Aryl Hydrocarbon Receptor Deficiency in Intestinal Epithelial Cells Aggravates Alcohol-Related Liver Disease

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SUMMARY

Herein, we identified that Ahr deficiency in intestinal epithelial cells enriched Helicobacter hepaticus and Helicobacter ganmani in the gut, promoted their translocation to liver, and aggravated alcohol-related liver disease (ALD) development. Dietary supplementation with AHR agonists effectively ameliorated ALD in mice, providing a new strategy for ALD treatment.

BACKGROUND & AIMS: The ligand-activated transcription factor, aryl hydrocarbon receptor (AHR) can sense xenobiotics, dietary, microbial, and metabolic cues. Roles of Ahr in intestinal epithelial cells (IECs) have been much less elucidated compared with those in intestinal innate immune cells. Here, we explored whether the IEC intrinsic Ahr could modulate the development of alcohol-related liver disease (ALD) via the gut–liver axis.

METHODS: Mice with IEC specific Ahr deficiency (Ahr<sup>IEC</sup>) were generated and fed with a control or ethanol diet. Alterations of intestinal microbiota and metabolites were investigated by 16S ribosomal RNA sequencing, metagenomics, and untargeted metabolomics. AHR agonists were used to evaluate the therapeutic potentials of intestinal Ahr activation for ALD treatment.

RESULTS: Ahr<sup>IEC</sup> mice showed more severe liver injury after ethanol feeding than control mice. Ahr deficiency in IECs altered the intestinal metabolite composition, creating an environment that promoted the overgrowth of Helicobacter hepaticus and Helicobacter ganmani in the gut, enhancing their translocation to mesenteric lymph nodes and liver. Among the altered metabolites, isobutyric acid was increased in the cecum of ethanol-fed Ahr<sup>IEC</sup> mice relative to control mice. Furthermore, both H. hepaticus and isobutyric acid administration aggravated ethanol-induced liver injury in vivo and in vitro. Supplementation with AHR agonists, 6-formylindolo[3,2-b]carbazole and indole-3-carbinol, protected mice from ALD.
development by specifically activating intestinal Ahr without affecting liver Ahr function. Alcoholic patients showed lower intestinal AHR expression and higher H. hepaticus levels compared with healthy individuals.

**CONCLUSIONS:** Our results indicate that targeted restoration of IEC intrinsic Ahr function may present as a novel approach for ALD treatment. (Cell Mol Gastroenterol Hepatol 2022;13:233–256; https://doi.org/10.1016/j.jcmgh.2021.08.014)

**Keywords:** Aryl Hydrocarbon Receptor; Alcohol-Related Liver Disease; Helicobacter hepaticus; Isobutyric Acid.

Alcohol-related liver disease (ALD) is a leading cause of liver-related morbidity and mortality worldwide, and approximately 2 million people die of liver diseases each year, of which up to 50% mortality from cirrhosis can be attributed to alcohol. Over the past 25 years, adult per capita alcohol consumption increased by 10%. The exact mechanisms underpinning the pathogenesis of ALD remain unclear. Therefore, elucidating the mechanism and exploring novel therapies for ALD urgently are needed given that the current treatments are very scarce.

Aryl hydrocarbon receptor (Ahr) is a highly conserved, ligand-inducible transcription factor that integrates environmental, dietary, microbial, and metabolic cues to control the adaptation of multicellular organisms to environmental challenges. The Ahr is expressed in many mammalian tissues, especially in the liver, intestine, and kidney. In the intestine, Ahr is expressed mainly by epithelial cells and innate immune cells, and plays an important role in the regulation of innate immunity. For example, it regulates the number of intraepithelial lymphocytes, controls the production of interleukin 22 by innate lymphoid cells, and senses the bacterial virulence factors then leads to antibacterial responses. Compared with the role of Ahr in innate immune cells in the intestine, the function of Ahr in intestinal epithelial cells (IECs) has not been studied. One previous study showed that Ahr in IECs was associated with maintenance of epithelial barrier function. Given that chronic alcohol consumption can disrupt the intestinal epithelial barrier and alter gut microecology, which contributes to ALD, we investigated whether intestinal epithelial Ahr affects the progression of ALD.

Gut dysbiosis, which contributes to the pathogenesis of ALD, could present as intestinal barrier dysfunction, gut microbiota alteration, and immune system dysregulation. Bacterial components and metabolites translocate from the leaky gut through blood and lymphatics to the liver in animal models and patients with ALD. Once the microbial products translocate to the liver, they activate the innate immune receptors and induce the increased expression of hepatic inflammatory cytokines and lipogenesis-related factors, which promote the development of ALD. Not only bacterial products, but viable microbiota also can translocate to the liver. Using regenerating islet-derived 3 gamma (Reg3g) or Reg3b-deficient mice, we previously showed that the translocation of intestinal mucosa-associated bacteria to mesenteric lymph nodes and the liver could aggravate the progression of ALD. In addition, translocating Enterococcus to the liver could increase interleukin 1β secretion via the pathogen-recognition receptor Toll-like receptor 2 in Kupffer cells, resulting in hepatic inflammation and hepatocytes death,

**Results**

Ahr Deficiency in IECs Aggravates Ethanol-Induced Liver Injury

We first showed that ethanol (EtOH) feeding mice subjected to the chronic-plus-binge model showed decreased messenger RNA (mRNA) levels of Ahr and its downstream target genes (Cyp1a1, Cyp1a2, and Cyp1b1) in IECs (Figure 1A–D). Furthermore, ethanol exposure could directly down-regulate Ahr expression in the murine

**Abbreviations used in this paper:** AHR, aryl hydrocarbon receptor; Ahrloxfl, Ahrfloxx; Ahrfl, intestinal epithelial cell-specific Ahr deficiency; ALD, alcohol-related liver disease; AML12, alpha mouse liver 12; ALT, alanine aminotransferase; DMSO, dimethyl sulfoxide; FFA, fatty acid; FICZ, 6-formylindolo[3,2-b]carbazole; IBC, iodine-3-carbonil; IBA, isobutyric acid; IEC, intestinal epithelial cell; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; TG, triglyceride; WT, wild-type.

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intestinal absorption and hepatic metabolism of ethanol promoted ethanol-induced liver disease without affecting intestinal microbiota in ethanol-fed mice clustered fatty acid protein 7 (*Ahr*fl) found that the expression of elongation of very long chain (TG) synthesis, elongation, and hydrolysis. Among these, we contributed to the increased lipid accumulation in ethanol-fed mice (Figure 1J–N). After ethanol administration, livers of *Ahr*fl mice also showed significantly higher mRNA expression of inflammation-related genes, including *Il1b*, *Il6*, *Cxcl5*, and *Ccxl10*, as compared with control mice (Figure 1O). However, we found that the hepatic mRNA levels of *Tnf*, *Adgre1* (also known as F4/80), and *Ly6g* in EtOH-fed *Ahr*fl mice were comparable with those in *Ahr*fl/fl mice (Figure 1P). Consistently, hepatic myeloperoxidase + neutrophils and tumor necrosis factor *α* protein expression in *Ahr*fl mice did not differ from *Ahr*fl/fl mice after alcohol feeding (Figure 1Q and R). To further clarify the crucial genes contributing to the increased lipid accumulation in ethanol-fed *Ahr*fl mice, we evaluated the hepatic expression of genes involved in free fatty acids (FFAs) and triglyceride (TG) synthesis, elongation, and hydrolysis. Among these, we found that the expression of elongation of very long chain fatty acid protein 7 (*Elolv7*), which encodes an enzyme that is responsible for the elongation of FFAs, was increased nearly 5-fold in the livers of *Ahr*fl mice than that in *Ahr*fl/fl mice after ethanol administration (Figure 10 and P), suggestive of its potential role in promoting lipogenesis in *Ahr*fl mice.

