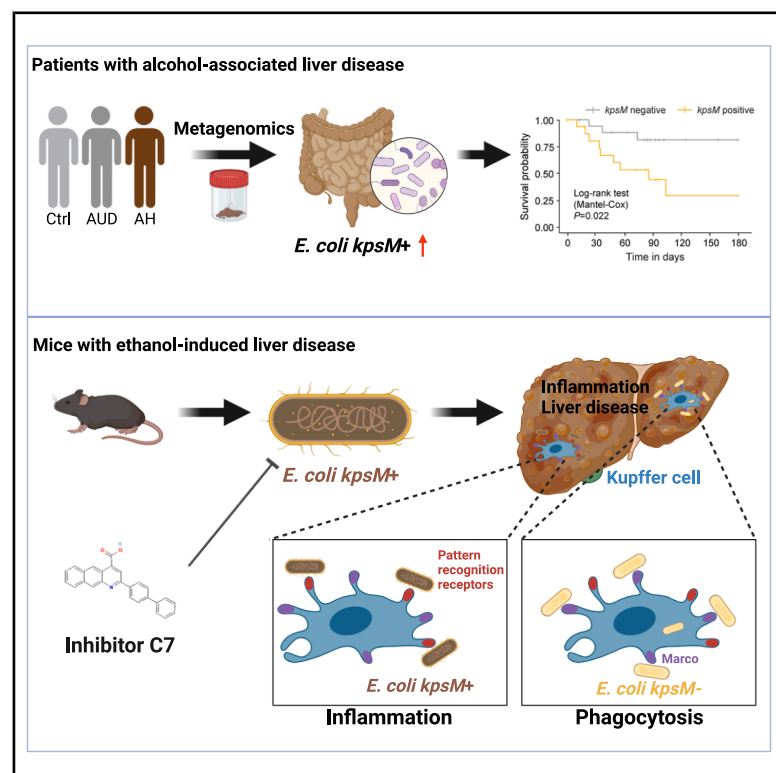


Cell Host & Microbe

Targeted inhibition of pathobiont virulence factor mitigates alcohol-associated liver disease

Graphical abstract



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In brief

Yang et al. show that the *E. coli* virulence factor KpsM is associated with increased mortality in patients with alcohol-associated hepatitis. In mice, KpsM allows evasion of phagocytosis by scavenger receptor Marco on Kupffer cells, causing bacterial spread and exacerbated disease. Small-molecule-mediated inhibition of KpsM ameliorates alcohol-associated liver disease.

Highlights

- *E. coli kpsM* correlates with mortality in patients with alcohol-associated hepatitis
- *kpsM*-positive *E. coli* exacerbate ethanol-induced liver disease in mice
- *kpsM*-positive *E. coli* evade phagocytosis by scavenger receptor Marco on Kupffer cells
- C7 inhibits *kpsM*-dependent capsules and reduces ethanol-induced liver disease



Article

Targeted inhibition of pathobiont virulence factor mitigates alcohol-associated liver disease

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SUMMARY

Alcohol-associated liver disease poses a global health burden with high mortality. Imbalances in the gut microbiota are important for disease progression. Using metagenomic sequencing of fecal samples from a multicenter, international cohort of patients with alcohol-associated hepatitis, we found that the presence of virulence factor KpsM, encoded in the genome of *Escherichia coli* (*E. coli*), correlated with patient mortality. Functional studies using gnotobiotic mouse models and genetic manipulation of bacteria demonstrated that *kpsM*-positive *E. coli* exacerbate ethanol-induced liver disease. The *kpsM* gene mediates the translocation of capsular polysaccharides to the cell surface. This enables *kpsM*-positive *E. coli* to evade phagocytosis by the scavenger receptor Marco on Kupffer cells in the liver, leading to bacterial spread. Importantly, inhibiting *kpsM*-dependent capsules with the small molecule 2-(4-phenylphenyl)benzo[g]quinoline-4-carboxylic acid (C7) attenuated ethanol-induced liver disease in mice. We show that precision targeting of the virulence factor KpsM is a promising approach to improve outcomes of patients with alcohol-associated hepatitis.



