Published in final edited form as:

Alcohol Clin Exp Res (Hoboken). 2023 May ; 47(5): 856–867. doi:10.1111/acer.15048.

Engineered bacteria producing aryl-hydrocarbon receptor agonists protect against ethanol-induced liver disease in mice

Tetsuya Kouno[1], **Suling Zeng**[1], **Yanhan Wang**[1], **Yi Duan**[1], **Sonja Lang**[1,2], **Bei Gao**[1], **Phillipp Hartmann**[1,3,4], **Noemí Cabré**[1], **Cristina Llorente**[1], **Chloé Galbert**[5,6], **Patrick Emond**[7,8], **Harry Sokol**[5,6,9], **Michael James**[10], **Chun Cheih Chao**[10], **Jian-Rong Gao**[10], **Mylene Perreault**[10], **David L. Hava**[10], **Bernd Schnabl**[1,11,*]

[1]Department of Medicine, University of California San Diego, La Jolla, CA, 92093, USA;

[2]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department of Gastroenterology and Hepatology, Cologne, Germany;

[3]Department of Pediatrics, University of California, San Diego, La Jolla, CA, USA;

[4]Division of Gastroenterology, Hepatology & Nutrition, Rady Children's Hospital San Diego, San Diego, CA, USA;

[5]Sorbonne Université, INSERM UMRS-938, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Paris, France;

[6]Paris Center for Microbiome Medicine (PaCeMM) FHU, Paris, France;

[7]UMR 1253, iBrain, University of Tours, Inserm, 37044 Tours, France;

[8]CHRU Tours, Medical Biology Center, 37000 Tours, France;

[9]INRAe, AgroParisTech, Micalis institute, Jouy-en-Josas, France;

[10]Synlogic Inc., Cambridge, MA, USA;

[11]Department of Medicine, VA San Diego Healthcare System, San Diego, CA, 92093, USA

Abstract

Background and purpose—Gut bacteria metabolize tryptophan into indoles. Intestinal levels of the tryptophan metabolite indole-3-acetic acid are reduced in patients with alcohol-associated hepatitis. Supplementation of indole-3-acetic acid protects against ethanol-induced liver disease in mice. The aim of this study was to evaluate the effect of engineered bacteria producing indoles as Aryl-hydrocarbon receptor (Ahr) agonists.

Methods—C57BL/6 mice were subjected to chronic-plus-binge ethanol feeding and orally given PBS, control *Escherichia coli* Nissle 1917 (EcN) or engineered EcN-Ahr. The effects of EcN and EcN-Ahr were also examined in mice lacking *Ahr* in Il22 producing cells.

Results—Through deletion of endogenous genes *trpR* and *tnaA*, coupled with overexpression of a feedback-resistant tryptophan biosynthesis operon, EcN-Ahr were engineered to overproduce

*Corresponding author: Bernd Schnabl, beschnabl@health.ucsd.edu.

tryptophan. Additional engineering allowed conversion of this tryptophan to indoles including indole-3-acetic acid and indole-3-lactic acid. EcN-Ahr ameliorated ethanol-induced liver disease in C57BL/6 mice. EcN-Ahr upregulated intestinal gene expression of *Cyp1a1*, *Nrf2*, *Il22*, *Reg3b*, and *Reg3g*, and increased Il22-expressing type 3 innate lymphoid cells (ILC3). In addition, EcN-Ahr reduced translocation of bacteria to the liver. The beneficial effect of EcN-Ahr was abrogated in mice lacking *Ahr* expression in Il22 producing immune cells.

Conclusions—Our findings indicate that tryptophan metabolites locally produced by engineered gut bacteria mitigate liver disease via Ahr-mediated activation in intestinal immune cells.

Keywords

Alcohol-related liver disease; synthetic biology; microbiome; microbiota

Introduction

Alcohol-associated liver disease is a spectrum of liver diseases ranging from simple steatosis to fibrosis, cirrhosis, and alcohol-associated hepatitis. Alcohol-associated liver disease is one of the most prevalent liver diseases worldwide, and a leading cause of mortality in the United States (Lozano et al., 2012, Rehm et al., 2014, Rehm et al., 2013, Tapper and Parikh, 2018). There are no effective treatments for alcohol-associated liver disease, especially for patients with advanced liver disease (Bataller et al., 2019, Hydes et al., 2019).

The aryl hydrocarbon receptor (Ahr), a ligand-activated transcriptional factor, plays a central role in the innate and adaptive immune system by regulating the functions of immune cells and production of cytokines (Agus et al., 2018, Ambrosio et al., 2019, Gutiérrez-Vázquez and Quintana, 2018). Although xenobiotic compounds were first identified as Ahr ligands (Poland and Knutson, 1982), the diet is an abundant source of Ahr ligands (Gutiérrez-Vázquez and Quintana, 2018). Recently, indoles as microbial-derived metabolites from dietary tryptophan, have been identified to activate Ahr (Hubbard et al., 2015, Zelante et al., 2013). Patients with alcohol use disorder and liver disease show changes in the gut microbiota composition (Bajaj, 2019), causing alterations in microbial metabolites including tryptophan and indole metabolites (Gao et al., 2020). Our previous study demonstrated that patients with alcohol-associated hepatitis have reduced fecal levels of the Ahr ligand indole-3-acetic acid, and oral indole-3-acetic acid administration reduces ethanol-induced liver disease in mice (Hendrikx et al., 2019). Indole-3-acetic acid induced intestinal interleukin-22 (Il22) in type 3 innate lymphoid cells (ILC3) and subsequently increased expression of the antimicrobials Regenerating islet derived 3 (Reg3) b and Reg3g in the intestine (Hendrikx et al., 2019). Whether Ahr is involved in this protective mechanism is not known.

The current study investigated the effect of *Escherichia coli* Nissle 1917- engineered to produce Ahr agonists (EcN-Ahr), on ethanol-induced liver disease in mice. EcN-Ahr lack two key enzymes and include two synthetic operons resulting in production of Ahr ligands, including indole-3-acetic acid. In addition, mice with $Il22^{+}$ cell-specific *Ahr* deficiency were created to examine the role of the Ahr-Il22 axis in mediating the effect of EcN-Ahr.

Methods

Mice

Male and female C57BL/6 mice were purchased from Charles River. To obtain Il22 expressing cell-specific Ahr deficient mice, which are homozygous for the floxed allele and hemizygous for the Cre transgene, $Ahr^{fl/fl}$ mice (C57BL/6; The Jackson Laboratories, Strain #006203) were bred with Il22-$Cre^{+/-}$ mice (The Jackson Laboratories, Strain #027524) to create $Ahr^{\Delta IL22+cells}$. $Cre^{+/-}$ and $Cre^{-/-}$ genotypes were assessed by Hot Shot DNA PCR assay. Mice that do not carry the Cre transgenes ($Ahr^{fl/fl}$) were used as a control.

For a chronic-binge ethanol feeding model, male C57BL/6 mice from Charles River were fed with Lieber-DeCarli diet for the first 5 days and then with ethanol containing diet (36% of total calories) from day 6 until the end of the study period. At day 16, mice were gavaged with a single binge of ethanol (5 g/kg body weight) and sacrificed 9 hours later (Bertola et al., 2013). Mice fed a control diet were given an isocaloric substitution of dextrose. Daily gavage of 200μl of PBS, isogenic *Escherichia coli* Nissle 1917 (EcN, 10^7 CFU/day), or engineered *Escherichia coli* Nissle 1917-Ahr (EcN-Ahr, 10^7 CFU/day) started from day 8 to day 15. Male and female $Ahr^{fl/fl}$ mice and $Ahr^{\Delta Il22+cells}$ mice were fed with 32% ethanol containing diet (based on calories), since this strain showed increased mortality with higher concentrations of ethanol. To measure tryptophan metabolites in colon contents, female C57BL/6 mice from Charles River were gavaged once with PBS, EcN (10^{10} CFU), or EcN-Ahr (10^{10} CFU) and harvested 2 hours later. All mice were between age 7–12 weeks at the time the experiment started. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (UCSD).

