

RESEARCH ARTICLE

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

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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 interleukin 22 (IL22)-producing cells.

Results: Through the deletion of endogenous genes *trpR* and *tnaA*, coupled with overexpression of a feedback-resistant tryptophan biosynthesis operon, EcN-Ahr were engineered to overproduce 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. 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, microbiome, microbiota, synthetic biology

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., 2013, 2014; Tapper & 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 & Quintana, 2018). Although xenobiotic compounds were first identified as Ahr ligands (Poland & Knutson, 1982), the diet is an abundant source of Ahr ligands (Gutiérrez-Vázquez & 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 (EtOH)-induced liver disease in mice (Hendriks et al., 2019). Indole-3-acetic acid induced intestinal interleukin-22 (IL22) in type 3 innate lymphoid cells (ILC3) and subsequently increased the expression of the antimicrobials Regenerating islet derived 3 (Reg3) b and Reg3g in the intestine (Hendriks et al., 2019). Whether Ahr is involved in this protective mechanism is not known.

This study investigated the effect of *Escherichia coli* Nissle 1917—engineered to produce Ahr agonists (EcN-Ahr), on EtOH-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^{ΔIL22+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 EtOH feeding model, male C57BL/6 mice from Charles River were fed with Lieber-DeCarli diet for the first 5 days and then with EtOH 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 EtOH (5 g/kg body weight) and sacrificed 9 h later (Bertola et al., 2013). Mice fed a control diet were given an isocaloric substitution of dextrose. Daily gavage of 200 μL of PBS, isogenic *Escherichia coli* Nissle 1917 (EcN, 10⁷ CFU/day), or engineered *Escherichia coli* Nissle 1917-Ahr (EcN-Ahr, 10⁷ CFU/day) started from Day 8 to Day 15. Male and female *Ahr^{fl/fl}* mice and *Ahr^{ΔIL22+cells}* mice were fed with 32% EtOH-containing diet (based on calories), since this strain showed increased mortality with higher concentrations of EtOH. To measure tryptophan metabolites in colon contents, female C57BL/6 mice from Charles River were gavaged once with PBS, EcN (10¹⁰ CFU), or EcN-Ahr (10¹⁰ CFU) and harvested 2 h later. All mice were between age 7 to 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.

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 & 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^{fbt}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_{trpS} promoter was synthesized by Gen9 and cloned into a low copy number, pSC101 origin, and ampicillin resistant vector. For constructing plasmid harboring the *aroG^{fbt}-trpDH-ipdC-iaa1* 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 *iaa1* 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 µg/mL).

Escherichia 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 subcultured for 3 h in LB medium without antibiotics. Bacteria were centrifuged at 8000 ×g for 3 min. 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 10⁷ 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 (Hendriks 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 min. 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 40s. Homogenized samples were spun down at 5000 ×g for 10 min. 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 10mM ammonium acetate containing 1 µg/mL tryptophan-d5 and 5 µg/mL indole-3-acetic acid-d5 as internal standards. Analytes were separated on a Waters Acquity HSS T3 C18, 2.1 × 100mm column running a gradient from 10 to 98% B over 2 min after a 2 µL injection. Mobile phase A was 10mM ammonium acetate while mobile phase B was 90% acetonitrile with 10mM 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) for 15 min, 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 EtOH was measured using EtOH 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:
GTCAGAAGCCAGCGTTCCACC