INTRODUCTION

Alcohol-associated liver disease is one of the most prevalent liver diseases and cause of liver-related mortality worldwide.^{1–4} Furthermore, it has become the leading cause of liver transplantation in the US.⁵ Patients may develop hepatic inflammation, fibrosis, and liver cirrhosis with an increased risk for hepatocellular carcinoma.^{6,7} Alcohol-associated hepatitis is a severe form within this spectrum, with 90-day mortality of 20%–50%.⁸ The global incidence of alcohol-associated hepatitis is on the rise, particularly among young adults and women.⁹ Liver transplantation is the only curative therapy but is only available at a limited number of centers and for a selected group of patients.^{10–12} Therefore, an urgent need exists for new therapeutic targets for this life-threatening disease.

Gut microbial dysbiosis has been linked to the development and progression of several chronic non-communicable diseases, including alcohol-associated liver disease.¹³ Chronic alcohol consumption alters gut microbiota composition.^{14–17} Transplantation of intestinal microbiota from patients with alcohol-associated hepatitis promotes ethanol-induced liver disease in mice.¹⁸ A promising pilot study showed improved survival rates in patients with severe alcohol-associated hepatitis following fecal microbiota transplantation.¹⁹

While many studies have explored the association between the gut microbiota and different diseases, focusing on diversity, relative abundance, and metabolic profiles of specific microorganisms,^{20–23} little is known about the role of specific pathobionts and their contributing factors in those diseases. Bacterial virulence factors include proteins or peptides that help the organisms colonize the intestine and can contribute to disease. Our recent findings highlighted the role of cytolysin, a virulence factor expressed by *Enterococcus faecalis* (*E. faecalis*), in causing direct lysis of hepatocytes and liver damage, likely through its ability to form pores.²⁴

In this study, we used an unbiased approach and analyzed intestinal bacterial metagenomes in fecal samples from a well-characterized, multicenter cohort of patients with alcohol-associated hepatitis to identify genes encoding potential virulence factors and determine their role in disease pathogenesis.