Bacterial strain construction

Escherichia coli Nissle 1917 (EcN) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, *E. coli* DSM 6601). The deletion of trp operon repressor gene, *trpR* and tryptophanase gene, *tnaA* was carried out by the lambda red recombineering method (Datsenko and Wanner, 2000). For deletion of the *trpR* and *tnaA* genes, a PCR amplification was performed using pKD3 or pKD4 as the template DNA. The primers were designed to generate a dsDNA fragment that contained homology adjacent to the target gene locus in the EcN chromosome and a chloramphenicol or kanamycin resistance gene flanked by flippase recognition target (frt) sites. EcN containing pKD46 was transformed with the *trpR* or *tnaA* knockout fragment by electroporation. Colonies were selected on LB agar containing chloramphenicol (30 μg/ml) or kanamycin (50 μg/mL). Finally, pCP20 was used to remove the chloramphenicol and kanamycin cassettes, to generate the final, antibiotic-free strain. Gene knockouts were verified by sanger sequencing.

The plasmids were made using isothermal assembly method (HiFI DNA Assembly Master Mix, NEB). For constructing plasmid harboring the $trpE^{fbr}DCBA$ operon, the *trpEDCBA* genomic fragment from EcN with an addition of C119T mutation resulting in S40F substitution in TrpE under the control of P_{fnrS} promoter was synthesized by

Gen9 and cloned into a low copy number, pSC101 origin and ampicillin resistant vector. For constructing plasmid harboring the *aroG*^{fbr}*-trpDH-ipdC-iad1* operon, *aroG* from EcN with a C1549T (P150L subs), *trpDH* from *Nostoc punctiforme* (GenBank accession number AGQ45832.1), ipdC from *Enterobacter cloacae* (GenBank accession number WP_073396207.1), and *iad1* from *Ustilago maydis* (GenBank accession number XP_011388928.1) were codon optimized, synthesized by IDT and cloned into a medium copy number, p15A origin and kanamycin resistant vector. To make EcN-Ahr, these plasmids were transformed into the EcN containing the *trpR* and *tnaA* gene knockouts by electroporation. Colonies were selected on LB agar containing carbenicillin (100 μg/ml) and kanamycin (50 mg/mL).

E. coli Nissle 1917 culture

EcN and EcN-Ahr strains were cultured overnight at 37°C in Luria-Bertani (LB) medium containing 300 μg/ml of streptomycin and 100 μg/ml of ampicillin, respectively, and sub-cultured for 3 hours in LB medium without antibiotics. Bacteria were centrifuged at 8,000g for 3 minutes. Bacteria were resuspended with PBS for the animal experiments and plated on antibiotics containing culture plates (streptomycin and ampicillin for EcN and EcN-Ahr strains, respectively) for estimating colony forming units (CFUs). After plates were incubated overnight at 37°C, the number of colonies was counted. To make 10^7 CFU in 200 μL, suspended bacteria were diluted with PBS.

Measurement of indoles in supernatant of bacteria

Indoles in culture supernatants of bacteria were measured by targeted LC-MS as described (Hendrikx et al., 2019).

Measurement of indoles in mouse serum or colon contents

Mouse serum was extracted with methanol to 80% v/v, vortexed, and centrifuged at 4000 g for 5 minutes. Supernatants were collected for analysis. Mouse colon contents were extracted using 1 mL 80% methanol with 0.1% formic acid in acid-washed steel homogenizer bead tubes then homogenized using a Fastprep-24 (MP bio) at 4 m/s for 40 seconds. Homogenized samples were spun down at 5000 g for 10 minutes. Supernatants were collected for analysis.

Tryptophan, indole-3-lactic acid, and indole-3-acetic acid were measured in mouse serum, cecum, or colon content extracts by targeted LC-MS/MS using a Thermo Vanquish UHPLC-Fortis TSQ MS/MS system. Extracted samples were diluted 10 fold with 10 mM ammonium acetate containing 1 ug/mL tryptophan-d5 and 5 ug/mL indole-3-acetic acid-d5 as internal standards. Analytes were separated on a Waters Acquity HSS T3 C18, 2.1 × 100 mm column running a gradient from 10 to 98% B over 2 minutes after a 2 uL injection. Mobile phase A was 10 mM ammonium acetate while mobile phase B was 90% acetonitrile with 10 mM ammonium acetate. Compounds were detected by selected reaction monitoring (SRM) using the following ion pairs in electrospray positive mode: Tryptophan 205/188; Tryptophan-d5 210/192; or electrospray negative mode: indole-3-lactic acid 204/158; indole-3-acetic acid 174/130; indole-3-acetic acid-d5 179/135. SRM chromatograms were integrated and analyte/

internal standard peak area ratios used to calculate unknown concentrations relative to standard dilutions analyzed concurrently as above.

Histological analysis of liver tissues

Liver was embedded in OCT compound, 10 μm frozen sections were stained with freshly prepared Oil Red O solution (Sigma-Aldrich, USA) for 15 minutes, washed with 60% isopropanol, and mounted in glycerol.

Biochemical analysis

Serum ALT level was determined using Infinity ALT kit (Thermo Scientific). Hepatic triglycerides were measured using Triglyceride Liquid Reagents Kit (Pointe Scientific). Serum concentration of ethanol was measured using Ethanol Assay kit (BioVision). Serum LPS was measured by ELISA (Cusabio).

Real-time quantitative PCR

RNA was extracted from liver and ileum using TRIzol (Invitrogen), and cDNA was generated using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Gene expression was determined with SYBR Green (Bio-Rad Laboratories) using ABI StepOnePlus real-time PCR system. Relative gene expression was determined by CT value and normalized to 18S as housekeeping gene. Primer sequences are as follows:

Mouse *18S*

Forward: AGTCCCTGCCCTTTGTACACA, Reverse: CGATCCGAGGGCCTCACTA

Mouse *Cxcl1*

Forward: TGCACCCAAACCGAAGTC, Reverse: GTCAGAAGCCAGCGTTCACC

Mouse *Ccl2*

Forward: ATTGGGATCATCTTGCTGGT, Reverse: CCTGCTGTTCACAGTTGCC

Mouse *Adh1*

Forward: GGGTTCTCAACTGGCTATGG, Reverse: ACAGACAGACCGACACCTCC

Mouse *Cyp2e1*

Forward: GGGACATTCCTGTGTTCCAG, Reverse: CTTAGGGAAAACCTCCGCAC

Mouse *Cyp1a1*

Forward: GGGTTTGACACAGTCACAACT, Reverse: GGGACGAAGGATGAATGCCG

Mouse *Nrf2*

Forward: TCTATGTCTTGCCTCCAAAGG, Reverse: CTCAGCATGATGGACTTGGA

Mouse *Il22*

Forward: GCTCAGCTCCTGTCACATCA, Reverse: TCGCCTTGATCTCTCCACTC

Mouse *Reg3b*

Forward: GGCTTCATTCTTGTCCTCCA, Reverse: TCCACCTCCATTGGGTTCT

Mouse *Reg3g*

Forward: AAGCTTCCTTCCTGTCCTCC, Reverse: TCCACCTCTGTTGGGTTCAT

Mouse *Ahr*

Forward: GGCTTTCAGCAGTCTGATGTC, Reverse: CATGAAAGAAGCGTTCTCTGG

Mouse *Cldn1*

Forward: ATGCCAATTACCATCAAGGC, Reverse: GAGGGACTGTGGATGTCCTG

16S

Forward: GTGSTGCAYGGYTGTCGTCA, Reverse: ACGTCRTCCMCACCTTCCTC

Ahr reporter assay

HepG2-Lucia™ Ahr Cells (InvivoGen) were treated with 0.5 μg/mL of FICZ (Med Chem Express, HY-12451) as Ahr ligand/positive control, or EMEM containing 10% of supernatant of bacteria for 24 hours. Luciferase activity was analyzed using QUANTI-Luc™ solution.

To prepare the supernatant of bacteria, EcN and EcN-Ahr strains were cultured overnight at 37°C in LB medium containing 300 μg/ml of streptomycin and 100 μg/ml of ampicillin, respectively, and sub-cultured for 3 hours in LB medium without antibiotics. 4×10^7 CFU/mL of EcN or EcN-Ahr were centrifuged at 8,000g for 3 minutes, and supernatants were used for reporter assay.

Lamina propria cell isolation

Lamina propria cells were isolated from the small intestine of mice as described previously (15). Cells were used for qPCR or flow cytometry.