To examine whether *Ahr* deficiency in IECs affects the absorption and metabolism of ethanol, we determined the level of ethanol in plasma and metabolism of ethanol in livers. The plasma ethanol level did not differ significantly between *Ahr*fl/fl and *Ahr*fl mice after ethanol feeding (Figure 2A), and the hepatic gene expression of *Adh1* and *Cyp2e1* (the 2 main primary enzymes that convert ethanol to acetaldehyde) were comparable between ethanol-fed *Ahr*fl/fl and *Ahr*fl mice (Figure 2B and C). These results indicate that *Ahr* deficiency in IECs promoted ethanol-induced liver disease without affecting intestinal absorption and hepatic metabolism of ethanol in mice.

**Ahr Deficiency in IECs Promotes Translocation of *H. hepaticus* and *H. ganmani to Liver in Mice**

Increasing evidence has shown that intestinal microbiota dysbiosis has been implicated in the progression of ALD. we thus used 16S ribosomal RNA (rRNA) gene sequencing to investigate the effects of IEC-specific *Ahr* disruption on gut microbiota. As shown by the principal coordinate analysis plot, we showed that the composition of intestinal microbiota in ethanol-fed mice clustered separately from that of pair-fed control mice (Figure 3A), while the overall microbiota composition of *Ahr*fl mice did not differ significantly from *Ahr*fl mice either on a control or ethanol diet (Figure 3A). Despite that the total number of intestinal bacteria remained unchanged between ethanol-fed *Ahr*fl and *Ahr*fl mice (Figure 3B), the cladogram (linear discriminant analysis effect size [LEfSe] analysis) showed that the abundance of *Helicobacter* (the family level is Helicobacteraceae, the order level is Campylobacterales) was enriched (linear discriminant analysis score > 2) in ethanol-fed *Ahr*fl mice compared with *Ahr*fl mice (Figure 3C, G, C, and D). *Helicobacter* also was increased slightly (*P = 0.076*) in control-fed *Ahr*fl mice in comparison with *Ahr*fl mice (Figure 3E). *Alistipes* (the family level is Rikenellaceae) was found to be enriched in the cecal content of EtOH-fed *Ahr*fl mice as evidenced by the 16S rRNA sequencing results (Figure 3C and D), although this increase could not be confirmed using a qPCR assay (Figure 3F). In addition, the 3 main species of *Alistipes*, including *Alistipes finegoldii*, *Alistipes timonensis*, and *Alistipes indistinctus*, did not alter significantly between ethanol-fed *Ahr*fl and *Ahr*fl mice (Figure 3F).

Furthermore, we also confirmed that the species of *H. hepaticus* and *H. ganmani* (2 species of Helicobacter) were increased in the cecum content of ethanol-fed *Ahr*fl mice relative to *Ahr*fl mice as determined by qPCR (Figure 3G). Accordingly, ethanol-fed *Ahr*fl mice showed significantly higher levels of *H. hepaticus* and *H. ganmani* both in mesenteric lymph nodes (MLNs) and livers compared with the ethanol-fed *Ahr*fl mice (Figure 3H and I), indicating that IEC-specific *Ahr* deficiency might facilitate the translocation of *H. hepaticus* and *H. ganmani* from the gut to liver after ethanol exposure. In addition, we also showed that levels of most commonly studied bacteria such as *Enterococcus*, *Bifidobacterium*, *Clostridium*, and *Prevotella* did not change obviously in livers between EtOH-fed *Ahr*fl and *Ahr*fl mice, although Firmicutes (phylum) and *Enterococcus* (genus) were increased in MLNs of *Ahr*fl mice (Figure 3F).

More importantly, we found that the relative level of *H. hepaticus* in cecum was correlated positively with hepatic steatosis, *Il6*, and *Ccxl5*, but not *Il1b* and *Ccxl10* expression (Figure 3K and L). Notably, the gene expression of gut barrier function–related proteins such as occludin (*Ocln*), tight junction protein 1 (*Tjp1*), *Tjp2*, and mucin 2 (*Muc2*) were reduced dramatically in the distal small intestine of *Ahr*fl mice relative to *Ahr*fl mice after ethanol feeding (Figure 4A). Consistently, lipopolysaccharide levels in plasma also were increased significantly in ethanol-fed *Ahr*fl mice compared with *Ahr*fl mice (Figure 4B), suggesting that *Ahr* deficiency in IECs disrupted intestinal epithelial barrier function. Of note, we failed to detect the higher mRNA levels of stem cell markers (*Lrig1* and *Lgr5*) in the intestine of ethanol-fed *Ahr*fl mice, although previous study showed that *Ahr* deletion in IECs promoted stem cell proliferation upon injury of infection or chemical insults, the gene expression of *Il22* and *Il17* in the proximal small intestine did not show the obvious difference either between ethanol-fed *Ahr*fl and *Ahr*fl mice.
Figure 2. Ahr deficiency in IECs causes the progression of ethanol-induced liver damage independent of ethanol absorption and metabolism. (A) Plasma ethanol level (n = 15 for Ahr/fl; n = 14 for AhrIEC) and (B) hepatic mRNAs of Cyp2e1 and Adh1 in Ahr/fl and AhrIEC mice fed an ethanol diet (n = 21 for Ahr/fl; n = 20 for AhrIEC). (C) Western blot of hepatic CYP2E1 and its quantification (n = 3 per group). Data are represented as means ± SEM. *P < 0.05, unpaired t test. Ctrl, control.

(Figure 4C).26 Taken together, Ahr deficiency in IECs enriched intestinal Helicobacter and aggravated their translocation to liver, eventually leading to enhanced alcohol-related liver injury.

IEC-Specific Ahr Deficiency Up-regulates IBA Level in the Intestines of Mice

To further decipher the underlying mechanism that IEC-specific Ahr deficiency promotes ethanol-induced liver damage, we also used untargeted metabolomics to assess the alteration of cecum metabolites between ethanol-fed Ahr/fl and AhrIEC mice. The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significant differences are shown in Figure 5A, and pathways such as protein digestion and absorption, aminoacyl-tRNA biosynthesis, and mineral absorption differed extensively between ethanol-fed AhrIEC and control mice. Although the tryptophan level was reduced in ethanol-fed AhrIEC mice, the level of kynurenine, which was converted from kynurenine, a main metabolite of tryptophan, was comparable between Ahr/fl and AhrIEC mice (Figure 5B). Indole and its derivatives, microbe-dependent products of tryptophan, did not alter dramatically either in these 2 groups of mice (Figure 5B). Instead, 64 metabolites with significant change were identified, among them, IBA, a short-chain fatty acid (SCFA), was increased notably in ethanol-fed AhrIEC mice compared with control mice (Figure 5C–E). The other SCFAs, including acetic acid, propionic acid, and butyric acid, were not different between the ethanol-fed AhrIEC mice and the Ahr/fl mice (Figure 5F). More importantly, we found that the increased level of IBA was correlated positively with hepatic steatosis and expression of inflammation-related genes (Figure 5G and H). Consistent with the increase of ethanol-fed AhrIEC and control mice. Although the tryptophan level was reduced in ethanol-fed AhrIEC mice, the level of kynurenine, which was converted from kynurenine, a main metabolite of tryptophan, was comparable between Ahr/fl and AhrIEC mice (Figure 5B). Indole and its derivatives, microbe-dependent products of tryptophan, did not alter dramatically either in these 2 groups of mice (Figure 5B). Instead, 64 metabolites with significant change were identified, among them, IBA, a short-chain fatty acid (SCFA), was increased notably in ethanol-fed AhrIEC mice compared with control mice (Figure 5C–E). The other SCFAs, including acetic acid, propionic acid, and butyric acid, were not different between the ethanol-fed AhrIEC mice and the Ahr/fl mice (Figure 5F). More importantly, we found that the increased level of IBA was correlated positively with hepatic steatosis and expression of inflammation-related genes (Figure 5G and H). Consistent with the increase of...
Figure 3. (See previous page). Ahr deficiency in IECs promotes expansion of cecal *H. hepaticus* and its translocation to the liver in ethanol-induced mice. (A) Principal coordinate analysis (PCoA) plot of cecal microbiota of Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice fed a control or ethanol diet (n = 6 for control [Ctrl]; n = 8 for EtOH). (B) Total bacteria in cecum of ethanol-fed mice (n = 18 for Ahr<sup>/fl/fl</sup>; n = 16 for Ahr<sup>/IEC</sup>). (C) The cladogram of gut microbiome in different taxonomic levels from ethanol-fed mice. The taxa of different abundance in Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> group are presented in blue and red, respectively (n = 8 per group). (D) Significantly altered bacterial taxa between ethanol-fed Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice (linear discriminant analysis [LDA] score, >2 or < -2). (E) The relative abundance of *Helicobacter* in cecum from control-fed mice detected by 16S rRNA gene sequencing (n = 6 per group). (F) The relative levels of Alistipes and the 3 main species of Alistipes in cecum from EtOH-fed Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice determined by qPCR (n = 6 for Ahr<sup>/fl/fl</sup>; n = 5 for Ahr<sup>/IEC</sup>). (G) The relative level of *H. hepaticus* and *H. ganmani* in cecum from control or ethanol-fed mice (n = 5–6 for Ctrl; n = 18–20 for EtOH) determined by qPCR. (H and I) *H. hepaticus* and *H. ganmani* in MLNs (n = 4–6 per group) and liver (n = 8–9 per group) of ethanol-fed Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice. (J) The relative levels of bacteria in MLNs and livers of EtOH-fed mice detected by qPCR (n = 3 per group for MLN; n = 7–9 for liver). (K) Correlation analysis between relative level of intestinal *H. hepaticus* with hepatic steatosis (left panel), Il6 (middle panel), and Cxcl5 expression (right panel). (L) Correlation analysis between the relative level of intestinal *H. hepaticus* with hepatic Il1b (upper) and Cxcl10 (lower) expression. Data are represented as means ± SEM. *P < 0.05, unpaired t test.