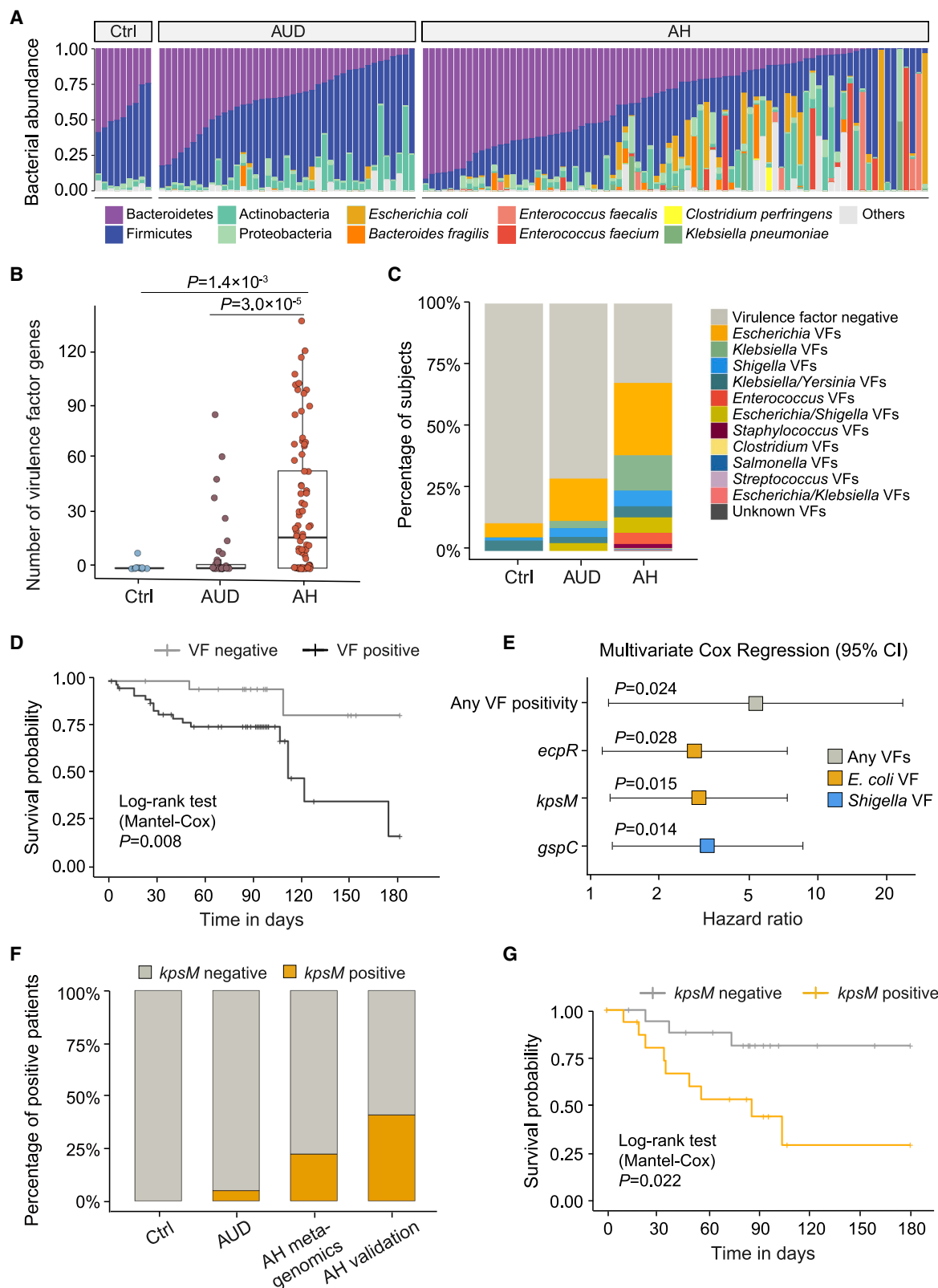
RESULTS

Increased carriage of virulence-related genes is associated with mortality in patients with alcohol-associated hepatitis

Using a cohort of subjects enrolled in a multicenter, international observational study, we performed shotgun metagenomic sequencing of fecal samples from 81 subjects with alcohol-associated hepatitis, 41 subjects with alcohol use disorder but no alcohol-associated hepatitis, and 9 subjects who did not have alcohol use disorder (controls) (Tables S1 and S2). We aligned sequences with those in the virulence factor gene database (VFDB).¹⁴ Compared with the other 2 groups, subjects with alcohol-associated hepatitis displayed distinct changes in microbial composition, with an enrichment of Proteobacteria at the phylum level and potential pathobionts such as *Enterococcus faecium*, *Escherichia coli* (*E. coli*), and *E. faecalis* at the species level (Figure 1A). Significantly more genes encoding

virulence factors were found in fecal metagenomes from subjects with alcohol-associated hepatitis than subjects with alcohol use disorder or controls (Figure 1B). Increased abundance of virulence factor genes was not associated with the duration of hospitalization, suggesting they were not acquired during hospital stays (Figures S1A and S1B). Overall, 68% of subjects with alcohol-associated hepatitis had at least one virulence factor gene, contrasting with 29% of subjects with alcohol use disorder without alcohol-associated hepatitis and 11% of controls ($p = 2.75 \times 10^{-4}$ and 2.27×10^{-3} , respectively, by Fisher's exact test; Figure 1C). The host origin of virulence factor genes varied among the study subjects. Subjects usually carried several virulence factor genes, originating from different host bacterial genera, independent of the centers where the subjects were enrolled (Figures S1C–S1E). The most frequently observed virulence factor genes were derived from *Escherichia*, followed by *Klebsiella*, *Shigella*, *Klebsiella/Yersinia*, and then *Enterococcus* (Figures 1C and S1F).