Flow cytometry

Lamina propria cells isolated from small intestine were incubated with 40 ng/mL of IL23 and BD GogiStop™ (BD Bioscience) at 37°C for 4 hours. Cells were blocked with CD16/32 (1:4000, BioLegend) and stained with CD3-FITC (1:400, BD Biosciences) and FVS575V (1:1000, BD Bioscicences) for 30 minutes. Intracellular staining was performed using RORgt-PE (1:200, eBioscience), Il22-APC (1:100, eBioscience), and Ahr-PE-Cy7 (1:200, eBioscience). Flow cytometry analysis was performed using NovoCyte (ACEA

Biosciences Inc.). Percentage of Ahr and Il22-expressing cells gated on $CD3^{-}RORgt^{+}$ cells were analyzed using FlowJo software.

Statistical analysis

All data are expressed as mean±S.E.M. For comparison of 2 groups, unpaired student t-test was performed. For multiple groups comparison within one experimental setting, one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or Turkey's post-hoc test was performed. A p value < 0.05 was considered to be statistically significant. Statistical analyses were conducted with GraphPad Prism (V.9.3.1).

Results

Engineered bacteria produce Ahr ligands

EcN was engineered to increase the production and secretion of Ahr ligands. First, modifications were made for EcN to overproduce tryptophan. This involved deletion of *trpR*, a transcriptional repressor controlling the endogenous tryptophan biosynthetic genes, and a deletion of *tnaA*, to ensure that EcN was unable to convert tryptophan to indole. Additionally, a feedback resistant tryptophan biosynthetic operon, $trpE^{fbr}DCBA$ was expressed from a plasmid to provide additional biosynthetic enzyme. The feedback resistant *trpE* variant ensured that tryptophan production was not subject to allosteric inhibition. A second set of modifications was made to facilitate the conversion of tryptophan to indole metabolites, and included the expression of the operon *aroGfbr-trpDH-ipdC-iad1* (Figure 1a). The concentration of tryptophan metabolites in culture medium of the isogenic control strain EcN and engineered bacteria, *Escherichia coli* Nissle 1917-Ahr (EcN-Ahr) was investigated by targeted LC-MS. EcN-Ahr had higher levels of indole-3-acetic acid (Figure 1b), indole-3-lactic acid (Figure 1c), and tryptophol (Figure 1d) than EcN. There was no significant difference in other metabolites between the groups, including tryptophan (Figure 1e), xanthurenic acid, indole-3-acetamide, kynurenic acid, indole-3-aldehyde, tryptamine, tryptophan, kynurenine, picolinic acid, indole-3-sulfate, quinolinic acid and 5-OH-tryptophan (not shown). Mice gavaged with EcN-Ahr once showed similar levels of indole-3-acetic acid (Figure 1f) and increased indole-3-lactic acid (Figure 1g) and tryptophan (Figure 1h) in colon contents. Ahr reporter assay indicated that both supernatant of EcN and EcN-Ahr activated Ahr, but EcN-Ahr showed significantly higher Ahr activity than EcN (Figure 1i).

EcN-Ahr protect mice against ethanol-induced liver disease

The effect of EcN-Ahr was examined in a mouse model of chronic-plus-binge ethanol-induced liver disease. There was no significant difference in body weight or food intake between the groups (Figure 2a, b). Ethanol-induced liver injury was significantly reduced in mice supplemented with EcN-Ahr compared with PBS and EcN gavaged mice as assessed by serum ALT level (Figure 2c). Hepatic triglycerides were also reduced in the EcN-Ahr group (Figure 2d), which was confirmed by Oil red O staining (Figure 2e). Gene expression of inflammatory chemokines such as C-X-C motif chemokine ligand 1 (*Cxcl1*) and C-C motif chemokine ligand 2 (*Ccl2*) was reduced in mice treated with EcN-Ahr (Figure 2f, g). Serum level of ethanol and hepatic expression of two enzymes that metabolize ethanol,

alcohol dehydrogenase 1 (*Adh1*) and cytochrome P450 family 2 subfamily e polypeptide 1 (*Cyp2e1*), were unchanged (Figure 3), indicating that ethanol metabolism is not affected by the treatment with engineered bacteria. CFUs of EcN and EcN-Ahr were similar in the cecum (Supplemental Fig. 1). These results indicate that EcN-Ahr reduce ethanol-induced liver disease.

EcN-Ahr activate Ahr in the intestine and prevent bacterial translocation to the liver

Since EcN-Ahr activates Ahr *in vitro* (Figure 1i), the gene expression of Ahr target genes was examined following chronic–binge ethanol diet. Gene expression of cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*), nuclear factor, erythroid derived 2, like 2 (*Nfe2l2,* also known as *Nrf2*), and *Il22* was significantly upregulated in the small intestine of mice gavaged with EcN-Ahr (Figure 4a–c), whereas no significant difference was observed in the hepatic expression of *Cyp1a1* or *Nrf2* (Figure 4f–g). Serum indole-3-acetic acid (Supplemental Figure 2a), indole (Supplemental Figure 2b) and tryptophan (Supplemental Figure 2c) were similar in all groups suggesting that the effect of EcN-Ahr is restricted to the intestine. We have previously demonstrated that Il22 secreted by type 3 innate lymphoid cells (ILC3) induces the expression of antimicrobials Reg3b and Reg3g in the intestine (Hendrikx et al., 2019, Wang et al., 2016). The proportion of Ahr^{+} $Il22^{+}$ ILC3 (gated on $CD3^{-}RORgt^{+}$) was increased in the intestinal lamina propria of ethanol-fed mice treated with EcN-Ahr. (Figure 4h). EcN-Ahr supplementation was associated with increased gene expression of antimicrobials *Reg3b* and *Reg3g* in the small intestine (Figure 4d, e). Translocation of gut bacteria to the liver was reduced following the gavage of EcN-Ahr as determined by qPCR for 16S rDNA (Figure 4i). Claudin-1 (Cldn1) is a tight junction molecule and is mainly expressed in epithelial cells (Garcia-Hernandez et al., 2017), and Ahr activation increases the expression of cldn1 (Yu et al., 2018). Intestinal *Cldn1* mRNA was similar in EcN-Ahr treated as compared with PBS or EcN treated mice after ethanol feeding (Fig. 4j). We also measured serum LPS levels as marker for paracellular intestinal permeability. Serum LPS levels were similar in EcN-Ahr treated as compared with PBS or EcN treated and ethanol-fed mice (Fig. 4k). These results indicate that intestinal epithelial cells are not the primary target for Ahr ligands secreted from engineered bacteria; EcN-Ahr activates intestinal Ahr-Il22-Reg3 pathway to prevent bacterial translocation from the intestine to the liver.

The beneficial effect of EcN-Ahr on ethanol-induced steatohepatitis is abrogated in mice lacking *Ahr* in Il22 producing cells

To further demonstrate that secreted Ahr ligands from our engineered bacteria activate Ahr and induce Il22, we generated mice lacking *Ahr* in Il22 producing cells ($Ahr^{\Delta Il22+cells}$). Immune cells, but not epithelial cells produce Il22 (Parks et al., 2015). There was no significant difference in the expression of *Ahr* in the intestinal epithelial cells (IECs) between $Ahr^{\Delta Il22+cells}$ and littermate $Ahr^{fl/fl}$ mice, but mice lacking *Ahr* in Il22 producing cells had lower gene expression of *Ahr* in immune cells of the lamina propria compared with control $Ahr^{fl/fl}$ mice (Figure 5a). Lamina propria cells were isolated from the small intestine, and the frequency of Il22-expressing cells was examined. The frequency of $Il22^{+}$ ILC3 (gated on $CD3^{-}RORgt^{+}$) was reduced in $Ahr^{\Delta Il22+cells}$ mice (Figure 5b). To investigate whether the effect of EcN-Ahr on ethanol-induced liver disease is mediated via the Ahr-Il22

axis, *Ahr*$^{\Delta Il22+cells}$ mice were subjected to the chronic-binge ethanol-induced liver disease model, and gavaged with PBS, EcN, or EcN-Ahr. As a result, there were no significant differences in liver injury or steatosis between the groups (Figure 5c–e). EcN-Ahr failed to induce the gene expression of *Cyp1a1*, *Nrf2*, *Il22*, *Reg3b*, or *Reg3g* in the small intestine (Figure 5f–j). Bacterial translocation was not significantly reduced by EcN-Ahr (Figure 5k). EcN-Ahr significantly reduced the serum ALT and hepatic triglycerides in control *Ahr*$^{fl/fl}$ mice (Supplemental Fig. 3). These results indicate that the Ahr/Il22 pathway in lamina propria immune cells mediates the beneficial effect of EcN-Ahr.