Figure 4. Ahr deficiency in IECs disrupted the intestinal epithelial barrier. (A) mRNA levels of Ocln, Tjp1, Tjp2, and Muc2 in the distal small intestine (n = 5–6 per group). (B) Plasma level of lipopolysaccharide (LPS) in EtOH-fed Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice (n = 13 for Ahr<sup>/fl/fl</sup>; n = 11 for Ahr<sup>/IEC</sup>). (C) mRNA levels of leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1), leucine-rich repeat containing G-protein–coupled receptor 5 (Lgr5), Il22, and Il17 in the proximal small intestine of ethanol-fed Ahr<sup>/fl/fl</sup> and Ahr<sup>/IEC</sup> mice (n = 5–16), Lrig1 and Lgr5 are the stem cell markers. Data are represented as means ± SEM. *P < 0.05, unpaired t test. Ctrl, control.
secondary bile acid lithocholic acid, which was reported to cause liver injury,27 nervous acid, and the stearamide, which was increased in patients with alcoholic cirrhosis,24 showed significant positive correlations with the abundance of *H. hepaticus* (Figure 6A and B), while the other 4 metabolites including hypoxanthine, phenylalanine-cysteine, acamprosate, and thymine showed negative correlations with *H. hepaticus* (Figure 6C). In addition, we found that metabolites such as adynacin, POPG, and adenosine monophosphate were correlated positively with *H. ganmani*, while linoleic acid was correlated negatively with *H. ganmani* (Figure 6D and E).

Subsequently, we evaluated the effects of the correlated metabolites on the growth of *H. hepaticus* in vitro. We found that only the lipid POPG could significantly promote the growth of *H. hepaticus* (Figure 6F). Interestingly, the untargeted metabolomics analysis showed that *H. hepaticus* per se could produce POPG as the metabolite (Figure 6G), although we cannot exclude that POPG also possibly was derived from the host. Consistently, the intestinal POPG level in mice fed with *H. hepaticus* also was higher than that in control mice (Figure 6H). More importantly, we also showed that POPG could enhance *H. hepaticus* growth in intestinal organoids (Figure 6I and J and Supplementary Video). Therefore, we provided mechanistic evidence that POPG might be the most important metabolite to promote the enrichment of *H. hepaticus* in the intestine.

**H. hepaticus Aggravates Ethanol-Induced Injury In Vivo and In Vitro**

To further prove that the IEC-specific *Ahr* deficiency–induced increase of *H. hepaticus* promotes the development of ALD, we challenged wild-type (WT) mice with *H. hepaticus* every other day in a chronic-plus-binge model. *H. hepaticus* administration markedly increased levels of *H. hepaticus* (>500-fold) in feces of mice (Figure 7A). In line with this, mice gavaged with *H. hepaticus* developed more severe ethanol-induced hepatic injury, indicated by increased ALT level (Figure 7B), increased hepatic steatosis (Figure 7C), as well as enhancement of hepatic *Cxcl5* and *Cxcl10* expression (Figure 7D). Consistently, both cultured hepatocytes and Kupffer cells stimulated with *H. hepaticus* in vitro recapitulated the effects of *H. hepaticus* in vivo. *H. hepaticus*–treated mouse hepatocyte alpha mouse liver 12 (AML12) cells showed more lipid droplet accumulation (Figure 7E and F) and increased expression of inflammation-related genes such as *Il1b*, *Il6*, *Cxcl5*, and *Cxcl10* in either basal or ethanol-stimulated conditions (Figure 7G). Notably, *H. hepaticus*–induced increment of *Cxcl10* expression became more obvious in the presence of ethanol (Figure 7G). In addition, *H. hepaticus* also could induce the increase of *Il1b*, *Il6*, *Cxcl5*, and *Cxcl10* gene expression in Kupffer cells (Figure 7H), which is comparable with that in hepatocytes. These results provided direct evidence that *H. hepaticus* could aggravate liver injury in vivo and in vitro.

**IBA Induces Liver Injury in Mice and Cultured Hepatocytes**

IBA level was increased in ethanol-fed *Ahr*flmice compared with *Ahr*flmice (Figure 5C), we thus investigated whether the increased IBA also could contribute to the progression of ALD. Administration of IBA in C57BL/6 mice showed obvious liver damage as indicated by the increased plasma ALT level, hepatic lipid accumulation, and higher hepatic TG level compared with control mice (Figure 8A–C). We also noticed that IBA could significantly increase hepatic *Cxcl5* gene expression (Figure 8D). Consistent with this, AML12 cells stimulated with IBA showed more lipid accumulation and increased gene expression of hepatic *Cxcl5* and *Cxcl10* (Figure 8E–G). Similarly, IBA also could increase *Cxcl5* and *Cxcl10* expression in Kupffer cells (Figure 8H). More interestingly, we found that *H. hepaticus* could boost the effects of IBA on lipid accumulation (Figure 8I and J). It should be noted that IBA treatment alone or in combination with ethanol significantly up-regulated *Elov17* expression in AML12 cells (Figure 8K), which is consistent with the increased *Elov17* expression in ethanol-fed *Ahr*flmice, which had the higher level of IBA in vivo (Figures 10 and 5C–F). We thus sought to determine the effect of *Elov17* on lipid accumulation in hepatocytes. Overexpression of *Elov17* resulted in more lipid droplet accumulation in AML12 cells, either in basal or alcohol-stimulated conditions (Figure 8L and M). All of these results showed that IBA could induce hepatic steatosis and liver damage in vivo and in vitro, while *H. hepaticus* was able to enhance the effects of IBA on lipogenesis.
also elucidated the crucial role of *Elovl7* in IBA-induced lipid accumulation.