Mouse *Ccl2*

Forward: ATTGGGATCATCTTGCTGGT, Reverse:
CCTGCTGTTCCACAGTTGCC

Mouse *Adh1*

Forward: GGGTTCTCAACTGGCTATGG, Reverse:
ACAGACAGACCCGACACCTCC

Mouse *Cyp2e1*

Forward: GGGACATTCTGTGTCCAG, Reverse:
CTTAGGGAAAACCTCCGCAC

Mouse *Cyp1a1*

Forward: GGGTTTGACACAGTCACAACCT, Reverse:
GGGACGAAGGATGAATGCCG

Mouse *Nrf2*

Forward: TCTATGTCTTGCTCCAAAGG, Reverse:
CTCAGCATGATGGACTTGGA

Mouse *Il22*

Forward: GCTCAGCTCTGTACATCA, Reverse:
TCGCCTTGATCTCTCCACTC

Mouse *Reg3b*

Forward: GGCTTCATTCTTGCTCTCCA, Reverse:
TCCACCTCCATTGGGTCT

Mouse *Reg3g*

Forward: AAGCTTCCTTCTGTCTCTCC, Reverse:
TCCACCTCTGTTGGGTTTCAT

Mouse *Ahr*

Forward: GGCTTTCAGCAGTCTGATGTC, Reverse:
CATGAAAGAAGCGTTCTCTGG

Mouse *Cldn1*

Forward: ATGCCAATTACCATCAAGGC, Reverse:
GAGGGACTGTGGATGTCCTG

16S

Forward: GTGSTGCAYGGYTGTCTGCA, Reverse:
ACGTCRTCCMCACCTTCTC

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 h. 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 subcultured for 3 h in LB medium without antibiotics. 4 × 10⁷ CFU/mL of EcN or EcN-Ahr were centrifuged at 8000 × g for 3 min, 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 (Hendrikx et al., 2019). 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 h. Cells were blocked with CD16/32 (1:4000, BioLegend) and stained with CD3-FITC (1:400, BD Biosciences) and FVS575V (1:1000, BD Biosciences) for 30 min. 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 two groups, unpaired Student's *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 EtOH-induced liver disease

The effect of EcN-Ahr was examined in a mouse model of chronic-plus-binge EtOH-induced liver disease. There was no significant difference in body weight or food intake between the groups (Figure 2A,B). EtOH-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 EtOH and hepatic expression of two enzymes that metabolize EtOH, alcohol dehydrogenase 1 (*Adh1*), and cytochrome P450 family

2 subfamily e polypeptide 1 (*Cyp2e1*), were unchanged (Figure 3), indicating that EtOH metabolism is not affected by the treatment with engineered bacteria. CFUs of EcN and EcN-Ahr were similar in the cecum (Figure S1). These results indicate that EcN-Ahr reduce EtOH-induced liver disease.

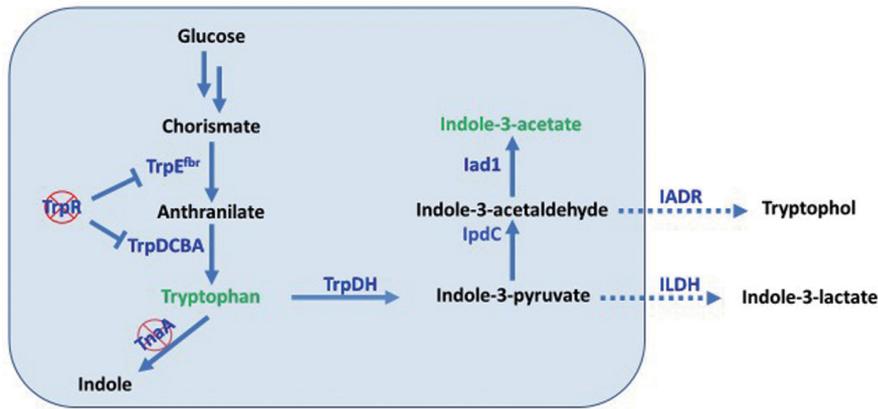
EcN-Ahr activate Ahr in the intestine and prevent bacterial translocation to the liver

Since EcN-Ahr activates Ahr in vitro (Figure 1I), the gene expression of Ahr target genes was examined following chronic-binge EtOH 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 (Figure S2a), indole (Figure S2b), and tryptophan (Figure S2c) 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 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 EtOH-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 EtOH feeding (Figure 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 EtOH-fed mice (Figure 4K). These results indicate that intestinal epithelial cells (IECs) 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.

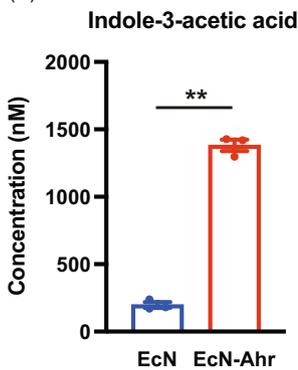
The beneficial effect of EcN-Ahr on EtOH-induced steatohepatitis is abrogated in mice lacking Ahr in *Il22*-producing cells

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^{ΔIl22+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 IECs between