Discussion

Ahr is ubiquitously expressed throughout the body, and activation of the Ahr pathway plays an important role in improving liver disease (Hendrikx et al., 2019) (Wrzosek et al., 2021). The current study is the first to show that engineered bacteria, EcN-Ahr, producing indoles as Ahr agonists protect against ethanol-induced liver disease by activating the Ahr pathway specifically in the gut.

EcN-Ahr activated the Ahr pathway in the intestine and increased the expression of *Il22*. This effect was abrogated in mice lacking Ahr in Il22^{+} cells. Although Ahr interacts with RORgt to promote Il22 expression in ILC3 (Qiu et al., 2012), we cannot rule out that the beneficial effect of EcN-Ahr is mediated via Il22^{+} immune cells other than ILC3. EcN-Ahr increased the expression of antimicrobial expression *Reg3b* and *Reg3g*, and reduced bacterial translocation to the liver. This is consistent with the findings that Il22 production maintains the epithelial barrier integrity (Li et al., 2021) and prevents bacterial infection by upregulating antimicrobial peptides (Moyat et al., 2017, Zheng et al., 2008). Bacterial translocation to the liver contributes to liver inflammation (Ponziani et al., 2018, Rodríguez-Laiz et al., 2019), which is a likely a contributing mechanism by which EcN-Ahr reduces ethanol-induced liver disease. Changes in the gut microbiota composition associated with EcN-Ahr supplementation might also contribute to the activation of IL-22 signaling and protection from ethanol-induced liver disease.

We supplemented engineered bacteria to obtain an intestine specific and gut restricted effect without activating Ahr in the liver. Hepatic expression of Ahr target genes, *Cyp1a1* or *Nrf2,* was not increased in EcN-Ahr treated mice. Similarly, hepatic expression of *Adh1* and *Cyp2e1,* and ethanol metabolism in the liver were not affected by EcN-Ahr supplementation. This is important as AhR activation in the liver reduces ethanol-induced liver disease via lowering ethanol-induced ROS generation and peroxidation, which contributes to a reduction of serum ethanol concentration (Dong et al., 2021).

EcN are safe and commercially available probiotics, which show some benefit for ulcerative colitis (Kruis et al., 2004, Kruis et al., 1997, Matthes et al., 2010, Schultz, 2008), infectious GI diseases (Boudeau et al., 2003, Henker et al., 2007), constipation (Möllenbrink and Bruckschen, 1994), and irritable bowel syndrome (Kruis et al., 2012). In our study, EcN showed a tendency to reduce liver disease as indicated by serum ALT. Since culture supernatant of EcN had higher level of AhR activity than control condition (Figure 1i), EcN have the potential to activate AhR. This observation is supported by the findings that

EcN elevates the level of serotonin (5-HT) in the gut (Nzakizwanayo et al., 2015), and 5-HT can potentiate the AhR activity to induce Cyp1a1 (Manzella et al., 2018), (Manzella et al., 2020). Importantly, EcN-Ahr designed to efficiently produce indoles activated the Ahr more than EcN did (Figure 1i). Different level of Ahr activation may contribute to the effect of probiotics on alcohol-associated liver disease. Although we have previously shown that supplementation with indole-3-acetic acid reduces ethanol-induced steatohepatitis in mice (Hendrikx et al., 2019), the use of orally administered probiotics has the potential advantage of delivering Ahr agonists in a local, more continuous and sustained fashion as opposed to a once daily oral administration of the metabolite itself. Considering that sustained activation of Ahr in the whole body might cause unintended side effects (Wang et al., 2020), an intestine specific Ahr activation in a controllable way can be a promising treatment for liver disease and possibly other diseases.

In conclusion, engineered bacteria EcN-Ahr protect against liver disease induced by ethanol. EcN-Ahr specifically activate the Ahr pathway in the intestine to regulate the expression of antimicrobial proteins, thereby preventing bacterial translocation to the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Statement of financial support:

This study was supported by the American Association for the Study of Liver Diseases (AASLD) Pinnacle Research Award in Liver Disease (8998GA), by the Southern California Research Center for Alcoholic Liver and Pancreatic Diseases (ALPD) and Cirrhosis (P50 AA011999) funded by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and its Animal Core facilities, and the Isenberg Endowed Fellowship jointly awarded by the Pilot/Feasibility Program of the San Diego Digestive Diseases Research Center (SDDRC) and the Hellman Family Foundation (P30 DK120515) (to C.L.). This study was supported in part by NIH grants R01 AA24726, R37 AA020703, U01 AA026939, U01 AA026939-04S1, by Award Number BX004594 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development, and a Harrington Discovery Institute Foundation Grant (to B.S.) and services provided by NIH centers P30 DK120515 and P50 AA011999. This study was supported in part by a laboratory service agreement from Synlogic Operating Company to the University of California San Diego.

Conflict of interest:

H.S. reports lecture fee, board membership, or consultancy from Carenity, AbbVie, Astellas, Danone, Ferring, Mayoly Spindler, MSD, Novartis, Roche, Tillots, Enterome, BiomX, Biose, Novartis, Takeda, Biocodex and is co-founder of Exeliom Biosciences. B.S. has been consulting for Ambys Medicines, Ferring Research Institute, Gelesis, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S.'s institution UC San Diego has received research support from Artizan Biosciences, Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals and Prodigy Biotech. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor related to this work. M.J., C.C.C, JR.G., M.P. and D.L.H. are employees of Synlogic Inc..

References

1. Agus A, Planchais J, Sokol H (2018) Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe 23:716–724. [PubMed: 29902437]
2. Ambrosio LF, Insfran C, Volpini X, Acosta Rodriguez E, Serra HM, Quintana FJ, Cervi L, Motrán CC (2019) Role of Aryl Hydrocarbon Receptor (AhR) in the Regulation of Immunity and Immunopathology During Trypanosoma cruzi Infection. Front Immunol 10:631. [PubMed: 30984194]

3. Bajaj JS (2019) Alcohol, liver disease and the gut microbiota. Nat Rev Gastroenterol Hepatol 16:235–246. [PubMed: 30643227]
4. Bataller R, Arteel GE, Moreno C, Shah V (2019) Alcohol-related liver disease: Time for action. J Hepatol 70:221–222. [PubMed: 30658723]
5. Bertola A, Mathews S, Ki SH, Wang H, Gao B (2013) Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat Protoc 8:627–637. [PubMed: 23449255]
6. Boudeau J, Glasser AL, Julien S, Colombel JF, Darfeuille-Michaud A (2003) Inhibitory effect of probiotic Escherichia coli strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. Aliment Pharmacol Ther 18:45–56.
7. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. [PubMed: 10829079]
8. Dong H, Hao L, Zhang W, Zhong W, Guo W, Yue R, Sun X, Zhou Z (2021) Activation of AhR-NQO1 Signaling Pathway Protects Against Alcohol-Induced Liver Injury by Improving Redox Balance. Cell Mol Gastroenterol Hepatol 12:793–811. [PubMed: 34082111]
9. Gao B, Duan Y, Lang S, Barupal D, Wu TC, Valdiviez L, Roberts B, Choy YY, Shen T, Byram G, Zhang Y, Fan S, Wancewicz B, Shao Y, Vervier K, Wang Y, Zhou R, Jiang L, Nath S, Loomba R, Abraldes JG, Bataller R, Tu XM, Stärkel P, Lawley TD, Fiehn O, Schnabl B (2020) Functional Microbiomics Reveals Alterations of the Gut Microbiome and Host Co-Metabolism in Patients With Alcoholic Hepatitis. Hepatol Commun 4:1168–1182. [PubMed: 32766476]
10. Garcia-Hernandez V, Quiros M, Nusrat A (2017) Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. Ann N Y Acad Sci 1397:66–79. [PubMed: 28493289]
11. Gutiérrez-Vázquez C, Quintana FJ (2018) Regulation of the Immune Response by the Aryl Hydrocarbon Receptor. Immunity 48:19–33. [PubMed: 29343438]
12. Hendrikx T, Duan Y, Wang Y, Oh JH, Alexander LM, Huang W, Stärkel P, Ho SB, Gao B, Fiehn O, Emond P, Sokol H, van Pijkeren JP, Schnabl B (2019) Bacteria engineered to produce IL-22 in intestine induce expression of REG3G to reduce ethanol-induced liver disease in mice. Gut 68:1504–1515. [PubMed: 30448775]
13. Henker J, Laass M, Blokhin BM, Bolbot YK, Maydannik VG, Elze M, Wolff C, Schulze J (2007) The probiotic Escherichia coli strain Nissle 1917 (EcN) stops acute diarrhoea in infants and toddlers. Eur J Pediatr 166:311–318. [PubMed: 17287932]
14. Hubbard TD, Murray IA, Bisson WH, Lahoti TS, Gowda K, Amin SG, Patterson AD, Perdew GH (2015) Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. Sci Rep 5:12689. [PubMed: 26235394]
15. Hydes T, Gilmore W, Sheron N, Gilmore I (2019) Treating alcohol-related liver disease from a public health perspective. J Hepatol 70:223–236. [PubMed: 30658724]
16. Kruis W, Chrubasik S, Boehm S, Stange C, Schulze J (2012) A double-blind placebo-controlled trial to study therapeutic effects of probiotic Escherichia coli Nissle 1917 in subgroups of patients with irritable bowel syndrome. Int J Colorectal Dis 27:467–474. [PubMed: 22130826]
17. Kruis W, Fric P, Pokrotnieks J, Lukás M, Fixa B, Kascák M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J (2004) Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut 53:1617–1623. [PubMed: 15479682]
18. Kruis W, Schütz E, Fric P, Fixa B, Judmaier G, Stolte M (1997) Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 11:853–858. [PubMed: 9354192]
19. Li YY, Wang XJ, Su YL, Wang Q, Huang SW, Pan ZF, Chen YP, Liang JJ, Zhang ML, Xie XQ, Wu ZY, Chen JY, Zhou L, Luo X (2021) Baicalein ameliorates ulcerative colitis by improving intestinal epithelial barrier via AhR/IL-22 pathway in ILC3s. Acta Pharmacol Sin.
20. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis

J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380:2095–2128. [PubMed: 23245604]

21. Manzella C, Singhal M, Alrefai WA, Saksena S, Dudeja PK, Gill RK (2018) Serotonin is an endogenous regulator of intestinal CYP1A1 via AhR. Sci Rep 8:6103. [PubMed: 29666456]
22. Manzella CR, Ackerman M, Singhal M, Ticho AL, Ceh J, Alrefai WA, Saksena S, Dudeja PK, Gill RK (2020) Serotonin Modulates AhR Activation by Interfering with CYP1A1-Mediated Clearance of AhR Ligands. Cell Physiol Biochem 54:126–141. [PubMed: 32017483]
23. Matthes H, Krummenerl T, Giensch M, Wolff C, Schulze J (2010) Clinical trial: probiotic treatment of acute distal ulcerative colitis with rectally administered Escherichia coli Nissle 1917 (EcN). BMC Complement Altern Med 10:13. [PubMed: 20398311]
24. Möllenbrink M, Bruckschen E (1994) [Treatment of chronic constipation with physiologic Escherichia coli bacteria. Results of a clinical study of the effectiveness and tolerance of microbiological therapy with the E. coli Nissle 1917 strain (Mutaflor)]. Med Klin (Munich) 89:587–593. [PubMed: 7815986]
25. Moyat M, Bouzourene H, Ouyang W, Iovanna J, Renauld JC, Velin D (2017) IL-22-induced antimicrobial peptides are key determinants of mucosal vaccine-induced protection against H. pylori in mice. Mucosal Immunol 10:271–281. [PubMed: 27143303]
26. Nzakizwanayo J, Dedi C, Standen G, Macfarlane WM, Patel BA, Jones BV (2015) Escherichia coli Nissle 1917 enhances bioavailability of serotonin in gut tissues through modulation of synthesis and clearance. Sci Rep 5:17324. [PubMed: 26616662]
27. Parks OB, Pociask DA, Hodzic Z, Kolls JK, Good M (2015) Interleukin-22 Signaling in the Regulation of Intestinal Health and Disease. Front Cell Dev Biol 3:85.
28. Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 22:517–554. [PubMed: 6282188]
29. Ponziani FR, Zocco MA, Cerrito L, Gasbarrini A, Pompili M (2018) Bacterial translocation in patients with liver cirrhosis: physiology, clinical consequences, and practical implications. Expert Rev Gastroenterol Hepatol 12:641–656. [PubMed: 29806487]
30. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, Zhou L (2012) The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity 36:92–104. [PubMed: 22177117]
31. Rehm J, Dawson D, Frick U, Gmel G, Roerecke M, Shield KD, Grant B (2014) Burden of disease associated with alcohol use disorders in the United States. Alcohol Clin Exp Res 38:1068–1077. [PubMed: 24428196]

32. Rehm J, Samokhvalov AV, Shield KD (2013) Global burden of alcoholic liver diseases. J Hepatol 59:160–168. [PubMed: 23511777]
33. Rodríguez-Laiz GP, Zapater P, Melgar P, Alcázar C, Franco M, Giménez P, Pascual S, Bellot P, Palazón JM, Rodríguez M, Carnicer F, Más-Serrano P, González-Navajas JM, Gómez L, Such J, Lluís F, Francés R (2019) Bacterial DNA translocation contributes to systemic inflammation and to minor changes in the clinical outcome of liver transplantation. Sci Rep 9:835. [PubMed: 30696924]
34. Schultz M (2008) Clinical use of E. coli Nissle 1917 in inflammatory bowel disease. Inflamm Bowel Dis 14:1012–1018. [PubMed: 18240278]
35. Tapper EB, Parikh ND (2018) Mortality due to cirrhosis and liver cancer in the United States, 1999–2016: observational study. Bmj 362:k2817. [PubMed: 30021785]
36. Wang L, Fouts DE, Stärkel P, Hartmann P, Chen P, Llorente C, DePew J, Moncera K, Ho SB, Brenner DA, Hooper LV, Schnabl B (2016) Intestinal REG3 Lectins Protect against Alcoholic Steatohepatitis by Reducing Mucosa-Associated Microbiota and Preventing Bacterial Translocation. Cell Host Microbe 19:227–239. [PubMed: 26867181]
37. Wang Z, Snyder M, Kenison JE, Yang K, Lara B, Lydell E, Bennani K, Novikov O, Federico A, Monti S, Sherr DH (2020) How the AHR Became Important in Cancer: The Role of Chronically Active AHR in Cancer Aggression. Int J Mol Sci 22.
38. Wrzosek L, Ciocan D, Hugot C, Spatz M, Dupeux M, Houron C, Lievin-Le Moal V, Puchois V, Ferrere G, Trainel N, Mercier-Nomé F, Durand S, Kroemer G, Voican CS, Emond P, Straube M, Sokol H, Perlemuter G, Cassard AM (2021) Microbiota tryptophan metabolism induces aryl hydrocarbon receptor activation and improves alcohol-induced liver injury. Gut 70:1299–1308. [PubMed: 33004548]
39. Yu M, Wang Q, Ma Y, Li L, Yu K, Zhang Z, Chen G, Li X, Xiao W, Xu P, Yang H (2018) Aryl Hydrocarbon Receptor Activation Modulates Intestinal Epithelial Barrier Function by Maintaining Tight Junction Integrity. Int J Biol Sci 14:69–77. [PubMed: 29483826]
40. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L (2013) Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 39:372–385. [PubMed: 23973224]
41. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W (2008) Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med 14:282–289. [PubMed: 18264109]

