice with FMT from OpnK\textsuperscript{KI} IEC showed higher mRNA expression of Ahr, Occludin, Claudin3, JamA, and Jam4 compared with ethanol-fed WT mice with no FMT (Figure 6, J). Analysis of total, cytosolic, and nuclear protein revealed that ethanol lowered AHR expression in IECs from jejunum and that FMT from Opn\textsuperscript{KI} IEC mice preserved protein expression (Figure 6, K). These data indicate that overexpression of OPN in IECs increases intestinal AMPs to maintain the gut microbiome, which maintains intestinal barrier function via Trp metabolites, SCFAs, and Ahr, all of which protect Opn\textsuperscript{KI} IEC mice from ALD.

**Oral Administration of mOPN maintains the gut microbiome and protects from ALD**

Our previous report proved that oral administration of mOPN during chronic ethanol feeding ameliorates ALD.\textsuperscript{1} To dissect if this occurred by preserving the gut microbiome, we performed FMT from ethanol-fed WT mice to WT mice and fed them with control or ethanol diet for 6 weeks in the presence or absence of mOPN. The H&E staining (Figure 7, A), the steatosis and inflammation scores, liver TGs, serum ALT activity, portal serum FITC-dextran (Figure 7, B), hepatic bacterial 16S rRNA (Figure 7, C), and hepatic mRNA expression of pro-inflammatory cytokines (Figure 7, D) revealed less liver injury in ethanol-fed WT mice with oral administration of mOPN in the presence or absence of FMT from ethanol-fed WT mice. The duodenum, jejunum, ileum, and colon from these mice did not show structural changes (not shown). To understand if protection from ALD by mOPN was mediated by preserving the gut microbiome, we analyzed the FM from WT mice fed the control or ethanol diet in the presence or absence of mOPN. The abundance of total bacteria and alpha diversity was higher in ethanol-fed WT mice with mOPN than in mice without mOPN (Figure 7, E–F), suggesting that mOPN maintains a rich, diverse, and equally distributed microbiome. Analysis of beta diversity showed that the fecal bacteria in mOPN plus ethanol-fed WT mice was different than that without mOPN but was comparable to control-fed WT mice with or without mOPN (Figure 7, G). Eight bacterial phyla were most abundant in mice with or without mOPN (Figure 7, H). Differential analysis of the bacterial genera (Figure 7, I) showed that chronic ethanol feeding without mOPN promoted the growth of pathogenic genera, such as Bacteroides, Alistipes, and Enterococcus (Figure 7, J). However, mOPN promoted the growth of bacteria, such as Bifidobacterium, Eubacterium, Desulfovibrio, Butyrivibrio, Butyrivibrio, Butyrivincoccus, and Roseburia, linked to intestinal Trp and SCFAs metabolism (Figure 7, J), which play a beneficial role in the gut.\textsuperscript{8,22-24} These findings suggest that mOPN protects from ALD by preserving the gut microbiome.

**Oral Administration of mOPN Induces AMPs in IECs, Which Preserve the Gut Microbiota and the Intestinal Barrier Function**

To understand if mOPN preserves the gut microbiome by inducing AMPs in IECs, we analyzed the expression of Reg3γ, Reg3γ, and IL33 in IECs from jejunum. Chronic ethanol feeding lowered Reg3γ, Reg3γ, and IL33 mRNAs, and mOPN induced them in IECs from jejunum (Figure 8, A). Similar findings occurred for REG3G protein (Figure 8, B). Moreover, mOPN increased the mRNA expression of Ahr and TJ proteins (Occludin, Claudin3, JamA) in IECs from jejunum (Figure 8, C). Analysis of total, cytosolic, and nuclear AHR protein in IECs from jejunum revealed that mOPN prevented the ethanol-mediated decrease in AHR (Figure 8, D). WT mice fed ethanol and mOPN also showed higher levels of Trp, its metabolites, and SCFAs in portal serum (Figure 8, E–H). In summary, these findings indicate that both over-
expression of OPN in IECs and oral administration of mOPN during chronic ethanol feeding preserve the gut microbiome, intestinal barrier integrity, and protect from ALD through similar mechanisms.

Discussion

The main findings of this study are that overexpression of OPN in the IECs protects from ALD and that oral administration of mOPN has therapeutic potential to prevent or delay ALD. In both cases, protection from ALD occurs by preserving the gut microbiome and the intestinal epithelial barrier function.