**AHR Agonists Ameliorate ALD in Mice**

Two widely used agonists for AHR, I3C and FICZ,\(^{12,29}\) whose activation effects on *Ahr* were confirmed in vitro by ourselves (Figure 9A and B), were selected to explore whether supplementation with AHR agonists could be a therapeutic approach for ALD. WT mice exposed to an ethanol diet were orally gavaged with I3C (50 mg/kg) and FICZ (50 µg/kg) daily for 15 days. Although body weight and food intake showed no significant differences compared with control mice (Figure 9C), both I3C and FICZ decreased hepatic steatosis (Figure 9D), decreased liver TG levels (Figure 9E), and reduced mRNA expression of *Elovl7, Il6, Cxcl5*, and *Cxcl10* (Figure 9F and G). Subsequently, we confirmed that I3C and FICZ activated AHR in the intestine as shown by the increased gene expression of *Cyp1a1* and *Cyp1b1* (Figure 9H), whereas they did not activate hepatic AHR (Figure 9I), suggesting that selectively activating intestinal AHR was sufficient to protect mice from alcohol-induced liver damage. Intriguingly, mice treated with I3C and FICZ dramatically decreased the abundance of intestinal *H. hepaticus* (Figure 9J). Collectively, our findings show the therapeutic potentials of AHR agonists for ALD treatment.

**Alcoholic Patients Showed Decreased Intestinal AHR Expression and Increased *H. hepaticus* Level**

To explore whether the decreased intestinal AHR expression was relevant to alcoholic patients, we analyzed their levels in the duodenal tissues. As expected, the mRNA and protein expression of intestinal AHR was decreased significantly in alcoholic patients compared with nonalcoholic individuals by qPCR analysis and immunohistochemical staining (Figure 10A and B). Intriguingly, compared with healthy controls, patients with alcoholic liver disease showed a higher level of fecal *H. hepaticus* (Figure 10C). Notably, 1 patient with an alcohol-use history for more than 30 years (2–3 bottles of liquor for daily drinking, equal to 400–600 g pure alcohol/d) and very severe aspartate aminotransferase, γ-glutamyl transferase, and Fibrosis-4 index levels, had a remarkably higher abundance of *H. hepaticus* in the stool sample (Figure 10C).

**Discussion**

*Ahr* is widely expressed throughout the body, and intestinal *Ahr* was reported to play an important role in enteric diseases such as inflammatory bowel disease and colitis by regulation of the immune response and intestinal barrier functions.\(^{6,8,12}\) However, the association between intestinal *Ahr* and liver diseases still remains largely unexplored. The AHR ligands constitute a large family that generally can be categorized into 4 major sources: xenobiotics (eg, dioxin), dietary metabolites (eg, I3C), endogenous metabolites (eg, indole acetic acid), and microbial derivatives (eg, indirubin).\(^{30,31}\) Although ethanol is not identified as a direct ligand of AHR, ethanol feeding was shown to reduce microbiota-dependent AHR ligand production from tryptophan in mice such as indole acetic acid.\(^{26}\) Notably, AHR activation by microbial tryptophan metabolites were shown to improve ALD in mice,\(^{52}\) highlighting the association of ALD with intestinal AHR and microbiota. We thus attempt to explore the crosstalk between intestinal AHR and gut microbiota, as well as their metabolites, and clarify the role of intestinal AHR in the progression of ALD.

Considering that IECs function as the first line sensing intestinal environment change derived from dietary, microbial, and metabolic cues, we generated IEC-specific *Ahr*-deficiency mice in which *Ahr* expression in lamina propria cells remained unchanged. We showed that *Ahr* deletion in IECs did not directly affect intestinal absorption and metabolism of ethanol. In addition, we could not find that a lack of *Ahr* in IECs altered the intestinal mRNA levels of stem cell markers, although 1 previous study showed that *Ahr* deletion in IECs enhanced stem cell proliferation upon injury through infection or chemical insults.\(^{12}\) The gene expression of *Il22* and *Il17* in the proximal small intestine was not different between ethanol-fed *Ahr*\(^{−/−}\) mice and control mice.

Here, we observed that alcohol depressed the expression of *Ahr* in IECs in mice and human beings, and its deficiency enhanced the susceptibility to ethanol-induced liver injury.

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**Figure 6.** (See previous page). *H. hepaticus* per se could produce POPG as the metabolite that enhances *H. hepaticus* growth. (A) The correlation analysis between stearamide, lithocholic acid, POPG, nervonic acid, and the relative abundance of intestinal *H. hepaticus*. (B) Spearman rank correlation between significantly altered metabolites and the intestinal relative abundance of *H. hepaticus*. (C) The correlation analysis between acamprosate, thymine, hypoxanthine, phenylalanine-cysteine (Phe-Cys) and the relative abundance of intestinal *H. hepaticus*. (D) Spearman rank correlation between significantly altered metabolites and the intestinal relative abundance of *H. ganmani*. (E) The correlation analysis between adynomin, POPG, adenosine monophosphate (AMP), linoleic acid, and the relative abundance of intestinal *H. ganmani* (Pearson correlation). (F) POPG was observed to boost the growth of *H. hepaticus* in vitro. *H. hepaticus* was cultured anaerobically in brucella agar plates containing 8% sheep blood with or without the metabolite for 6 days. Bacteria were collected and suspended in medium and their growth were determined by OD\(_{600}\) measurement. n = 4 independent experiments performed in 2 replicates in each experiment. (G) The relative level of POPG in control or *H. hepaticus* by untargeted metabolomics (n = 2). (H) The level of POPG in the cecal content from mice fed with *H. hepaticus* or broth (n = 5–6). (I) Schematic representation of microinjection of *H. hepaticus* into the intestinal organoid. (J) POPG enhanced the growth of *H. hepaticus* in the intestinal organoid isolated from WT mice. *H. hepaticus* exposed to POPG or control medium was microinjected into intestinal organoid. After incubation for 4 days in the lumen of the organoid, bacteria were released and cultured anaerobically in brucella agar plates for 6 days and determined by OD\(_{600}\) analysis. n = 3 independent experiments performed in 2 replicates in each experiment. Data are represented as means ± SEM. *P* < 0.05, unpaired *t* test.
Compared with control mice, intestinal levels of Helicobacter (H. hepaticus and H. ganmani) and IBA were up-regulated in ethanol-fed Ahr-IEC mice, which were accompanied by increased intestinal permeability, and the abundance of H. hepaticus and H. ganmani in MLNs and liver also were increased accordingly in these mice. Of note, H. hepaticus per se could produce POPG as the metabolite that, in turn, enhanced the bacteria growth and promoted its translocation from gut to liver on the basis of the intestinal barrier disruption caused by Ahr deficiency. H. hepaticus and IBA aggravated ethanol-induced liver injury by eliciting hepatic inflammation and steatosis. Importantly, oral supplementation of AHR agonists FICZ and I3C (a derivative from cruciferous vegetables) markedly ameliorated ALD by activating intestinal Ahr and reducing H. hepaticus in a mouse model, suggesting a new way for the treatment of ALD (Figure 10D). Consistently, a recent study showed that the prebiotic, pectin, could improve alcohol-induced liver injury by increasing the bacterial tryptophan metabolites, and proposed that targeting intestinal AHR activation could improve alcoholic liver disease, however, our study, through using IEC-specific Ahr-deficiency mice, showed elaborately that it is the IEC-intrinsic Ahr that mediated the beneficial effects of AHR activation on the progression of ALD.

In this study, we showed that a specific dysbiosis, increased intestinal Helicobacter species, contributed to the progression of ALD in mice. Previously, Helicobacter species...
were not regarded as pathogens for human beings because the majority of colonized individuals were asymptomatic.\textsuperscript{13} However, increasing evidence has shown a strong association of intestinal \textit{Helicobacter} species with enteric diseases such as Crohn’s disease and inflammatory bowel disease in patients, indicating its pathogenicity in the digestive diseases.\textsuperscript{14,15} \textit{H. hepaticus}, one of the main species of \textit{Helicobacter}, also could be detected in patients’ bile and liver samples, and was associated with chronic liver diseases, including primary hepatocellular carcinoma and bile duct cancer.\textsuperscript{16–18} Here, we identified that the \textit{H. hepaticus} level was increased markedly in patients with ALD compared with that in healthy individuals. In addition, \textit{H. ganmani}, another important species of \textit{Helicobacter} with increased abundance in ethanol-induced \textit{Ahr}\textsuperscript{IEC} mice, was not explored in this study because of its current unavailability, however, the role of \textit{H. ganmani} in ALD also deserves further investigation.