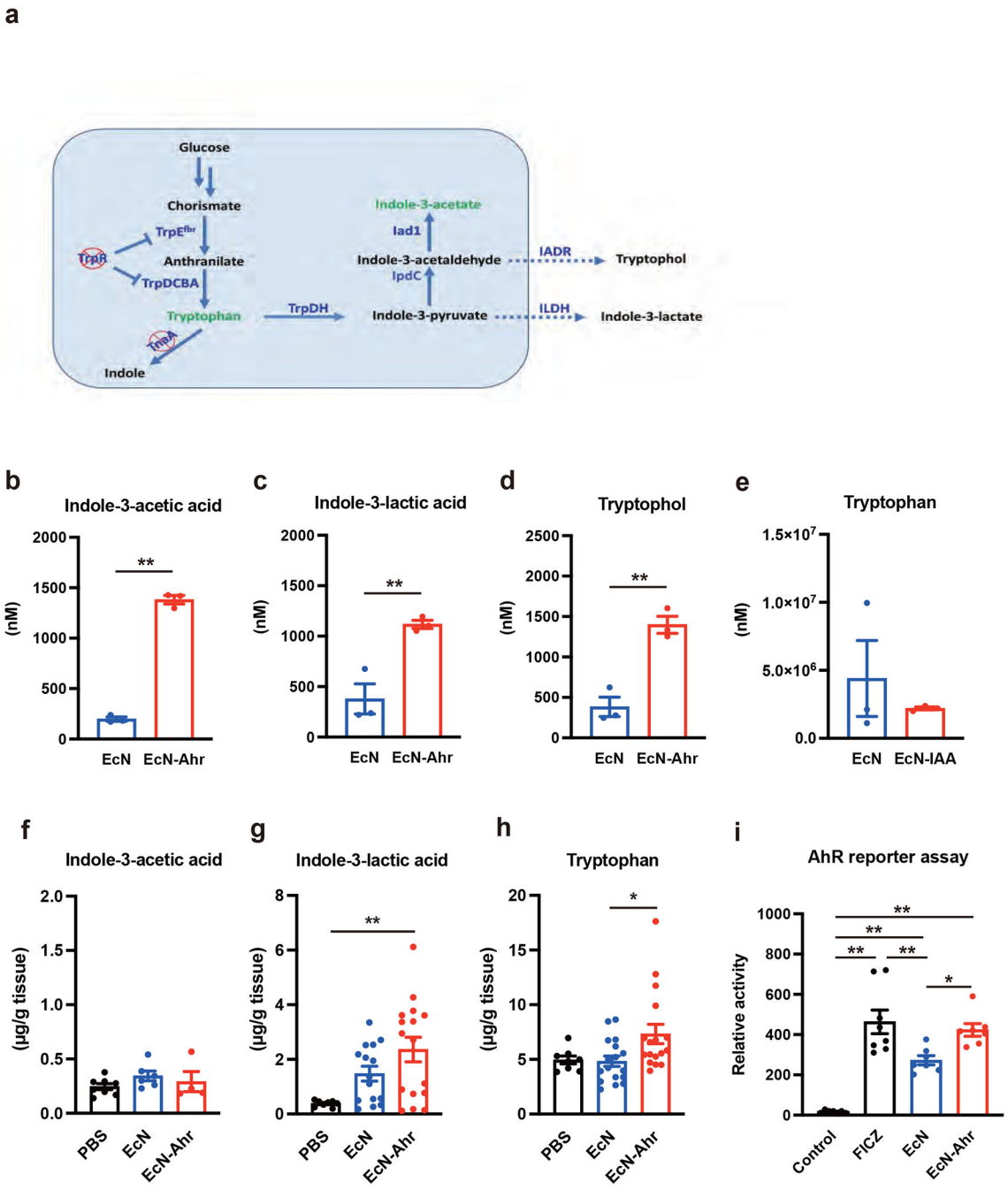

Figure 1. Characterization of EcN-Ahr.

(a) Schematic of the engineered EcN-Ahr in tryptophan metabolism. EcN-Ahr lack tryptophan transcriptional receptor (TrpR), and tryptophanase A (TnaA), and the *aroGfbr-trpDH-ipdC-iad1* operon was added. Concentrations of indole-3-acetic acid (b), indole-3-lactic acid (c), tryptophol (d), and tryptophan (e) in culture medium. (f-h) To measure tryptophan metabolites in colon contents, mice were gavaged once with PBS (n = 8), EcN (10^{10} CFU) (n = 17), or EcN-Ahr (10^{10} CFU) (n = 17) and harvested 2 hours later. (f) indole-3-acetic acid, (g) indole-3-lactic acid, and (h) tryptophan. (i) A luciferase reporter

assay in HepG2-Lucia™ Ahr cells treated with 0.5 μg/mL of the Ahr agonist FICZ, or 10% supernatant of bacteria for 24 hours. Data are presented as mean±S.E.M. *p<0.05 and **p<0.01 denotes the significant difference between the groups.

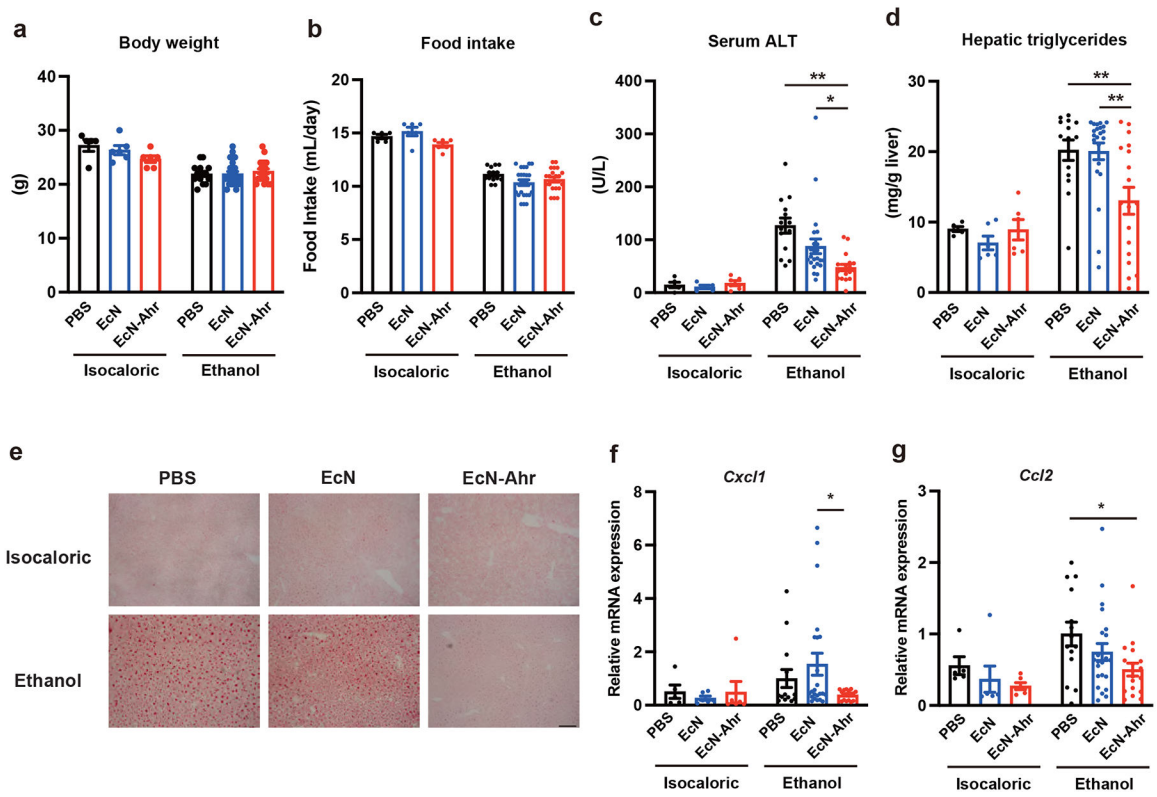

Figure 2. Engineered EcN-Ahr reduce ethanol-induced steatohepatitis.

Male C57BL/6 mice were gavaged with PBS (n = 5), EcN (n = 6), or EcN-Ahr (n = 6) during the control (isocaloric) diet or with PBS (n = 14), EcN (n = 23), or EcN-Ahr (n = 18) during chronic–binge ethanol diet. Body weight (a) and food intake (b) of mice subjected to chronic-plus-binge ethanol feeding. (c) Serum level of ALT. (d) Hepatic triglycerides content. (e) Representative Oil red O-stained liver sections. (f, g) Gene expression of *Cxcl1* and *Ccl2* in the liver of mice fed with ethanol diet. Data are presented as mean±S.E.M. of 3 technical replicates. *p<0.05 and **p<0.01 denotes the significant difference between the groups. Bar size = 100 μm.