A key finding is that OPN prevents alcohol-induced bacterial dysbiosis in the intestine, central to the pathogenesis of ALD. Intestinal dysbiosis participates in the onset and progression of ALD as a result of effects in the gut-liver axis. The intestinal epithelium is in close
Figure 5. Continued.
contact with pathogenic and beneficial microorganisms.\textsuperscript{27,28}

The host evolves unique regulatory mechanisms to maintain a homeostatic balance between tolerance and immunity to the microbiota.\textsuperscript{19} A vital regulatory mechanism is intestinal AMPs, such as Reg3\textbeta, Reg3\textgamma, and Il33 in IECs isolated from jejunum. Data were normalized with Gapdh as housekeeping gene and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (A). Western blot of REG3G and total AHR in IECs isolated from jejunum (n = 3 males/group) (B). Western blot of STAT3 and pSTAT3 in IECs isolated from jejunum (n = 3 males/group) (C). Levels of Trp, its metabolites kynureline and indole metabolites (3-indoleacetic acid + 3-indolepropionic acid + 3-indoxyl sulfate + indole + indole-3-lactic acid + tryptamine + tryptophol) in portal serum of WT and Opn\textsuperscript{OE} IEC mice were fed control or ethanol diet for 6 weeks. Data are expressed as mean ± standard error of the mean (SEM). *P < .05; **P < .01; and ***P < .001 vs control; \#P < .01 vs WT control; \#\#P < .01 vs ethanol control. WT and Opn\textsuperscript{OE} IEC mice were fed control or ethanol diet for 6 weeks to provoke ALD. mRNA expression of Reg3\textbeta, Reg3\textgamma, and Il33 in IECs isolated from jejunum (n = 3 males/group) (D). mRNA expression of Ahr, Occludin, Claudin3, JamA, and Jam4 in IECs from jejunum, Data were normalized with Gapdh as housekeeping gene and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (E). Levels of SCFAs (acetic acid + propionic acid + butyric acid) in portal serum of WT and Opn\textsuperscript{OE} IEC mice were fed control or ethanol diet for 6 weeks to provoke ALD. mRNA expression of Ahr, Occludin, Claudin3, JamA, and Jam4 in IECs from jejunum, Data were normalized with Gapdh as housekeeping gene and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (F). Western blot of total, cytosolic, and nuclear AHR in IECs from jejunum (n = 3 males/group) (G). Data were expressed as mean ± SEM. *P < .05 and \#P < .01 vs control. WT and Opn\textsuperscript{OE} IEC mice were fed control or ethanol diet for 6 weeks to provoke ALD. mRNA expression of Reg3\textbeta, Reg3\textgamma, and Il33 in IECs isolated from jejunum (n = 3 males + 3 females)/group) (H). Data are expressed as mean ± standard error of the mean (SEM). *P < .05; **P < .01; and ***P < .001 vs control; \#P < .01 vs WT control; \#\#P < .01 vs ethanol control. Western blot of total, cytosolic, and nuclear AHR in IECs from jejunum (n = 3 males + 3 females)/group) (I). Data were expressed as mean ± SEM. *P < .05 and **P < .01 vs control; \#P < .01 vs no-FMT ethanol control. C, Control diet; E, ethanol diet.