Besides the changes of gut microbiota, another explanation for the link between gut dysbiosis and liver diseases is the alteration of metabolites produced by the intestinal microbiota. Well-known metabolites such as SCFAs (e.g., butyrate, acetate) could exert immunomodulatory effects both inside and outside of the intestine.\textsuperscript{40,41} Here, we showed that the SCFA IBA was increased significantly in IEC-specific \textit{Ahr}-deficiency mice compared with control mice after alcohol drinking. Shotgun metagenomics analysis indicated that the pathway contributing to IBA synthesis was more activated in \textit{Ahr}\textsuperscript{IEC} mice than that in \textit{Ahr}\textsuperscript{fl/fl} mice as evidenced by the increased bacterial gene expression of \textit{ileV}, \textit{bkdA}, and \textit{pdhD}. Notably, the bacterial gene \textit{ileV}, coding the enzyme responsible for catalyzing valine to 3-methyl-2-oxobutanoate, also could be found in \textit{H. hepaticus}. Thus, we presumed that the increased abundance of \textit{H. hepaticus} in \textit{Ahr}\textsuperscript{IEC} mice also may contribute to the increased IBA. Regarding the role of IBA in liver disease, we showed that both IBA administration in vivo and treatment in cultured hepatocytes could induce liver injury when used alone or in combination with alcohol. Although we failed to detect IBA in mice serum, it still is possible that IBA could go to the liver from the portal vein to aggravate the alcohol-induced liver disease in \textit{Ahr}\textsuperscript{IEC} mice given that these mice had a disrupted intestinal barrier. Our findings also are consistent with findings in nonalcoholic steatohepatitis patients containing a higher fecal IBA level.\textsuperscript{12} These results therefore suggest that fecal IBA might be developed as a new biomarker to predict the progression of ALD.

Mechanistically, we used the combination of 16S rRNA sequencing, metagenomics, and untargeted metabolomics to show that IEC-specific \textit{Ahr} deficiency induced the alteration of intestinal metabolites, these metabolites, such as the anionic phospholipid POPG and the secondary bile acids lithocholic acid, neroenic acid, and deoxycholic acid (increased in patients with alcoholic cirrhosis),\textsuperscript{28} correlated positively with the abundance of \textit{H. hepaticus}, while metabolites such as hypoxanthine, phenylalanine–cysteine, acamprosate, and thymine correlated negatively with the abundance of \textit{H. hepaticus}. We further showed that the lipid POPG, which was produced by \textit{H. hepaticus}, could in turn promote the growth of the bacteria itself. In addition, acamprosate, a drug for alcohol use disorder treatment,\textsuperscript{43,44} was identified as decreased in cecal content from EtOH-fed \textit{Ahr}\textsuperscript{IEC} mice by untargeted metabolomics. It showed a negative correlation with \textit{H. hepaticus}, although validation of this metabolite remains to be addressed by targeted metabolomics analysis. All of these changes in metabolite composition might directly promote the overgrowth of intestinal \textit{H. hepaticus}, facilitating its translocation to the liver. At the gene level, we showed that the increased bacterial gene expression of \textit{ilvE}, \textit{bkdA}, and \textit{pdhD} contributed to the increase of IBA in ethanol-fed \textit{Ahr}\textsuperscript{IEC} mice relative to control mice, suggesting that these genes might be good targets for regulating the intestinal IBA level. More interestingly, we showed that IBA and \textit{H. hepaticus} could have a synergistic effect on lipogenesis in hepatocytes. Overall, we elucidated the balance of interactions between IEC-intrinsic \textit{Ahr}, gut metabolites, and microbiota, the disruption of this balance came from IEC-specific \textit{Ahr} deficiency, which would lead to the alteration of gut metabolites and microbiota as well as the development of ALD in this study.

\textit{Elovl7}, one enzyme responsible for the elongation of saturated FFAs, was reported to trigger lipid accumulation in differentiated adipocytes, leading to oxidative damage and inflammation.\textsuperscript{45,46} In our study, compared with \textit{Ahr}\textsuperscript{fl/fl} mice, we found an up-regulated gene expression of hepatic \textit{Elovl7} in ethanol-induced \textit{Ahr}\textsuperscript{IEC} mice, which had more severe steatotic livers. This was consistent with the in vitro results that overexpression of \textit{Elovl7} resulted in lipid accumulation in cultured hepatocytes. Mechanistically, we identified that IBA, which was increased in \textit{Ahr}\textsuperscript{IEC} mice

\textbf{Figure 8. (See previous page.) IBA induces liver damage in vivo and in vitro.} (A–D) Mice were injected intraperitoneally with vehicle or IBA for 24 or 48 hours. (A) Plasma ALT level at 24 hours (n = 8 per group). (B) Representative H&E staining of liver sections at 48 hours. (C) Hepatic TG content (n = 7–8 per group) at 48 hours. (D) Hepatic Cxcl5 mRNA at 24 hours (n = 8 per group). (E) AML12 cells were seeded in 12-well plates overnight, and were treated with control (PBS) or ethanol (100 mmol/L), and stimulated with vehicle (DMSO) or IBA (1 and 4 mmol/L) at the same time for 24 hours, and the cells were stained with Oil red O. (F) Quantification of the Oil red O staining. (G and H) mRNA levels of \textit{Cxc5} and \textit{Cxc10} in AML12 cells (G) treated with vehicle or IBA for 24 hours and isolated Kupffer cells, or (H) treated with vehicle or IBA for 4 hours. n = 3 independent experiments performed in 2 replicates each experiment. (I and J) AML12 cells were seeded in 12-well plates overnight and treated with medium, IBA, \textit{H. hepatitis}, and IBA + \textit{H. hepatitis} for 24 hours. The cells were finally stained with Oil red O. (I) Representative images of the Oil red O staining. (J) Quantification of Oil red O staining. (K) \textit{Elovl7} mRNA levels in AML12 cells exposed to EtOH (100 mmol/L), IBA (4 mmol/L), or EtOH (100 mmol/L) + IBA (4 mmol/L). (L) Cells were transfected with empty vector or \textit{Elovl7}, and exposed to control or EtOH (100 mmol/L) for 24 hours. Cells were stained with Oil red O. (M) Quantification of the Oil red O staining. For cell culture experiments, at least 3 independent experiments were performed with 2 replicates in each experiment. Scale bar: 50 \mu m. Data are represented as means ± SEM. *P < 0.05, unpaired t test. Ctrl, control.
relative to control mice, could directly up-regulate Elovl7 mRNA expression in hepatocytes. Our observations thus proved that Elovl7 might play an important role in regulating hepatic lipid accumulation in the context of alcohol-related liver disease.

In conclusion, we showed an essential role for intestinal epithelial cell intrinsic Ahr in regulating hepatic lipid accumulation and inflammation in ALD through affecting H. hepaticus and IBA levels, which might serve as predictive biomarkers for the progression of ALD. Moreover, dietary
supplementation with AHR agonists provided a new therapeutic strategy for treating ALD.

**Methods**

**Animals**

Ahr<sup>fl/fl</sup> (stock no. 006203) and Villin-Cre (stock no. 021504) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house at China Pharmaceutical University. For IEC-specific Ahr disruption, Ahr<sup>fl/fl</sup> mice were crossed with Villin-Cre mice to obtain Ahr<sup>IEC</sup> mice. WT mice on a C57BL/6 background were obtained from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). All mice were housed in the specific pathogen-free facility and maintained under a temperature-controlled (22°C–23°C) room with a 12:12-hour light/dark cycle. All animal procedures were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China).