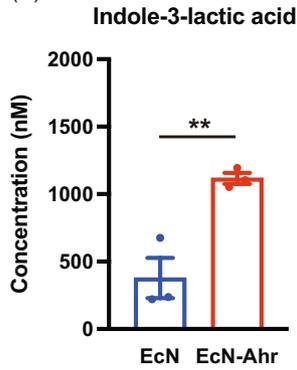
(A)



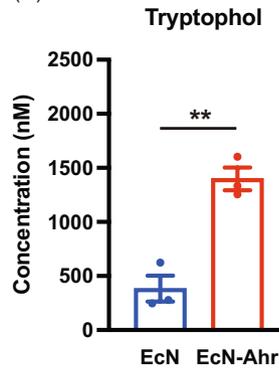
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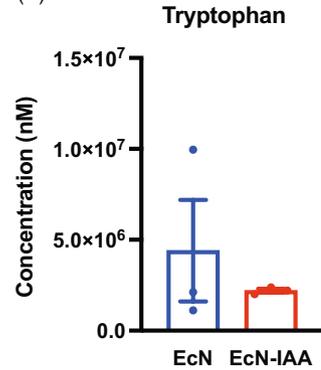
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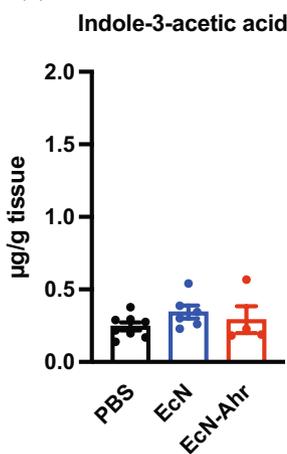
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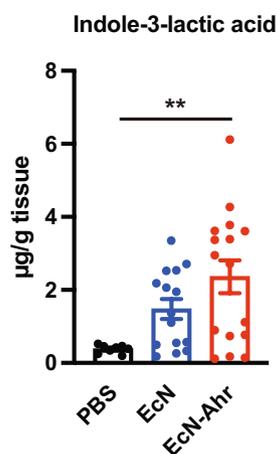
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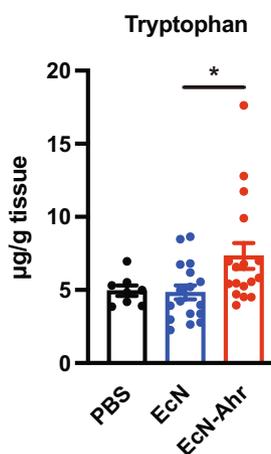
(F)



(G)



(H)



(I)

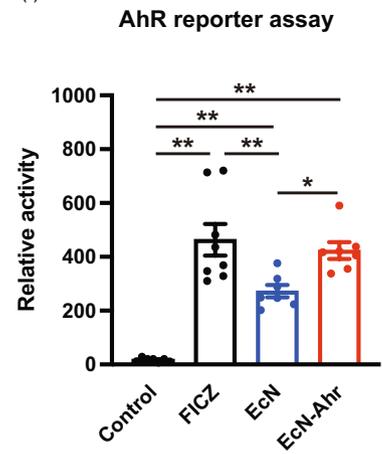


FIGURE 1 Characterization of EcN-Ahr. (A) Schematic of the engineered EcN-Ahr in tryptophan metabolism. EcN-Ahr lack tryptophan transcriptional receptor (TrpR), and tryptophanase A (TnaA), and the *aroGfbr-trpDH-ipdC-iad1* operon was added. Concentrations of indole-3-acetic acid (B), indole-3-lactic 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 h later. Two technical replicates. (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 $\mu\text{g}/\text{mL}$ of the Ahr agonist FICZ, or 10% supernatant of bacteria for 24 h. Data are presented as mean \pm S.E.M. * $p < 0.05$ and ** $p < 0.01$ denotes the significant difference between the groups.