In subjects with alcohol-associated hepatitis, higher proportions of subjects with intestinal carriage of virulence factors died within 180 days (Figure 1D). 82% of virulence factor-negative subjects were alive after 180 days following enrollment, compared with 18% of virulence factor-positive subjects ($p = 0.008$). In a univariate Cox regression analysis, a fecal sample with any virulence factor gene, with any *Escherichia* virulence factor gene, or with 1 of 6 specific virulence factor genes was associated with increased 180-day mortality (Table S3). After adjustment for antibiotic treatment in the multivariate Cox regression analysis as well as the sequence batch, fecal samples with any virulence factor gene remained associated with increased risk of death, with an adjusted hazard ratio of 5.67 (95% confidence interval [CI], 1.26–25.52; $p = 0.024$; Figure 1E). Specifically, among all 350 core virulence factor genes, fecal samples containing *kpsM* or *ecpR* of *E. coli* were independently associated with increased mortality (Figure 1E). Similarly, *gspC* of *Shigella* or *E. coli* was associated with higher mortality. The *gspC* gene and genomes of *Shigella* and *Escherichia* are similar, and patients did not exhibit clinical signs of dysenteric diarrhea, making it likely that the *Shigella* association is a misalignment. We subsequently focused on *E. coli* virulence factors. In our cohort, *kpsM* and *ecpR* were present in 22.2% and 30.9% of patients with alcohol-associated hepatitis, respectively (Table S1; Figure 1F). The strain-level phylogeny of *ecpR*- and *kpsM*-positive *E. coli* revealed high diversity, which grouped into distinct lineages (Figures S2A and S2B; $p = 0.035$ and $p = 0.013$, respectively, by Fisher's exact test). These virulence factor genes were not associated with infection immediately before or during hospitalization (Figures S2C and S2D). Subjects positive for *ecpR* and/or *kpsM* (32% of subjects with alcohol-associated hepatitis) had a microbiome that differed significantly from that of non-carriers, based on the relative abundance of fecal bacterial populations in the metagenomic dataset (Figure S2E). In particular, the presence of genes encoding virulence factors from *E. coli* was associated with increased relative abundance of *E. coli* (Figure S2F). Whole-genome sequencing of *E. coli* strains isolated from patients with alcohol-associated hepatitis validated the finding that *kpsM*-positive strains of *E. coli* exhibited high diversity and belonged to different sequence types, indicating that the prevalence of *kpsM* was not due to clonal



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dissemination (Figure S2G). Colonies from the same patient were identical, indicating that *kpsM*-positive strains within the host were monophyletic. Furthermore, most *kpsM*-positive *E. coli* strains were multidrug resistant, predicted to carry antimicrobial resistance genes conferring resistance to different classes of antimicrobial agents (aminoglycosides, cephalosporins, sulfamethoxazole, etc.) (Figure S2G). Analysis of 31,247 publicly available *E. coli* genomes revealed that *kpsM* was present in 7,942 (25%) of these genomes across 381 different sequence types. *kpsM* was prevalent in extraintestinal pathogenic *E. coli* (ExPEC) lineages, including the globally dominant multidrug-resistant extended-spectrum beta-lactamase-producing *E. coli* ST131²⁵ (Figure S2H). Multiple sequence alignment of *KpsM* amino acid sequences revealed that *KpsM* is also present in other species, but it shows high identity (>97%) within *E. coli* strains and limited similarity (<50%) across different species (data not shown).

Higher *E. coli* abundance itself was independently associated with increased 180-day mortality. However, when we adjusted for the presence of *ecpR* and/or *kpsM*, this association was no longer statistically significant (Table S4). This indicates that the abundance of *E. coli* is associated with increased mortality dependent on the carriage of genes encoding virulence factors. The presence of cirrhosis was not associated with higher numbers of virulence factor genes (Figures S3A and S3B). Furthermore, levels of zonulin (a marker for gut barrier dysfunction) and lipopolysaccharide-binding protein (LPS-BP) (a marker of the systemic immune response to LPS) in serum samples were not associated with an increased number or presence of any virulence factor genes (Figures S3C–S3F). Virulence factors, therefore, do not seem to be associated with increased intestinal permeability or translocation of LPS, a feature of alcohol-associated hepatitis. In addition, the overall abundance and spectrum of fecal virulence factor genes were not different between patients with alcohol-associated hepatitis treated with antibiotics or not (Figure S3G).

Given the strong association of bacterial virulence factor genes with mortality and the greater importance of *kpsM* compared with *ecpR* observed in our mouse studies (see below), we validated our results for one of the virulence factors, *kpsM*, in an independent cohort using qPCR analyses in fecal samples from patients with

alcohol-associated hepatitis (Table S5). Subjects who were positive for *kpsM* (40.8%; Figure 1F) had a higher risk for mortality within 180 days compared with subjects negative for *kpsM*, with a hazard ratio of 4.10 (95% CI 1.11–15.19; $p = 0.022$; Figure 1G), which remained significant after adjustment for antibiotic use, with an adjusted hazard ratio of 3.86 (95% CI 1.04–14.32; $p = 0.044$).