**Figure 10. Decreased intestinal AHR level and increased H. hepaticus in alcoholic patients.** (A) mRNA and (B) protein levels of AHR in duodenal tissues from controls and alcoholic patients (n = 12 controls; n = 6 alcoholic patients) determined by qPCR and immunohistochemical staining. (C) The relative level of H. hepaticus in healthy controls and patients with alcoholic liver disease (n = 13 per group). (D) A schematic diagram summarizing our findings that alcohol abuse down-regulates AHR expression in IECs, which contributes to ALD progression. Alcohol depresses the expression of Ahr in IECs, and subsequently enriches intestinal H. hepaticus, H. ganmani, and IBA levels. Ahr deficiency in IECs exacerbates intestinal H. hepaticus and H. ganmani translocation through MLNs to liver via the disrupted gut barrier. POPG, 1 metabolite of H. hepaticus, in turn boosts H. hepaticus growth. H. hepaticus and IBA augment ethanol-induced liver injury by eliciting hepatic inflammation and steatosis. Supplementation with AHR agonists, FICZ and indole-3-carbinol (abundant in cruciferous vegetables), protects mice from ALD development by activating intestinal Ahr. Data are represented as means ± SEM. *P < 0.05, Mann–Whitney test.
Animal Models

Age-matched female Ahr/fl/f and Ahr/fl/littermates (age, 8–10 wk; weight, 20–22 g) were subjected to the chronic-plus-binge model. In this model, 8-week-old mice (female) received a Lieber-DeCarli control liquid diet (F1259SP; Bio-Serv, Flemington, NJ) for 5 days and an ethanol liquid diet (F1258SP; Bio-Serv) for 10 days, and then were administered with a single binge of 5 g/kg ethanol (459844; Sigma-Aldrich, St. Louis, MO) on day 16. Pair-fed control mice (Ahr/fl/f and Ahr/fl/littermates) received an isocaloric substitution of dextrose diet. For AHR agonist treatment, WT female mice were gavaged daily with a volume of 100 µL vehicle (dimethyl sulfoxide [DMSO] suspended in corn oil), 50 mg/kg I3C (105220; Aladdin, Shanghai, China), or 50 µg/kg FICZ (synthesized by Professor Yinan Zhang, Nanjing University of Chinese Medicine, Nanjing, China), starting on day 1 of the binge ethanol feeding model. I3C and FICZ were dissolved in a small volume of DMSO initially, and adjusted with corn oil to prepare the final concentrations before use. When the mice were killed, plasma and appropriate tissues including liver, MLNs, intestine, and cecum were harvested. For IBA (11754; Sigma-Aldrich) injection, WT female mice (age, 10 wk) were injected intraperitoneally with vehicle (phosphate-buffered saline [PBS]) or IBA (2.5% IBA diluted in PBS) at a dose of 2.5 mL/kg. Blood and liver tissues were collected at 24 or 48 hours after injection.

Human Samples

Alcoholic patients were enrolled in this study according to the pathologic examination results and inclusion criteria as described previously, and written informed consent was signed by each patient and control. All of these participants did not take any antibiotics during the 2 weeks preceding the enrollment.

For H. hepaticus abundance in feces, patients with ALD (n = 13; 12 males/1 female; mean age, 52.8 y) were enrolled in this study. Thirty healthy volunteers (social drinkers consuming <20 g/d; 12 males/1 female; mean age, 49.07 y) were recruited as controls. Baseline features of these subjects are shown in Table 1. Fecal samples were collected, frozen immediately, and stored at −80°C. The protocol was approved by the Ethics Committee of the Beijing Ditan Hospital, Capital Medical University (Beijing, China).

For intestinal AHR level analysis, duodenal tissues were collected with endoscopically normal duodenum from alcoholic patients (n = 6) and individuals without alcohol consumption (controls, n = 12). Patient characteristics are summarized in Table 2. This study protocol was approved by the Ethics Committee of the Sir Run Run Shaw Hospital, Nanjing Medical University (Nanjing, China).

Bacterial Cultures

H. hepaticus (ATCC51449) was a gift from Professor Quan Zhang’s laboratory (Institute of Comparative Medicine, College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, China). The strain was cultured anaerobically on brucella agar (8241972; BD Difco, Detroit, MI) plates with 8% sheep blood (TX0030; Solarbio, Beijing, China) for 6 days. Bacteria collected and suspended in PBS (adjusted to optical density analysis at 600 nm [OD600] = 1.0) were used for untargeted metabolomics.

### Table 1. Demographic and Clinical Parameters of Controls and Patients With Alcoholic Liver Disease

| Variables                          | Controls (n = 13) | Alcoholic liver disease (n = 13) |
|-----------------------------------|------------------|---------------------------------|
| Sex, male, n (%)                  | 12 (92.3)        | 12 (92.3)                       |
| Age, y, n = 26                    | 49.07 (33–66)    | 52.8 (36–78)                    |
| ALT level, U/L, n = 23            | 19.6 (11.9–37.4) | 30.5 (6.6–83.0)                 |
| AST level, U/L, n = 23            | 21.5 (13.0–28.0) | 61.2 (19.6–237.0)               |
| GGT level, U/L, n = 11            | 259.3 (18.3–932.1)|                                 |
| Albumin level, g/dL, n = 13       | 32.5 (24.5–41.5) |                                 |
| Bilirubin level, mg/dL, n = 13    | 40.4 (13.1–92.7) |                                 |
| Creatinine level, mg/dL, n = 13   | 65.9 (34.1–123.7)|                                 |
| INR, n = 11                       | 1.3 (0.83–1.79)  |                                 |
| Platelet count, 10⁹/L, n = 13     | 115.6 (24.5–402.0)|                                 |
| MELD, n = 11                      | 12.8 (7.0–18.0)  |                                 |
| FIB-4, n = 11                     | 7.2 (0.6–21.2)   |                                 |
| FIB-4 > 3.25, n (%)               | 6 (46.2)         |                                 |
| Prior length of alcohol abuse, n = 11 |                 |                                 |
| 10–20 y                           | 2 (18.2%)        |                                 |
| 20–30 y                           | 4 (36.4%)        |                                 |
| >30 y                             | 5 (45.4%)        |                                 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, fibrosis-4 index; GGT, γ-glutamyl transferase; INR, international normalized ratio; MELD, model for end-stage liver disease.
The total RNA from livers of mice was isolated using RNAiso Plus (9109; TaKaRa, Dalian, Liaoning, China) to obtain total RNA. The RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). RNase-free DNase (TaKaRa, Dalian, Liaoning, China) was used to remove genomic DNA contamination, and the RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (5541-4148; Thermo Scientific, Waltham, MA). Subsequently, qPCR was performed as described in our previous work.49,50

**Table 2. Demographic and Clinical Parameters of Controls and Alcoholic Patients**

| Variables                          | Controls (n = 12) | Alcoholic patients (n = 6) |
|-----------------------------------|------------------|---------------------------|
| Sex, male, n (%)                  | 6 (50.0)         | 6 (100.0)                 |
| Age, y                            | 36.7 (21–59)     | 51.2 (37–57)              |
| ALT level, U/L                    | 17.7 (8.0–25.0)  | 22.3 (15.0–29.0)          |
| AST level, U/L                    | 19.3 (14.0–29.0) | 17 (15.0–20.0)            |
| GGT level, U/L                    | 26.1 (11.0–56.0) | 30.7 (18.0–44.0)          |
| Albumin level, g/dL               | 44.9 (41.2–48.8) | 45.4 (43.3–51.2)          |
| Bilirubin level, µmol/L           | 9.4 (5.8–13.2)   | 8.8 (5.3–14.3)            |
| Creatinine level, µmol/L          | 69.3 (58.0–79.0) | 62.2 (56.0–82.0)          |
| INR                               | 0.97 (0.89–1.10) | 0.99 (0.89–1.04)          |
| Platelet count, 10^9/L            | 225.7 (148.0–297.0) | 215.7 (173.0–335.0)     |
| FIB-4                             | 0.71 (0.36–1.37) | 1.19 (0.52–1.71)          |
| Prior length of alcohol abuse, n  | 10–20 y          | 2 (16.7%)                 |
|                                  | 20–30 y          | 1 (33.3%)                 |
|                                  | >30 y            | 3 (50.0%)                 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, fibrosis-4 index; GGT, γ-glutamyl transferase; INR, international normalized ratio.