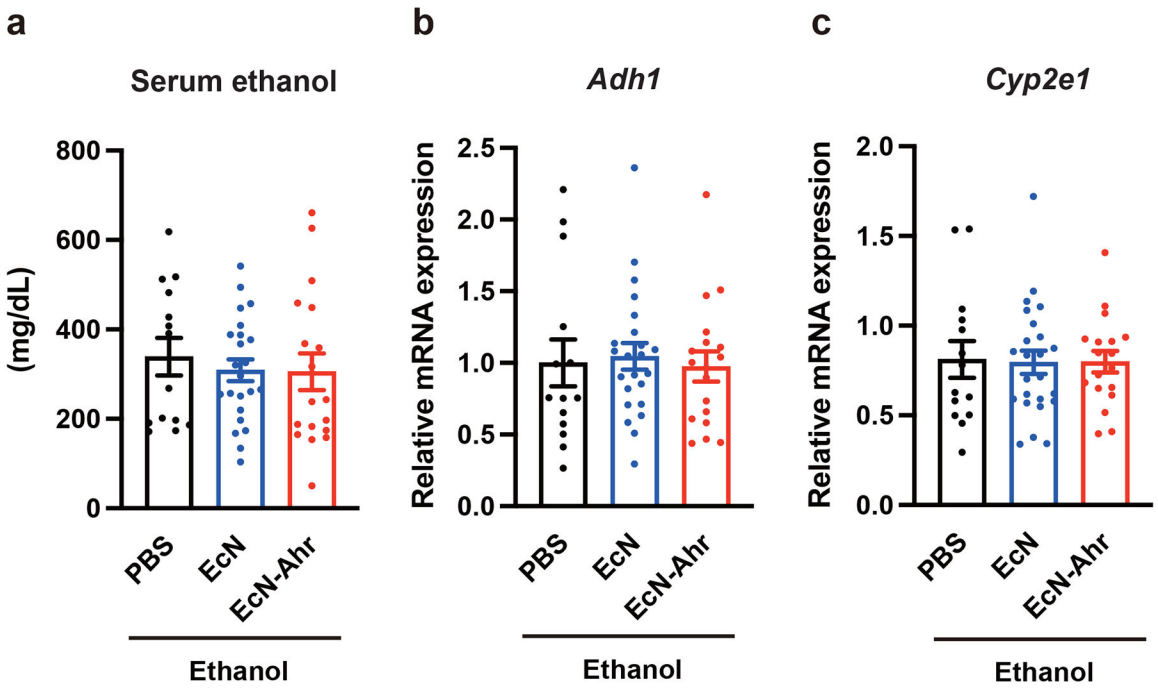

Figure 3. Effect of EcN-Ahr on ethanol metabolism in a chronic-plus-binge ethanol feeding model.

Male C57BL/6 mice were gavaged with PBS (n = 14), EcN (n = 23), or EcN-Ahr (n = 18) during chronic–binge ethanol diet. Serum concentration of ethanol (a) and gene expression of *Adh1* and *Cyp2e1* in the liver (b, c) of mice subjected to chronic-plus-binge ethanol feeding. Data are presented as mean±S.E.M. of 3 technical replicates.

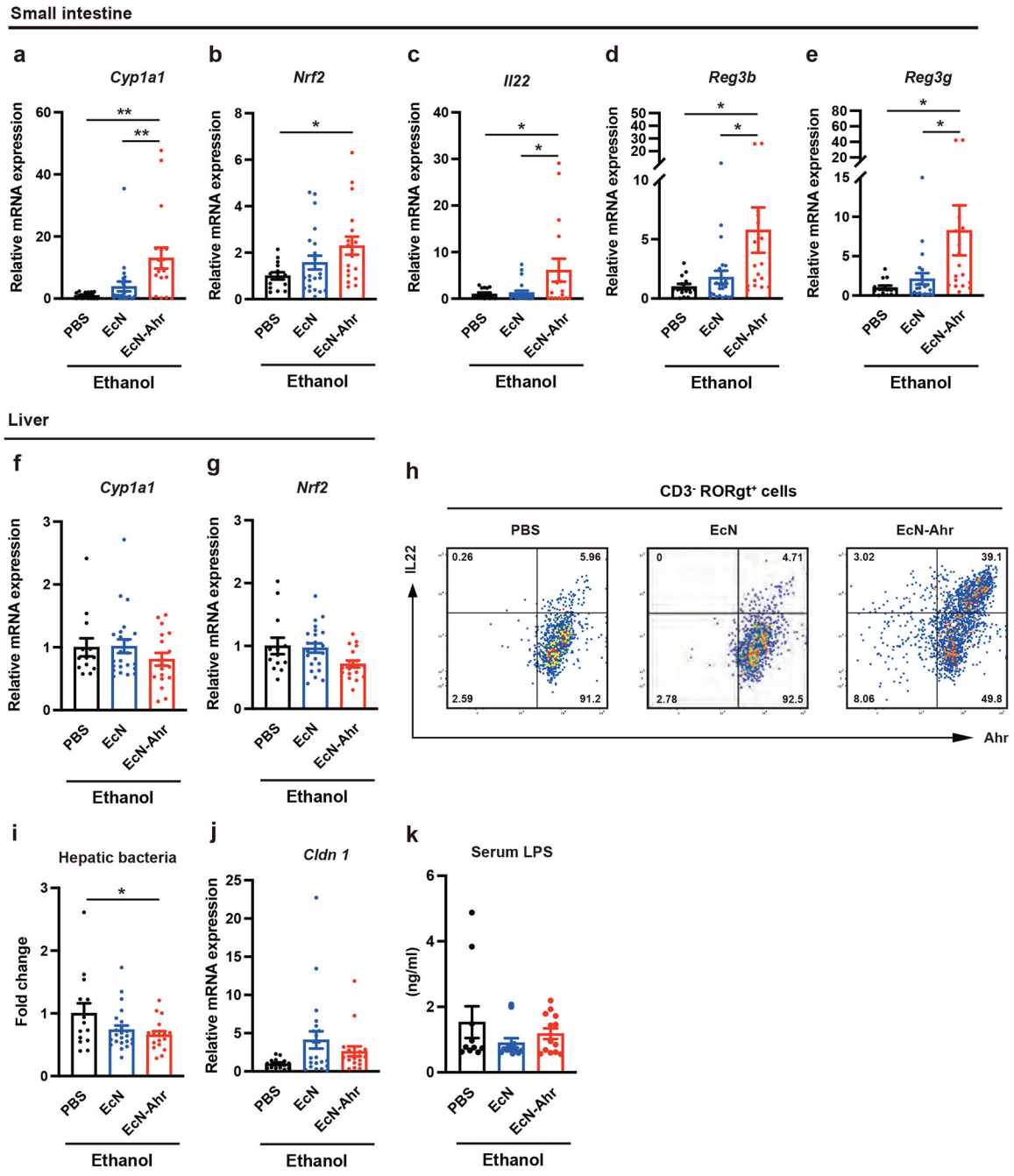

Figure 4. Effect of EcN-Ahr on intestinal Ahr-Il22 pathway and bacterial translocation in a chronic-plus-binge ethanol feeding model.

Male C57BL/6 mice were gavaged with PBS (n = 14), EcN (n =23), or EcN-Ahr (n = 18) during chronic–binge ethanol diet. Gene expression of *Cyp1a1*, *Nrf2*, *Il22*, *Reg3b,* and *Reg3g* in the ileum (a-e). Gene expression of *Cyp1a1* and *Nrf2* in the liver (f, g). (h) Frequency of Il22- and Ahr-expressing ILC3 (gated on $CD3^{-}RORgt^{+}$) after stimulation with IL23 for 4 hours in lamina propria cells. (i) Total bacteria in the liver of mice subjected to chronic-plus-binge ethanol feeding as determined by qPCR. (j) Gene expression of *Cldn1*

in the ileum. (k) Serum level of LPS. Data are presented as mean±S.E.M. of 3 technical replicates. *p<0.05 and **p<0.01 denotes the significant difference between the groups.