Figure 6. OPN preserves the gut microbiome by inducing AMPs expression in IECs, which maintains the intestinal barrier function and protects from ALD. WT and Opn\textsuperscript{OE} IEC mice were fed the control or ethanol diet for 6 weeks to provoke ALD. mRNA expression of Reg3\textbeta, Reg3\textgamma, and Il33 in IECs isolated from jejunum. Data were normalized with Gapdh as housekeeping gene and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (A). Western blot of REG3G and total AHR in IECs isolated from jejunum (n = 3 males/group) (B). Western blot of STAT3 and pSTAT3 in IECs isolated from jejunum (n = 3 males/group) (C). Levels of Trp, its metabolites kynureline and indole metabolites (3-indoleacetic acid + 3-indolepropionic acid + 3-indoxyl sulfate + indole + indole-3-lactic acid + tryptamine + tryptophol) in portal serum (n = 3 males/group) (D-E). Levels of SCFAs (acetic acid + propionic acid + butyric acid) in portal serum (n = 3 males/group) (F-G). mRNA expression of Ahr, Occludin, Claudin3, JamA, and Jam4 in IECs from jejunum. Data were normalized with Gapdh as housekeeping gene and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (H). Data are expressed as mean ± standard error of the mean (SEM). *P < .05; **P < .01; and ***P < .001 vs control; \#P < .01 vs WT control; \#\#P < .01 vs ethanol control. WT and Opn\textsuperscript{OE} IEC mice were fed control or ethanol diet for 6 weeks to provoke ALD. mRNA expression of Reg3\textbeta, Reg3\textgamma, and Il33 in IECs isolated from jejunum. Data were normalized with Gapdh as housekeeping gene, and fold change (FC) was calculated against WT control (n = 6 [3 males + 3 females]/group) (I). Data are expressed as mean ± SEM. *P < .05 and **P < .01 vs control; \#P < .01 vs no-FMT ethanol control. C, Control diet; E, ethanol diet.
in IECs to induce the production of AMPs needs further investigation. More importantly, this study reveals that the IM in Opn\textsuperscript{KI IEC} mice is resistant to alcohol-induced bacterial dysbiosis and explains why these mice are protected from ALD. This is also supported by less alcohol-induced liver injury in Opn\textsuperscript{KI IEC} mice and WT mice with FMT from Opn\textsuperscript{KI IEC} mice.

A second finding is that OPN preserves the gut microbiome and the intestinal epithelial barrier function by maintaining TJs, and therefore, protects from ALD. The intestinal epithelium, a physical barrier of IECs sealed through TJs\textsuperscript{27,32} maintains gut barrier integrity and is the first line of defense against bacterial toxins and pathogens in the intestinal lumen.\textsuperscript{32,33} Intestinal bacteria are involved in

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**Figure 6.** Continued.
metabolism\textsuperscript{8,34} and signal to IECs to maintain gut barrier integrity.\textsuperscript{8} An essential player in this scenario is the Ahr, which supports TJs in IECs\textsuperscript{8} and protects from ALD.\textsuperscript{7} Small-molecules such as SCFAs (acetate, propionate, butyrate) and Trp metabolites (indole metabolites and others), synthesized by intestinal bacteria, are secreted into the intestinal

<table>
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<th>SCFAs (mg/L)</th>
<th>WT Control (n=3)</th>
<th>WT EtOH (n=3)</th>
<th>Opn\textsuperscript{KIIEC} Control (n=3)</th>
<th>Opn\textsuperscript{KIIEC} EtOH (n=3)</th>
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<td>Acetic acid</td>
<td>77.7±4.8</td>
<td>50.3±4.9 *</td>
<td>111.3±33.4</td>
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<td>0.5 0.2 ^</td>
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Figure 6. Continued.
lumen and act as endogenous agonists of Ahr.\(^8,21,22\) Intestinal dysbiosis due to alcohol consumption results in impaired bacterial metabolism, including Trp metabolism\(^7,34\) and SCFAs synthesis.\(^{35}\) As a result, there is reduced Ahr signaling, loss of TJs, impairment of intestinal barrier function, and worsening of ALD.\(^7,18\) The present study highlights that overexpression of OPN in IECs or oral administration of mOPN prevents the alcohol-mediated downregulation of Ahr signaling in IECs. Previous studies demonstrated that alcohol consumption lowers the abundance of bacteria associated with Trp metabolism and synthesis of SCFAs.\(^7,35\) We show that OPN stimulates the