Ahr ^{Δ 122+cells} and littermate *Ahr*^{fl/fl} mice, but mice lacking *Ahr* in I122-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 I122-expressing cells was examined. The frequency of I122⁺ ILC3 (gated on CD3⁻ ROR γ t⁺) was reduced

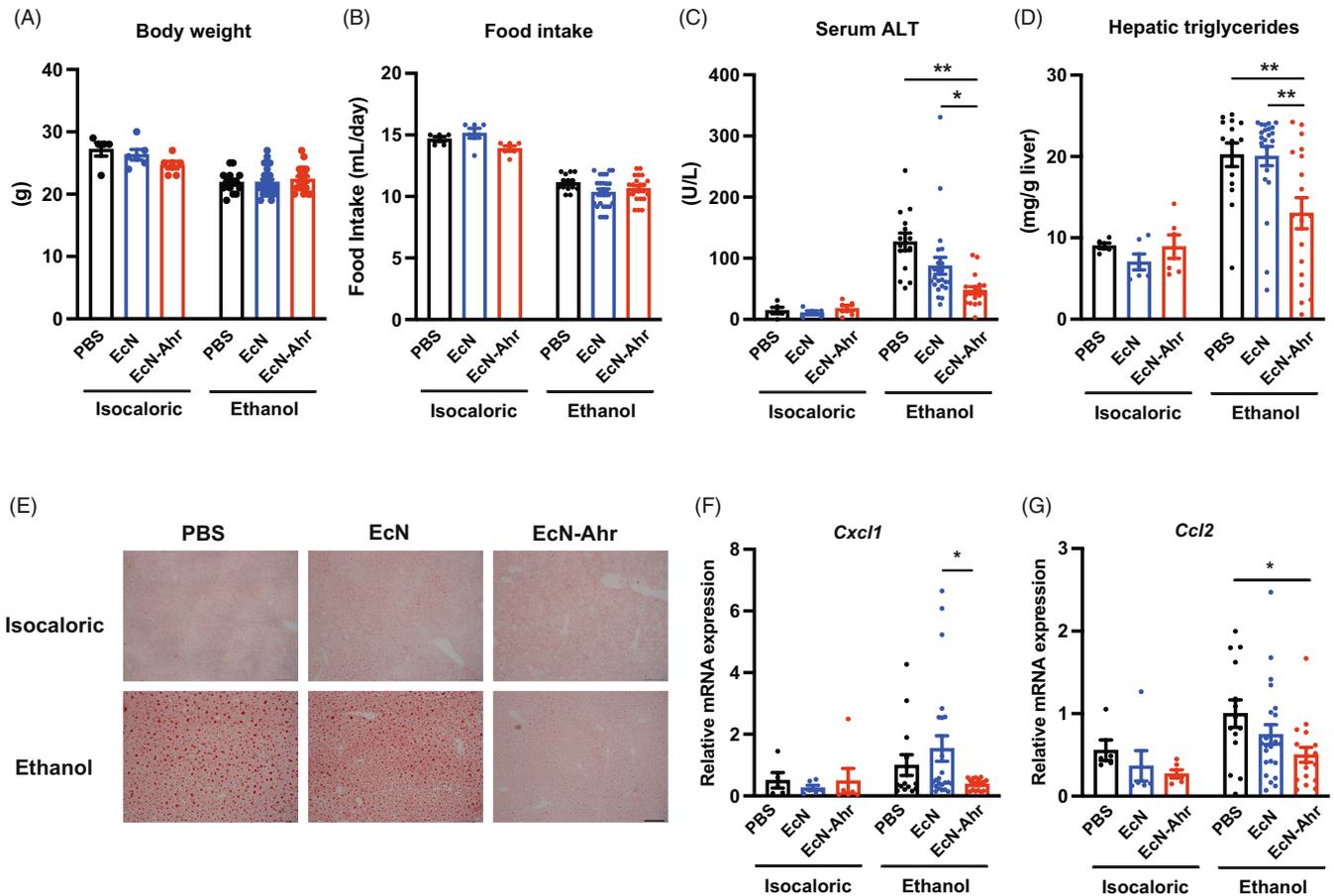


FIGURE 2 Engineered *EcN-Ahr* reduce EtOH-induced steatohepatitis. Male C57BL/6 mice were gavaged with PBS ($n = 5$), *EcN* ($n = 6$), or *EcN-Ahr* ($n = 6$) during the control (isocaloric) diet or with PBS ($n = 14$), *EcN* ($n = 23$), or *EcN-Ahr* ($n = 18$) during chronic-binge EtOH diet. Body weight (A) and food intake (B) of mice subjected to chronic-plus-binge EtOH 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 EtOH diet. Data are presented as mean \pm S.E.M. of three technical replicates. * $p < 0.05$ and ** $p < 0.01$ denotes the significant difference between the groups. Bar size = 100 μ m.