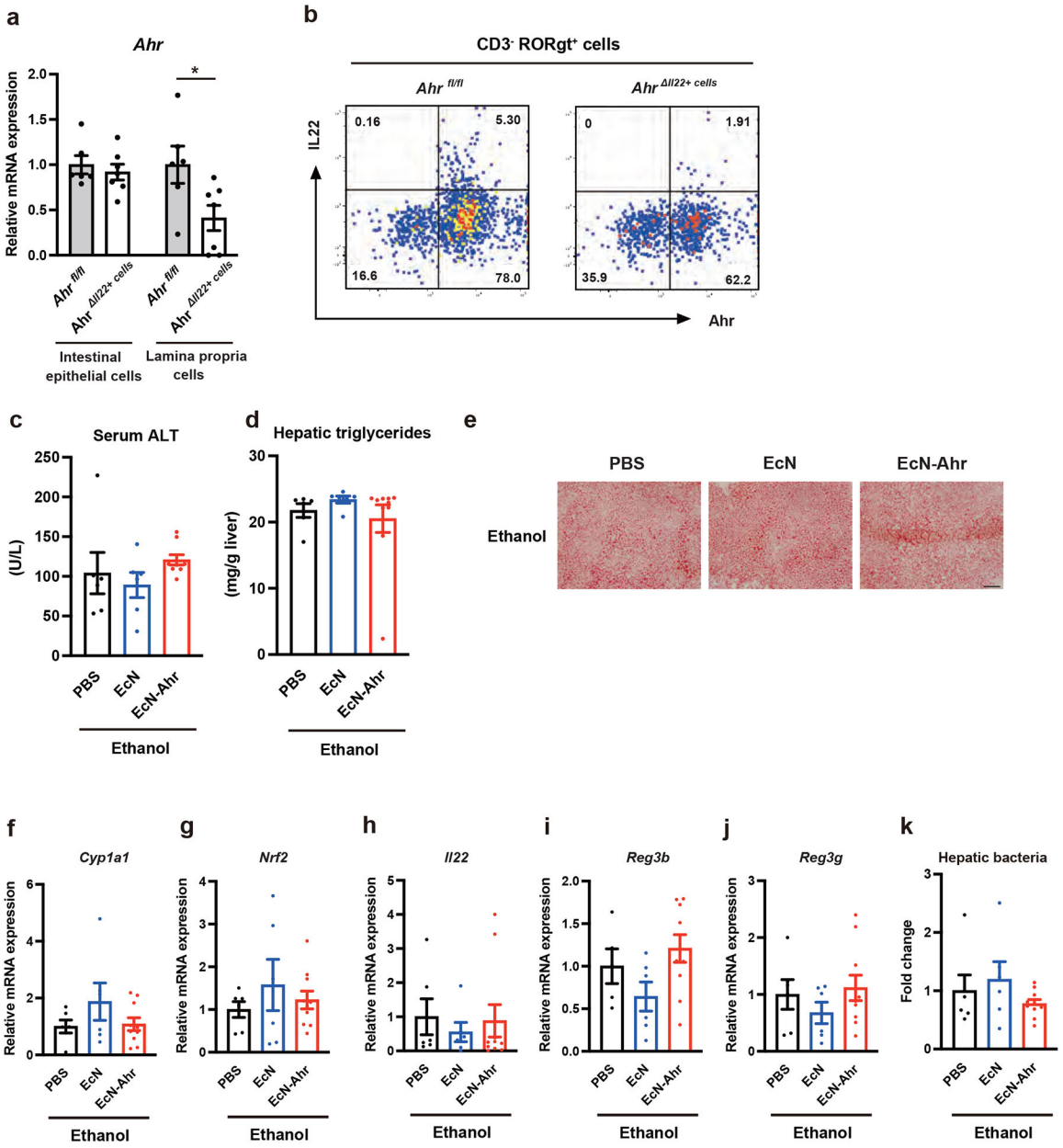

Figure 5. Effect of EcN-Ahr in mice lacking Ahr in Il22^{+} immune cells in a chronic-plus-binge ethanol feeding model.

(a) Gene expression of Ahr in small intestinal epithelial cells and lamina propria cells isolated from *Ahr*$^{fl/fl}$ or *Ahr*$^{\Delta Il22+cells}$ mice. (b) Frequencies of Il22-expressing and Ahr-expressing cells in the gated ILC3 population (CD3^{-}RORgt^{+}) after stimulation with IL23 for 4 hours in lamina propria cells of *Ahr*$^{fl/fl}$ or *Ahr*$^{\Delta Il22+cells}$ mice. (c-k) Male and female *Ahr*$^{\Delta Il22+cells}$ mice were gavaged with PBS (n = 6), EcN (n = 6), or EcN-Ahr (n = 9) during chronic–binge ethanol diet. Serum level of ALT (c), hepatic triglycerides (d), representative Oil red O-stained liver sections (e), gene expression of *Cyp1a1*, *Nrf2*, *Il22*, *Reg3b,* and *Reg3g* in the ileum (f-j), and total bacteria in the liver (k). Data are presented as

mean±S.E.M. of 2 technical replicates. *p<0.05 denotes the significant difference between the groups. Bar size = 100 μm.

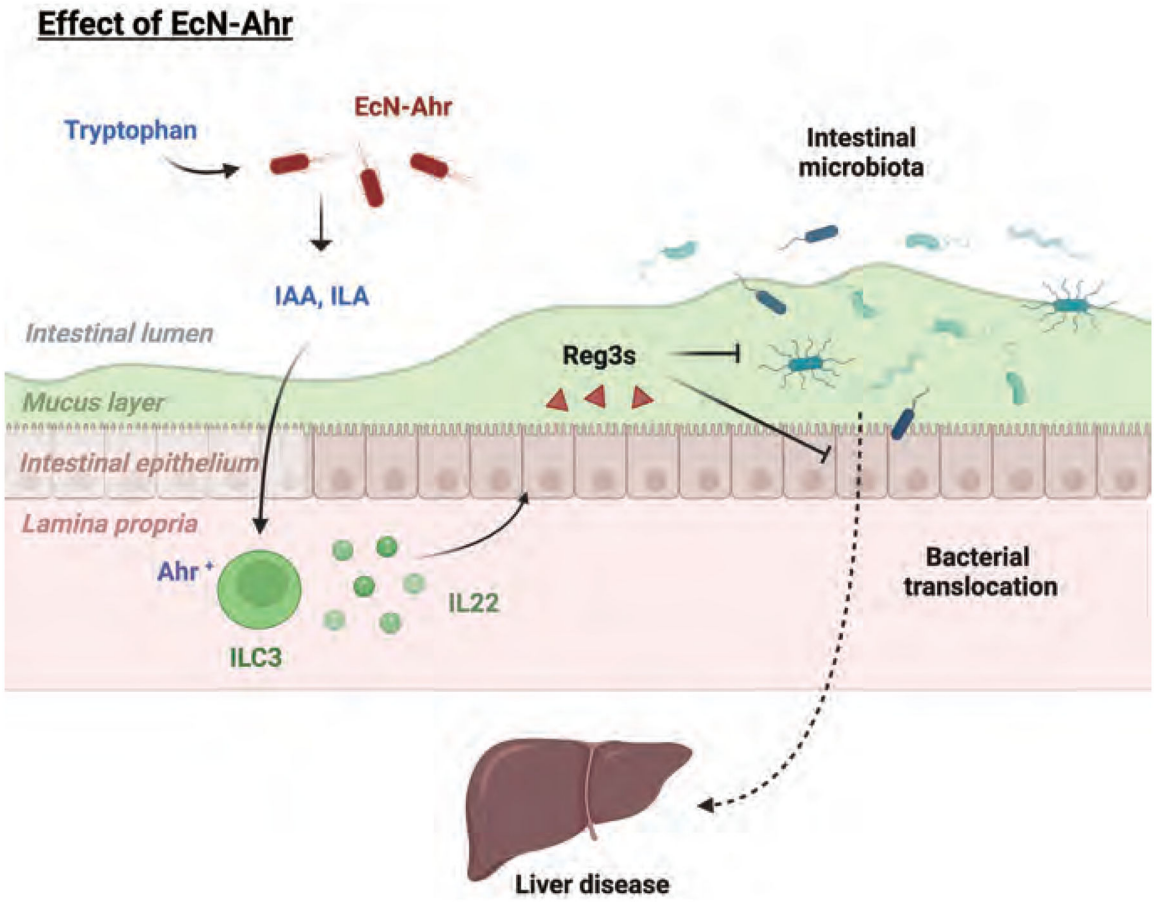

Figure 6. Model for the effects of Ahr agonists producing engineered bacteria.

Engineered EcN-Ahr produce Ahr agonists such as indole-3-acetic acid (IAA) or indole-3-lactic acid (ILA), which bind to Ahr^{+} immune cells in the lamina propria and increase Il22 production. Il22 induces the expression of antimicrobials Reg3b and Reg3g in intestinal epithelial cells, which reduce bacteria in the mucus layer, suppress bacterial translocation and ameliorate ethanol-induced liver disease. Cartoon was created with a license from BioRender.com