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<td>Control (n=4)</td>
<td>EtOH (n=4)</td>
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Figure 6. Continued.
Figure 7. Oral administration of mOPN preserves the gut microbiome and protects from ALD. FM from 6-week ethanol-fed WT mice was transplanted to WT mice, and then mice were fed the control or ethanol diet for 6 weeks in the presence or absence of mOPN. Liver H&E staining (green arrows, macrovesicular steatosis; yellow arrows, microvesicular steatosis; red arrows, inflammatory foci; CV, central vein; PV, portal vein) (A). Body weight, liver weight, liver to body weight ratio, pathology scores, liver TGs, serum ALT activity, serum alcohol levels, and portal serum FITC-dextran (B). Hepatic bacterial 16S rRNA. Data were normalized with 18S as housekeeping gene, and fold change (FC) was calculated against WT control (I). Liver mRNA expression of pro-inflammatory cytokines. Data were normalized with β-actin as housekeeping gene, and FC was calculated against WT control (D). Total bacterial abundance (E). Shannon Index for alpha diversity (F). Principal components analysis plot of weighted UniFrac distances for beta diversity (G). Operational taxonomic unit (%) of bacterial phyla (H). Volcano plot of bacterial genera (I). Differential analysis of bacterial genera (J). n = 4/group; data are presented as mean ± standard error of the mean. *P < .05; **P < .01; and ***P < .001 vs control; #P < .05 vs no-FMT control; ^P < .05 vs FMT control; ¶P < .05 vs no-FMT ethanol; ¶¶P < .05 and §§P < .01 vs FMT ethanol. ¥P < .05 vs without mOPN ethanol. *False discovery rate (FDR) < .05; **FDR < .01; and ***FDR < .001 vs without mOPN ethanol. C, Control diet; E, ethanol diet. 

WT without FMT

WT with FMT from EtOH-fed WT

mOPN

WT with FMT from EtOH-fed WT + mOPN
growth of Trp-metabolizing and SCFAs-synthesizing bacteria in the intestine under alcohol feeding. These include *Bifidobacterium*,23,36,37 *Eubacterium*,23,37 *Prevotella*,38,39 *Allloprevotella*,40 *Desulfovibrio*,36,41 *Butyricoccus*,42 *Butyrivibrio monas*,43 and *Roseburia*.23,44,45 An increase in these bacteria enhances the synthesis of SCFAs from undigested

**Figure 7.** Continued.
carbohydrates and indole derivatives from Trp catabolism
in the gut. This is revealed by high levels of SCFAs (acetate,
propionate, butyrate) and of indole compounds (3-
indoleacrylic acid, 3-indoleacrylic acid, 3-indolepropionic
acid, 3-indoxyl sulfate, indole) in portal serum from
Opn
KI IEC mice and from WT mice with oral administration
of mOPN. These SCFAs and indole derivatives are well-
known ligands for Ahr, which activate it in IECs. SCFA
and indole signaling through Ahr in IECs increases the
expression of TJ proteins, as shown by increased mRNA
expression of
Occludin,
JamA,
and
Jam4
in IECs from
jejunum of Opn
KI IEC and
Claudin-3
and
JamA
in IECs from
jejunum of WT mice with oral administration of mOPN.
This preserves the gut barrier function and limits the
translocation of bacteria and bacterial products from the
gut to the portal blood.

In ALD, disruption of the intestinal epithelial barrier
enhances intestinal permeability
1
and allows translocation
of bacteria and bacterial products from the gut lumen to the
portal circulation.
2 Upon reaching the liver, they stimulate
KCs, infiltrating MFs and neutrophils to produce
proinflammatory cytokines, which damage hepatocytes.
3 We
demonstrate that OPN preserves the gut barrier function
and blocks the translocation of bacterial products from the
gut to the liver as seen by reduced portal serum LPS and
RNA expression of
Tnfα, interleukin-1β (Il1β), Ccl2,
and
Ccl3
in the liver from ethanol-fed Opn
KI IEC mice and ethanol-fed
WT mice with oral administration of mOPN.

Additionally, we demonstrate comparable alcohol-
induced liver injury between male and female mice; this
could be because of the genetic background of the mice and/
or the ethanol feeding model. However, ethanol-fed WT and
Opn
KI IEC females show higher liver TGs than ethanol-fed
males. Ethanol-fed WT females show higher Il1β expres-
sion in liver, whereas ethanol-fed WT and Opn
KI IEC males
show increased gut permeability and higher
Tnfα,
Ccl2,
and
Ccl3
expression in liver than ethanol-fed females. Further,
based on liver TG and serum ALT activity, the protective
effects of OPN overexpression are greater in males than in
females.

Our study has some limitations. First, the 2 mouse
models, Opn
KI IEC and Opn
ΔIEC, show heterogeneity in the
extent of alcohol-induced liver injury. Overexpression of OPN in IECs prevents the alcohol-induced increase in gut permeability, and deletion of OPN from IEC increases gut permeability after alcohol exposure but does not show further worsening of gut permeability. Second, deletion of OPN from IECs does not downregulate the expression of AMPs, Ahr, and TJs in IECs from jejunum. Third, FMT from ethanol-fed WT mice to OPNKI IEC and WT mice lack the OPN KI IEC and WT mice without FMT as control. Last, in the FMT experiment, only male mice were used; therefore, these results may be gender-specific.