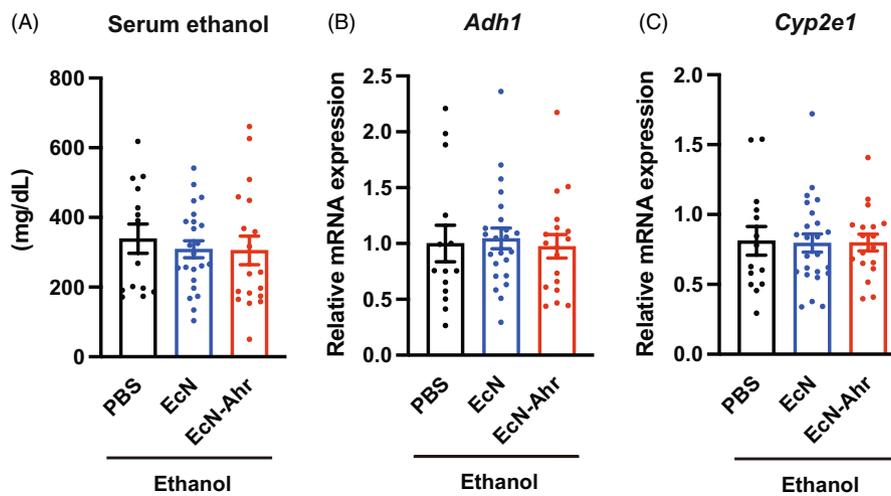
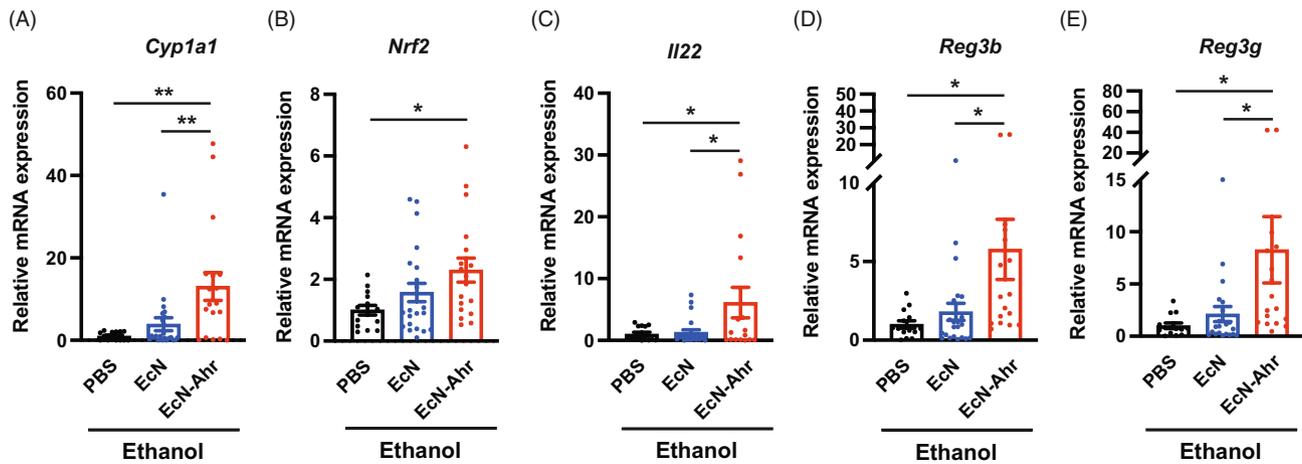


FIGURE 3 Effect of *EcN-Ahr* on EtOH metabolism in a chronic-plus-binge EtOH feeding model. Male C57BL/6 mice were gavaged with PBS ($n = 14$), *EcN* ($n = 23$), or *EcN-Ahr* ($n = 18$) during chronic-binge EtOH diet. Serum concentration of EtOH (A) and gene expression of *Adh1* and *Cyp2e1* in the liver (B, C) of mice subjected to chronic-plus-binge EtOH feeding. Data are presented as mean \pm S.E.M. of three technical replicates.