To determine the effect of metabolites for *H. hepaticus* growth in vitro, *H. hepaticus* was cultured on brucella agar plates containing 8% sheep blood with or without the metabolite. After 6 days of incubation, bacteria were collected and suspended in medium and growth was determined by OD₆₀₀ measurement.

For *H. hepaticus* administration, WT female mice (age, 8 wk) subjected to the chronic-plus-binge model were orally administered 1.0 or medium, respectively, as described.49 At the end of the experiment, mice were killed and appropriate tissues were harvested.

**Biochemical Assays**

Blood was harvested from the inferior caval vein of mice to tubes containing anticoagulant (0.5 mol/L EDTA-Na₂), and centrifuged for 5 minutes (11,292 × g, 4°C) to obtain plasma. The levels of ALT, aspartate aminotransferase in plasma, and triglycerides in liver were determined using commercial kits (C009-2-1, C010-2-1, and A110-1-1, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).50

**RNA Extraction and Real-Time qPCR**

The total RNA from livers of mice was isolated using RNAiso Plus (9109; TaKaRa, Dalian, Liaoning, China) according to the methods described previously.50 The complementary DNA was synthesized from 1 µg RNA by using a High Capacity complementary DNA reverse-transcription kit (R312-02; Vazyme, Nanjing, Jiangsu, China). qPCR was performed as described in our previous work.49,50 using SYBR Premix (Q331-02; Vazyme) according to the manufacturer’s instructions on the ABI StepOnePlus real-time PCR machine (Applied Biosystems, Foster City, CA). All qPCR primers were synthesized by Sangon Biotech (Shanghai, China) and are shown in Table 3. The relative gene expression was calculated by the 2–ΔΔCT method.

**Bacterial DNA Isolation and qPCR for 16S, *H. hepaticus*, and *H. ganmani***

Bacterial genomic DNA was extracted from mice cecum content, livers, and MLNs as previously described.19,50 The bacterial abundance was quantified by qPCR, and the value of the 16S rRNA gene was normalized to cecum weight, and the relative abundance of *H. hepaticus* and *H. ganmani* in cecum, livers, and MLNs was normalized to 16S.

**16S rRNA Gene Sequencing and Analysis**

Cecal DNA samples from each group were selected randomly for 16S rRNA gene sequencing and analysis.19 The V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina NovaSeq platform (Illumina, San Diego, CA) in Novogene Technology Co, Ltd (Beijing, China). Raw sequence data were analyzed with a pipeline51 based on USEARCH52 v10.0.240 (http://www.drive5.com/usearch) and VSEARCH53 v2.13.6 (https://github.com/torognes/vsearch). Briefly, after a combination of sequences and removal of barcodes, UNOISE35 was performed to generate amplicon sequence variants. After sequence alignment by SILVA database55 (Silva_123, http://www.drive5.com/usearch/manual/sintax_downloads.html), β-diversity was calculated with USEARCH. The principal coordinate analysis plot was generated based on Bray-Curtis distance. Then LEfSe56 (http://huttenhower.sph.harvard.edu/galaxy) and the R package edgeR57 were obtained for detecting differential taxons. All plots were drawn by R version 3.6.1 except those of LEfSe. The raw data reported here have been deposited in the National Center for Biotechnology Information Sequence Read Archive database (accession no. PRJNA663684).

**Untargeted Metabolomics**

Mice cecal contents were analyzed for untargeted metabolomics by using an ultrahigh performance liquid chromatography (UHPLC, 1290 Infinity LC; Agilent Technologies, Santa Clara, CA) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600; Applied Protein Technologies, Shanghai, China). The analytes were separated on a 2.1 mm × 100 mm ACQUITY UPLC BEH 1.7-µm C18 column (Waters, Dublin, Ireland) and analyzed in both electrospray-positive and electrospray-negative ionization modes.
Metagenomics

DNA sample testing, library construction, and sequencing with an Illumina HiSeq platform were conducted at Novogene Technology Co, Ltd. The analyzing steps were described previously.51 In short, quality control was performed with Kneaddata pipeline (https://bitbucket.org/biobakery/kneaddata) to exclude the host genome. Then, these clean reads were assembled to contigs via MEGAHIT,58 and Prokka59 was used for contigs identification in a conda environment of MetaWrap,60 and CD-HIT61 was used for nonredundant genes. A gene abundance table was generated through Salmon62 and functional annotations of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology by eggNOG-mapper 63 with its related database.

SCFA Quantification

Cecum content of SCFAs were analyzed based on previous method.64 Briefly, samples (50 mg) were suspended in 200 μL distilled water and acidified with 50 μL (50%) sulfuric acid. Then, the solution was vortexed vigorously and extracted with diethyl ether. SCFAs were quantified by a gas chromatography Nexis GC-2030 (Shimadzu, Kyoto, Japan).

Table 3. Primers Used for Real-Time Reverse-Transcription PCR

| Name for mouse | Sequence, 5’-3’ |
|----------------|----------------|
| Acaca-f | GGACAGAGCTGATCGCGAGGAAAG |
| Acaca-r | TGGAGAGCCCGCCACACCA |
| Acly-f | AGCGAGATAGTCAAAGTCCAG |
| Acly-r | AAGATTCAGTCCCAAGTCCAG |
| Accox1-f | TCGCAGACCCTGAAGAAATC |
| Accox1-r | CCTGATTCAGCAAGGTAGGG |
| Adh1-f | AGCGGATTGTTGAGACGCT |
| Adh1-r | CGGAGATTGTTGAGACGCT |
| Ahf-r | CTCTCTTGCAAAACTCGC |
| Ahf-r | CGGAGATTGTTGAGACGCT |
| Cadb-f | GCCATGTTGACAGTCTCC |
| Cadb-r | TGTGAGGATGCTCTGATTC |
| Cpy1-f | ATCGTGGAGATCTCAAGCATA |
| Cpy1-r | TATGTGAGGATGCTCTGATTC |
| Cpy2-f | CCAATATGCTGGTTGCAATC |
| Cpy2-r | GTAGTGGAGATCTCAAGCATA |
| Dgat2-f | GAAGAGTCTTGCGAGGCT |
| Dgat2-r | GGACAGAGCTGATCGCGAGGAAG |
| Elv1-f | CACATATTGCCAGATTTGAGG |
| Elv1-r | ACGCAATGACACGATTGAG |
| Elv5-f | CCTGTTAAGTATTGTCAATAG |
| Elv5-r | GTAGTGGAGATCTCAAGCATA |
| Elv7-f | GCAATATGCTGGTTGCAATC |
| Elv7-r | GTAGTGGAGATCTCAAGCATA |
| Fasn-f | CTCTCTTGCTGCTGAGT |
| Fasn-r | GTGAGGATGCTCTGCTGAGT |
| II1-f | TGTGAAATGCCACGATTGAG |
| II1-f | TGTGAAATGCCACGATTGAG |
| II6-f | TAGCTCTTCCTACCCCAATTC |
| II6-r | GCAATATGCTGGTTGCAATC |
| II7-f | GCAATATGCTGGTTGCAATC |
| II7-r | GCAATATGCTGGTTGCAATC |
| II2-f | AGCGGATTGTTGAGACGCT |
| II2-r | CGGAGATTGTTGAGACGCT |
| Lgr-f | GGAGAGGATGCATGAGG |
| Lgr-f | GGAGAGGATGCATGAGG |
| Lpr-f | GCCATGTTGAGATCTCAAGCATA |
| Lpr-r | TCTCTCTTGCTGCTGAGT |
| Lrl-f | TTGAGACATTGGAAGGAT |
| Lrl-f | TTGAGACATTGGAAGGAT |
| Ly6-f | CATCCTCTCTCTGCTGGT |
| Ly6-f | CATCCTCTCTCTGCTGGT |
| Muc2-1-f | GAGATATGCTGGTTGCAATC |
| Muc2-1-r | TCTCTCTTGCTGCTGAGT |
| Muc2-2-f | GAGATATGCTGGTTGCAATC |
| Muc2-2-r | TCTCTCTTGCTGCTGAGT |
| Ocfn-f | ATTTATGATGAACTGAC |
| Ocfn-r | CATGCAATGATGGGGTGGA |
| Sreb1-f | ACAGAGCTGATCGCGAGGAAAG |
| Sreb1-r | GCTCTCAGAGGAGTGGG |
| Tgl1-f | TGCAATTCCCACAATGG |
| Tgl1-r | GAGAGGATGCATGAGG |
| Tgl2-f | GAGAGGATGCATGAGG |
| Tgl2-r | GAGAGGATGCATGAGG |
| Tgl3-f | GAGAGGATGCATGAGG |
| Tgl3-r | GAGAGGATGCATGAGG |
| Tgl4-f | GAGAGGATGCATGAGG |
| Tgl4-r | GAGAGGATGCATGAGG |