In conclusion, we show that natural induction plus overexpression of OPN in IECs or oral administration of mOPN during ethanol feeding protect from ALD by preserving the gut-liver axis. OPN increases AMPs such as Reg3β and Reg3γ in IECs, which regulate the IM and prevent alcohol-mediated loss of Trp-metabolizing and SCFAs-synthesizing bacteria in the gut. This increases Ahr signaling in IECs, prevents disruption of the intestinal epithelial barrier, and protects from ALD. Therefore, induction of OPN in the intestine or oral administration of mOPN could benefit patients with ALD. The latter therapeutic approach is feasible, as administration of OPN has proven safe, for example, incorporated in infant formulas as Laprodan OPN-10 (Arla Foods) or as bovine mOPN in clinical trials to study its effect on growth, health, and immune function in infants (NCT04306263, NCT03331276, NCT00970398).

Materials and Methods

General Methodology

Details on general methodology, such as immunohistochemistry for OPN, H&E staining, measurement of serum ALT activity, liver TGs, serum alcohol, total RNA extraction, quantitative real-time polymerase chain reaction analysis, and measurement of FITC-dextran and LPS in portal serum are described in previous publications.5,46-50 The sequence of the primers used is listed in Table 1. Cytosolic and nuclear proteins were isolated with the compartmental protein extraction kit (Millipore, Burlington, MA). Western blot analysis of STAT3, pSTAT3, Histone H3, AHR, REG3G, and GAPDH was performed using antibodies 9132S, 9145S, and 9715S (Cell Signaling Technology, Inc, Danvers, MA).
514 and PA5-50450 (Invitrogen, Carlsbad, CA), and sc-32233 (Santa Cruz, Dallas, TX), respectively.

**Mice**

\(Opn^{\text{loxP}}\) mice were generated in our laboratory. The \(Opn^{\text{loxP}}\) allele was created inserting \(\text{loxP}\) sites to remove exons 4–7. \(Opn\text{-Stop}^{\text{fl}}\) mice were donated by Dr Panoutsakopoulou (Biomedical Research Foundation, Academy of Athens, Greece). Both \(Opn\text{-Stop}^{\text{fl}}\) and \(Opn^{\text{fl}}\) mice were bred with \(\text{Villi-Cre}\) (JAX004586, the Jackson Laboratory, Bar Harbor, ME) to generate IEC-specific knock-in (\(\text{Opn}\text{KI IEC}\)) and knock-out (\(\text{Opn}\text{KO IEC}\)) mice, respectively. \(\text{Villi-Cre}\) mice were used as controls and referred to as WT on the text or figures for simplicity purposes only. The targeting strategy was validated by immunohistochemistry in the jejunum (Figure 9, A–B). We also confirmed that overexpression or deletion OPN in IECs does not affect OPN...
expression in the liver (Figure 9, C–D). All mice were in C57BL/6J background and lacked a liver or intestine phenotype in the absence of treatment.

**Model of Alcohol-induced Liver Injury**

The LDC model was used to provoke early alcohol-induced liver injury. The control and ethanol LDC diets (Bio-Serv Inc, Frenchtown, NJ) are equicaloric and have the same composition of fat (42% of calories) and protein (16% of calories). The content of carbohydrates is 42% of total calories (dextrin-maltose) in the control diet and 12% of total calories in the ethanol diet, where up to 30% of carbohydrate-derived calories are replaced by ethanol.

Equal number of male and female mice (12 weeks old) were acclimatized to the liquid diet by feeding control diet for 7 days. Then, the percentage of ethanol-derived calories was progressively increased from 10% (1 week) to 20% (1 week), 25% (2 weeks), and 30% (2 weeks) in the ethanol group. The control groups remained on control diet. Mice were gavaged with 4 μL/g of body weight of FITC-dextran 4 hours before sacrifice. Liver and body weight were recorded upon sacrifice to calculate the liver-to-body weight ratio. Systemic and portal blood were drawn under anesthesia from the submandibular and portal veins, respectively, to obtain serum. Serum, liver, intestine and stool samples were collected and stored at −80 °C for further analysis.