Small intestine



Liver

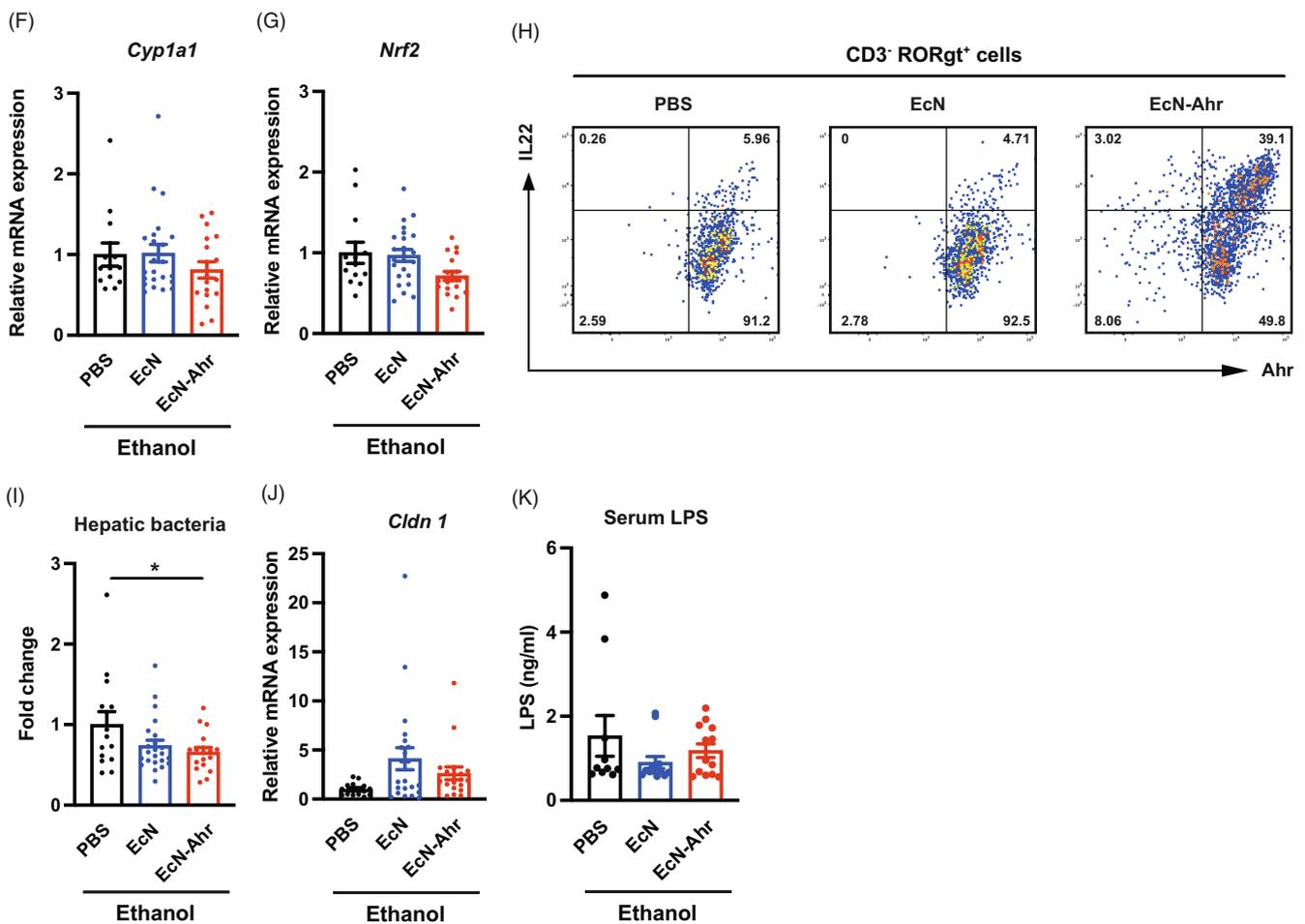


FIGURE 4 Effect of EcN-Ahr on intestinal Ahr-IL22 pathway and bacterial translocation in a chronic-plus-binge EtOH feeding model. Male C57BL/6 mice were gavaged with PBS ($n = 14$), EcN ($n = 23$), or EcN-Ahr ($n = 18$) during chronic-plus-binge EtOH diet. Gene expression of *Cyp1a1*, *Nrf2*, *Il22*, *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 h in lamina propria cells. (I) Total bacteria in the liver of mice subjected to chronic-plus-binge EtOH feeding as determined by qPCR. (J) Gene expression of *Cldn1* in the ileum. (K) Serum level of LPS. Data are presented as mean \pm S.E.M. of three technical replicates. * $p < 0.05$ and ** $p < 0.01$ denotes the significant difference between the groups.