Table 3. Continued

| Name for bacteria | Sequence, 5’-3’ |
|------------------|----------------|
| Alistipes-f | AGATGATCTGACTGCTGGG |
| Alistipes-r | CTGTCGACTTGGTTGAGATG |
| A. finegoldii-f | TCGAGGAACCGGCTAACA |
| A. finegoldii-r | GCTCCTACAGCAGAGG |
| A. Indistinctus-f | GTGAGGTAACGCTACCAA |
| A. Indistinctus-r | CGGACCTTTCAACAGATTCAAGC |
| Bacteroidetes-f | CGGACCCGGGCGCGCGG |
| Bacteroidetes-r | GGCAGAGGAGGGCGG |
| Bilidobacterium-f | TGGGCTGCGGCTGAAAG |
| Bilidobacterium-r | CCAACATCAACCTTCG |
| Clostridium-f | AGCTCTTCATGAGAGG |
| Clostridium-r | GAGCCAGTCGGTCTTTG |
| Enterococcus-f | AACCTACCATGACAGG |
| Enterococcus-r | GACGTTGACTTAAAG |
| Firmicutes-f | GGAGYATGTGGTTTAATC |
| Firmicutes-r | GGAGYATGTGGTTTAATC |
| H. hepaticus-f | TGGTGCTGAGATGTTGG |
| H. hepaticus-r | TGGTGCTGAGATGTTGG |
| H. gannani-f | TGGGAGGCTGCTCTTGAG |
| H. gannani-r | CCAACATCCATCAGACAGA |
| Prevotella-f | CACCTGATAAACGTG |
| Prevotella-r | GGTCGCGG TTCGACAG |

f, forward; r, reverse.
**Staining Procedures**

The liver samples were formalin-fixed and sectioned for H&E staining as described.\textsuperscript{19} Frozen liver sections were stained with Oil red O.\textsuperscript{19} Formalin-fixed intestinal samples were embedded in paraffin and stained with anti-AHR antibody (ab84833, 1:200; Abcam, Cambridge, MA) and anti-myeloperoxidase antibody (ab9535, 1:200; Abcam). All sections were scanned by Nano Zoomer 2.0 RS (Hamamatsu).\textsuperscript{55}

**Western Blot Analysis**

Western blot analysis was performed as described in our previous work.\textsuperscript{19} Briefly, intestine or liver tissues were lysed and the extracted protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transferring into a nitrocellulose membrane. The membranes were blocked and probed with antibodies against CYP2E1 (AB1252, 1:1000; Millipore, Bedford, MA) or actin (A5441, 1:5000; Sigma-Aldrich) overnight and then incubated with the corresponding secondary antibodies. The protein bands were quantified using Image J (National Institutes of Health, Bethesda, MD), normalized to actin.

**Cell Culture Experiments**

MODE-K cells (C495; Shanghai Hongshun Biotechnology Co, Ltd, Shanghai, China) were treated with vehicle (PBS) or ethanol (86, 100, and 200 mmol/L) for 24 hours for extraction of RNA and qPCR analysis. For *H. hepaticus* coculture experiments, AML12 cells (CRL-2254; ATCC, Rockville, MD) were seeded in 12-well plates overnight, and subsequently exposed to *H. hepaticus* collected from the plates (OD\textsubscript{600} = 1, suspended in cell culture medium, 100 \(\mu\)L/well). After incubation for 2 hours anaerobically, the plate was subjected to aerobic conditions for a total of 24 hours.\textsuperscript{66} The treated cells were stained with Oil red O or extracted RNA for qPCR analysis.

**Luciferase-Reporter Assay**

AML12 cells were seeded in a 24-well plate and cultured for 12 hours before transfection with *Ahr* reporter plasmid (kindly provided by Professor Dalei Wu, Shandong University, China)\textsuperscript{67} and pRL-TK Vector (E2241; Promega, Madison, WI). After 24 hours, cells were treated with AHR agonists (suspended in DMSO initially and diluted with medium). With another 24 hours of incubation, cells were washed with PBS and then lysed with lysis buffer (E1910; Promega). Luciferase activity was measured using a microplate reader (Tecan Spark, Männedorf, Switzerland) after adding luciferase reagent (E1910; Promega).

**Isolation of IECs and Lamina Propria Cells**

Small intestines were harvested immediately once mice were killed. After removal of all visible fat and Peyer’s patches, all intestines were cut open longitudinally. Then, IECs and lamina propria cells were collected based on the protocol as described previously.\textsuperscript{26}

**Isolation of Kupffer Cells**

Kupffer cells were isolated as described in our previous work.\textsuperscript{58}

**Organoid Culture**

Intestinal organoids were derived from WT mice and cultured as described previously.\textsuperscript{69–71} In brief, small intestine was harvested and flushed with cold Dulbecco’s phosphate-buffered saline (DPBS). The intestine was cut longitudinally followed by removal of intestinal contents and villi. Intestine was washed and cut into 2- to 4-mm pieces, then tissues were collected after filtering through a 70-\(\mu\)m cell strainer. After digestion in 2 mmol/L EDTA of DPBS for 30 minutes, intestine pieces were transferred into DPBS and shaken vigorously. After filtration through a 70-\(\mu\)m cell strainer 3 times, the crypts were harvested by centrifuging (600 \(\times\) g, 5 min). Crypts were suspended in Matrigel (3432-010-01; R&D Biosystems, Minneapolis, MN) and cultured with IntestiCult OGM mouse basal medium (06000; Stemcell Technologies, Vancouver, Canada).

**Microinjection of *H. hepaticus* Into Organoids**

*H. hepaticus* was microinjected into the intestinal organoids as previously described.\textsuperscript{71,72} Antibiotic-free mouse basal medium was refreshed every 3 days to remove antibiotics before injection. *H. hepaticus* was collected and suspended in antibiotic-free medium (OD\textsubscript{600} = 0.4). Then, *H. hepaticus* with or without POPG (5 \(\mu\)g/mL) was microinjected into lumens of organoids with FastGreen dye (0.05%, wt/vol, F7252; Sigma-Aldrich), which is used to track the injected organoids. After 2 hours, fresh medium with nonpermeant gentamicin (5 \(\mu\)g/mL) was added to prevent the overgrowth of bacteria outside the organoids. After 4 days of incubation, the injected organoids were dissociated with 200 \(\mu\)L broth containing 0.5% saponin (SAE0073; Sigma-Aldrich) for 15 minutes with repeated pipetting on ice. The bacteria were released and cultured on plates with 8% sheep blood for 6 days to determine OD\textsubscript{600} analysis.

**Synthesis of FICZ**

The starting materials were 1-Boc-3-(2-ethoxyl-2-oxoethyl)-indole and 1-Boc-2-chloro-3-formyl-indole, and FICZ was synthesized as described in the previous work.\textsuperscript{73,74}

**Statistical Analysis**

Results in this study are expressed as means ± SEM. Statistical significance between 2 groups was determined by the Mann–Whitney test or an unpaired t test. All analyses were performed using GraphPad PRISM 7.0 (San Diego, CA). \(P < 0.05\) was considered statistically significant. For untargeted metabolomics, significance between groups was identified based on the combination of variable influence on projection values (>1.0) and \(P\) values (<0.1).

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