**Pathology**

Sections from left liver lobe, duodenum, jejunum, ileum, and colon were obtained, fixed in 10% neutral formalin buffer, processed, and sectioned (4 μm) for H&E staining. Liver injury was determined by a liver pathologist according to the Brunt classification. The steatosis grade was 0 <5%; 1 = 5% to 33%; 2 = >33% to 66% and 3 = >66%. Steatosis was noted to be macrovascular, microvascular, or both. Inflammation was noted based on the presence of inflammatory foci and was scored as follows: 0 for none; 1 for <2 foci/field; 2 for 2 to 4 foci/field; and 3 for >4 foci/field. Ten fields per section were observed to semi-quantitative scoring of liver steatosis and inflammation.

**FMT**

Three independent FMT experiments were performed in 12-week-old male mice. First, FMT from OpnKO mice to WT (C57BL/6J, Stock No. 000664) mice. Second, FMT from WT fed ethanol for 6 weeks to OpnKO mice. Third, FMT from WT fed ethanol for 6 weeks to WT mice. Briefly, feces (100 mg) were dissociated in 1 mL of sterile saline solution by vigorous shaking followed by centrifugation at 800 x g for 3 minutes. The supernatant containing the FM suspension was stored in aliquots (200 μL) at −80 °C until FMTs were performed. Mice were fasted for 1 hour, and the bowel was cleansed with polyethylene glycol (Macrogol 4000, Therma...
Figure 8. Continued.
Polyethylene glycol (200 μL of a solution of 425 g/L in water) was administered by oral gavage. After 4 hours, mice received 200 μL of the FM suspension by oral gavage. Mice had free access to control or ethanol diet for 6 weeks. In the third FMT experiment, mice received control or ethanol diet supplemented with purified bovine mOPN (gift from Arla Foods, Viby, Denmark) at a concentration of 200 μg/mL. Experimental design of the FMT (E).

Figure 9. Validation of the targeting strategy and experimental design of the FMT. OPN immunohistochemistry in the jejunum from male and female mice fed with control diet (orange arrows indicate OPN staining in IECs) (A–B). Opn mRNA expression in liver from male and female OpnKI IEC and OpnMEC mice fed with control diet. Data were normalized with Gapdh as housekeeping gene, and fold change (FC) was calculated against WT (n = 6/group) (C–D). Experimental design of the FMT (E).

Fisher Scientific, Carlsbad, CA). Polyethylene glycol (200 μL of a solution of 425 g/L in water) was administered by oral gavage. After 4 hours, mice received 200 μL of the FM suspension by oral gavage. Mice had free access to control or ethanol diet for 6 weeks. In the third FMT experiment, mice received control or ethanol diet supplemented with purified bovine mOPN (gift from Arla Foods, Viby, Denmark) at a concentration of 200 μg/mL. Experimental design of the FMT (E).
twice a week for 6 weeks, and bowel cleansing was done once before the first FMT (Figure 9, E).

**Metagenomics**

DNA was isolated from fecal samples using the DNeasy power soil kit (Qiagen, Germantown, MD). Sequencing of 16S rRNA was performed at the Argonne National Laboratory (Lemont, IL) using an Illumina micro MiSeq platform. The V4 region of the 16S rRNA gene was amplified using specific barcodes and primer sequences listed in Table 2. Data were analyzed using the Illumina 16S metagenomics App in BaseSpace. Demultiplexed fastq sequences were processed for quantitative and qualitative insight into the microbial ecology. Alpha and beta diversity were determined based on the Shannon’s index and weighted UniFrac distances, respectively. For comparisons, nonparametric 1-way analysis of variance followed by the Fisher LSD post-hoc tests or a combination of fold change analysis and t tests (volcano plot), were used, and a cutoff of \( P \leq 0.05 \) adjusted P-value (false discovery rate) or P-value was considered for the statistical significance.

**Measurement of Trp Metabolites and SCFAs**

Trp and its metabolites (kynurene and indoles) and SCFAs were measured in portal serum by targeted metabolite profiling at the Metabolomics Core from the Roy J.