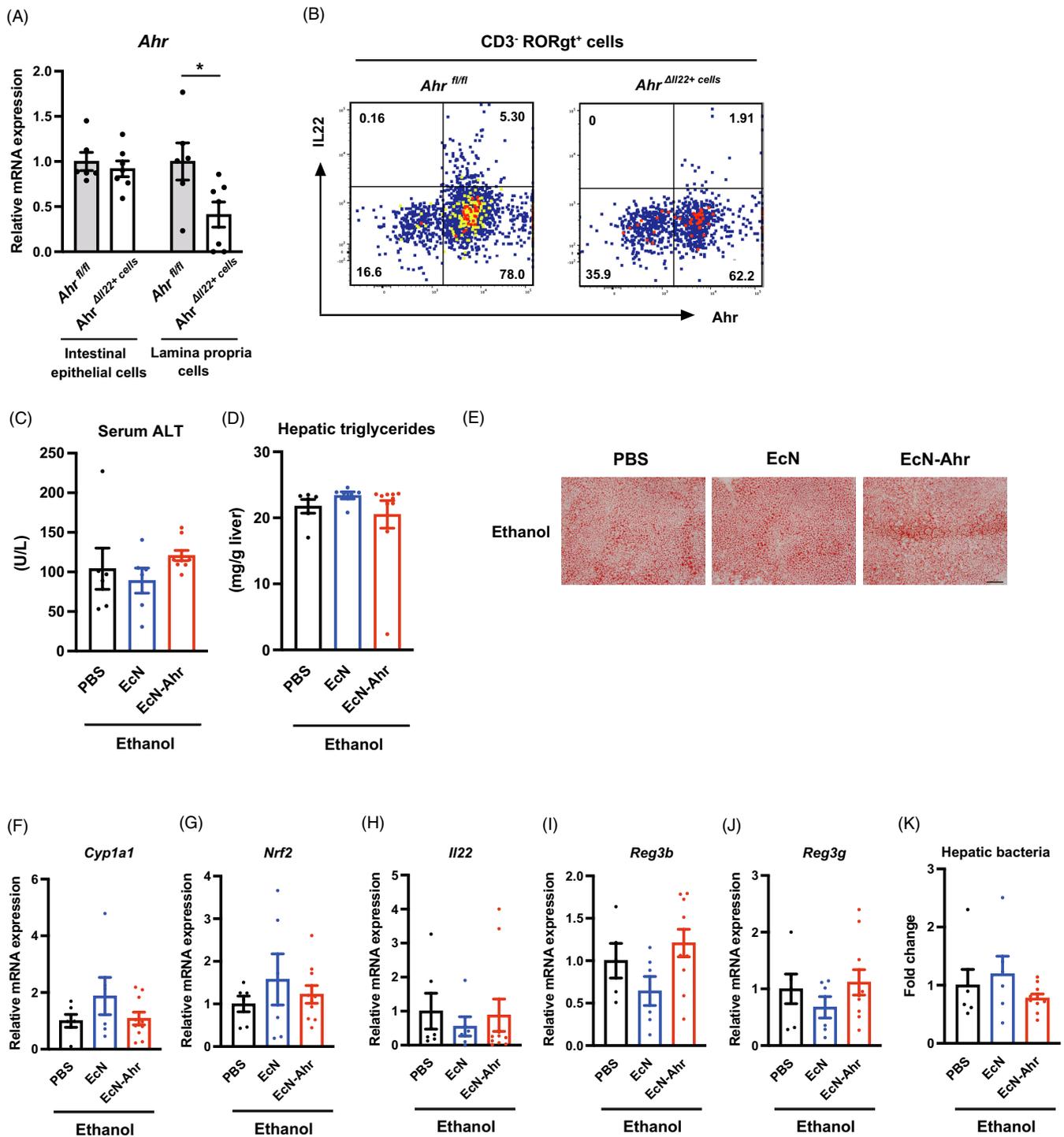


FIGURE 5 Effect of EcN-Ahr in mice lacking Ahr in IL22⁺ immune cells in a chronic-plus-binge EtOH feeding model. (A) Gene expression of Ahr in small intestinal epithelial cells and lamina propria cells isolated from $Ahr^{fl/fl}$ or $Ahr^{\Delta IL22+ cells}$ mice. (B) Frequencies of IL22-expressing and Ahr-expressing cells in the gated ILC3 population (CD3⁺RORgt⁺) after stimulation with IL23 for 4 h 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 EtOH 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 \pm S.E.M. of 2 technical replicates. * $p < 0.05$ denotes the significant difference between the groups. Bar size = 100 μ m.

in $Ahr^{\Delta IL22+ cells}$ mice (Figure 5B). To investigate whether the effect of EcN-Ahr on EtOH-induced liver disease is mediated via the Ahr-IL22 axis, $Ahr^{\Delta IL22+ cells}$ mice were subjected to the chronic-binge

EtOH-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

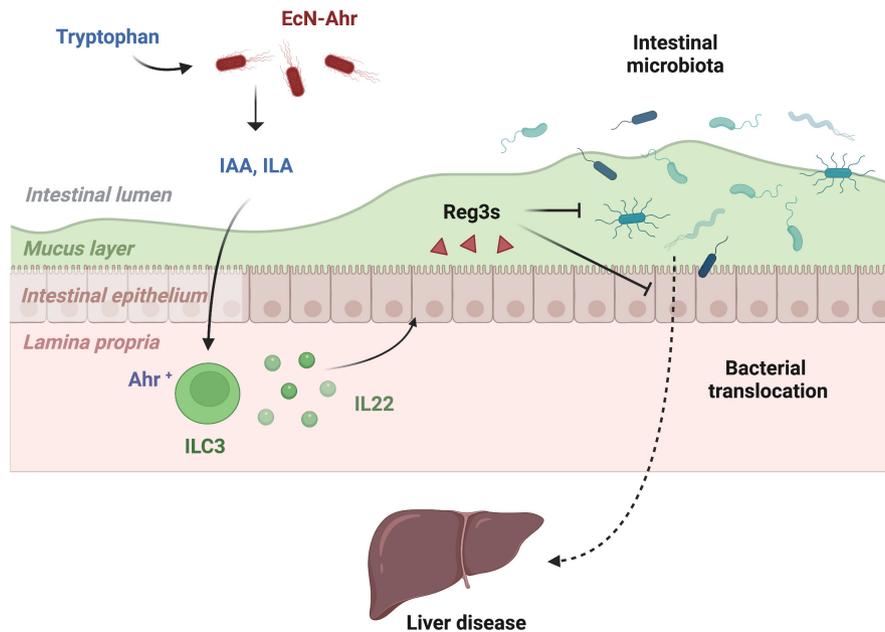
Effect of EcN-Ahr

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 EtOH-induced liver disease. Cartoon was created with a license from [BioRender.com](https://www.biorender.com/).

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 (Figure S3). 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 EtOH-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 ROR γ t 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 mechanism by which EcN-Ahr reduces EtOH-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 EtOH-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 EtOH metabolism in the liver were not affected by EcN-Ahr supplementation. This is important as Ahr activation in the liver reduces EtOH-induced liver disease via lowering EtOH-induced ROS generation and peroxidation, which contributes to a reduction of serum EtOH concentration (Dong et al., 2021).

EcN are safe and commercially available probiotics, which show some benefit for ulcerative colitis (Kruis et al., 1997, 2004; Matthes et al., 2010; Schultz, 2008), infectious GI diseases (Boudeau et al., 2003; Henker et al., 2007), constipation (Möllenbrink & 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 11), 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, 2020). Importantly, EcN-Ahr designed to efficiently produce indoles activated the Ahr more than EcN did (Figure 1). 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 EtOH-induced steatohepatitis in mice (Hendriks 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 EtOH. EcN-Ahr specifically activate the Ahr pathway in the intestine to regulate the expression of antimicrobial proteins, thereby preventing bacterial translocation to the liver (Figure 6).

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CONFLICT OF INTEREST STATEMENT

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 Y.D., C.L. and B.S. as inventors related to this work. M.J., C.C.C., J.R.G., M.P., and D.L.H. are employees of Synlogic Inc.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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