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**Table 1. Sequence of Primer Pairs Used in qRT-PCR Analysis**

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qRT-PCR, Quantitative real-time polymerase chain reaction.
Table 2. Sequence of Barcodes and Primers Used in 16s rRNA Sequencing

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Table 2. Continued

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<th>Sl.No</th>
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</table>

Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

Trp and its metabolites were quantified using liquid chromatography–mass spectrometry (LC/MS). Briefly, 30 µL of serum were deproteinized with 70 µL of methanol and centrifuged to obtain the supernatant. The supernatants and the standards for Trp and its metabolites were spiked with 5 µL of the internal standard (DL-Chlorophenyl alanine, 0.01 mg/ml) and quantified by LC/MS in a Vanquish high-performance liquid chromatography (Thermo Fisher Scientific, Waltham, MA) with a Polaris 3 C18-Ether 2x100 mm (3 µ) column at a flow rate of 500 µL/min. As mobile phases, 0.1% formic acid in water (A) and methanol (B) were used with the following gradient: 0 to 0.5 minutes–0% B, 3 minutes–100% B, 3 to 4.5 minutes–100% B, and 4.5 to 6 minutes–0% B. Mass spectrometry was performed in a TSQ Altis LC-MS/MS (Thermo Fisher Scientific). Data were acquired in both negative and positive SRM modes. Integration and quantification of the peaks were performed using Thermo TraceFinder. For Trp, 3-Indoleacrylic acid and 3-Indoxyl, a standard curve 200 to 0.5 µg/mL was used, and for the rest of the metabolites, a standard curve of 500 to 0.1 ng/mL was used.

After the LC/MS measurements, the same samples were used to quantify SCFAs by gas chromatography/MS on an Agilent 7890 gas chromatograph, with a 5977A mass selective detector and a 5977A mass spectrometer. Brieﬂy, 200 µL of sample was injected and analyzed on a 30-m HP-INNOWAX column with a 0.25 mm inner diameter and a 0.25 µm ﬁlm thickness (Agilent). An injection temperature of 200 °C, mass selective detector transfer line of 200 °C, adjusted ion source of 230 °C, and a constant ﬂow rate of 1 mL/min of helium carrier gas were used for the analysis. The temperature program was 2 minutes at 70 °C, followed by an oven temperature ramp of 10 °C/minute to 190 °C and 40 °C to 240 °C for a ﬁnal 2 minutes. The MS was performed in positive electron impact mode at 69.9 eV ionization energy in m/z 30 to 300 scan range in combined scan and selected ion monitoring modes targeted for m/z 43, 45, 46, 60, and 74. Using Mass Hunter Quantitative Analysis B.08.00 (Agilent) software, targeted peaks were evaluated. To generate standard curves, 100 to 0.01 mg/L concentrations were used.
Isolation of IECs

IECs were isolated from the jejunum. In this protocol, first the adhesions between the IECs were disrupted by chelating Ca\(^{2+}\) ions, and then mechanical separation of IECs was performed using a vortex.\(^\text{58}\)

Study Approval

All mice received humane care according to the criteria outlined in the guide for the care and use of laboratory animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Housing and husbandry conditions were approved by the Institutional Animal Care and Use Committee office at the University of Illinois at Chicago prior to initiation of the studies. All in vivo experiments were carried out according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism v7 with statistical significance set at \(P < .05\). Comparisons between 2 groups were performed using the nonparametric \(t\) tests (Mann-Whitney \(U\) test). For comparisons between 3 groups, nonparametric 1-way analysis of variance (Kruskal-Wallis test) followed by post hoc comparisons by the Bonferroni method were used.

References


Cassard AM, Ciocan D. Microbiota, a key player in individual susceptibility to alcoholic liver disease. Nutrients 2021;13:3170.


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Zhuolu Song, PhD (Conceptualization: Supporting; Writing – review & editing: Supporting)
Hui Han, PhD (Conceptualization: Supporting; Writing – review & editing: Supporting)
Xiaodong Ge, PhD (Conceptualization: Supporting; Writing – review & editing: Supporting)
Romain Desert, PhD (Conceptualization: Supporting; Writing – review & editing: Supporting)
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Fernando Magdaleno, PhD (Conceptualization: Supporting; Methodology: Supporting; Writing – review & editing: Supporting)
Wei Chen, PhD (Writing – review & editing: Supporting)
Daniel Lantvit (Methodology: Supporting)
Grace Guzman, MD (Methodology: Supporting)
Natalia Nieto, PhD (Conceptualization: Lead; Funding acquisition: Lead; Supervision: Lead; Writing – review & editing: Lead)

Conflicts of interest
The authors disclose no conflicts.

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