E. coli kpsM promotes ethanol-induced liver disease

To study the contribution of virulence factor-positive bacteria to liver disease, germ-free C57BL/6 mice were colonized with stool from three groups of patients with alcohol-associated hepatitis: (1) *E. coli kpsM*-negative and *ecpR*-negative, (2) *E. coli kpsM*-positive and *ecpR*-positive, and (3) *E. coli kpsM*-positive and *ecpR*-negative. Colonized mice were then placed on a chronic-binge ethanol diet and examined for the occurrence of ethanol-induced liver disease (Figure 2A). All fecal samples were selected to be negative for *E. faecalis* cytolysin.²⁴

Compared with mice colonized with feces from *kpsM*-negative and *ecpR*-negative patients with alcohol-associated hepatitis, mice fed ethanol after they were colonized with *kpsM*-positive and *ecpR*-positive feces developed more severe liver injury, as indicated by higher levels of serum alanine amino-transferase (ALT) (Figure 2B), increased hepatic steatosis (Figures 2C–2E), and more severe liver inflammation with increased expression of the inflammatory cytokines and chemokines interleukin-1 beta (*Il1b*), chemokine (C-X-C motif) ligand-1 (*Cxcl1*), and *Cxcl2* (Figures 2F–2H) compared with mice colonized with *kpsM*-negative and *ecpR*-negative feces. Mice colonized with *kpsM*-positive and *ecpR*-negative feces also displayed significantly more ethanol-induced liver injury, steatosis, and inflammation (Figures 2B–2H) compared with mice colonized with *kpsM*-negative and *ecpR*-negative feces, indicating that *kpsM* rather than *ecpR* is responsible for liver disease exacerbation. Gene expression of *F4/80*, a marker for Kupffer cells, did not differ significantly among ethanol-fed groups (Figure S4A). *kpsM* was detectable in livers of mice colonized with *kpsM*-positive feces following chronic ethanol administration but not when fed an isocaloric (control) diet (Figure 2I), indicating that ethanol-induced changes in the gut

Figure 1. Increased carriage of virulence gene *kpsM* in *E. coli* is associated with increased mortality in patients with alcohol-associated hepatitis

(A) Relative taxa abundance by shotgun metagenomic sequencing is shown for fecal samples from 81 subjects with alcohol-associated hepatitis (AH), 41 subjects with alcohol use disorder and no alcohol-associated hepatitis (alcohol use disorder [AUD]), and 9 subjects without AUD (controls, Ctrl). Results present different taxonomic levels, with each phylum level having its relative abundance adjusted by subtracting the abundance of the shown species levels. (B) Presence of 350 different virulence-related core genes in fecal samples from 81 subjects with AH, 41 subjects with AUD, and 9 subjects without AUD (controls, Ctrl). p values were determined by Kruskal-Wallis test with Dunn's post hoc test followed by false discovery rate (FDR) procedures. (C) Relative abundance of virulence factors found in fecal samples from 81 subjects with AH, 41 subjects with AUD, and 9 subjects without AUD (controls, Ctrl). Factors are color-coded by genus of bacteria that carry them. (D) Kaplan-Meier curve of survival of subjects with AH whose fecal samples were virulence factor (VF)-positive ($n = 55$) or VF-negative ($n = 26$), $p = 0.008$. For the only subject who underwent liver transplantation, the transplantation date was considered as the date of death. Patients were censored at the time point they were last seen alive. p values were determined by a two-sided log-rank (Mantel-Cox) test. (E) Multivariate Cox regression adjusted for treatment with antibiotics and the sequence batch. Only significant virulence factors are shown. Squares identify hazard ratios, with lower and upper 95% confidence interval (CI) values shown as lines. Colors indicate the genus of bacteria that produces each specific virulence factor. p values were determined by a two-sided Wald test. (F) Proportions of subjects positive for *kpsM* using metagenomics data (Ctrl = 0%, AUD = 4.9%, AH metagenomics = 22.2%, $p = 0.017$) or qPCR data (AH validation = 40.8%). p values were determined by two-sided Fisher's exact test. (G) Kaplan-Meier survival curve for subjects with AH in the validation cohort. The subjects were assigned to groups based on the fecal presence of *kpsM*-positive ($n = 20$) and *kpsM*-negative ($n = 29$) *E. coli* as assessed by qPCR. p values were determined by a two-sided log-rank (Mantel-Cox) test.

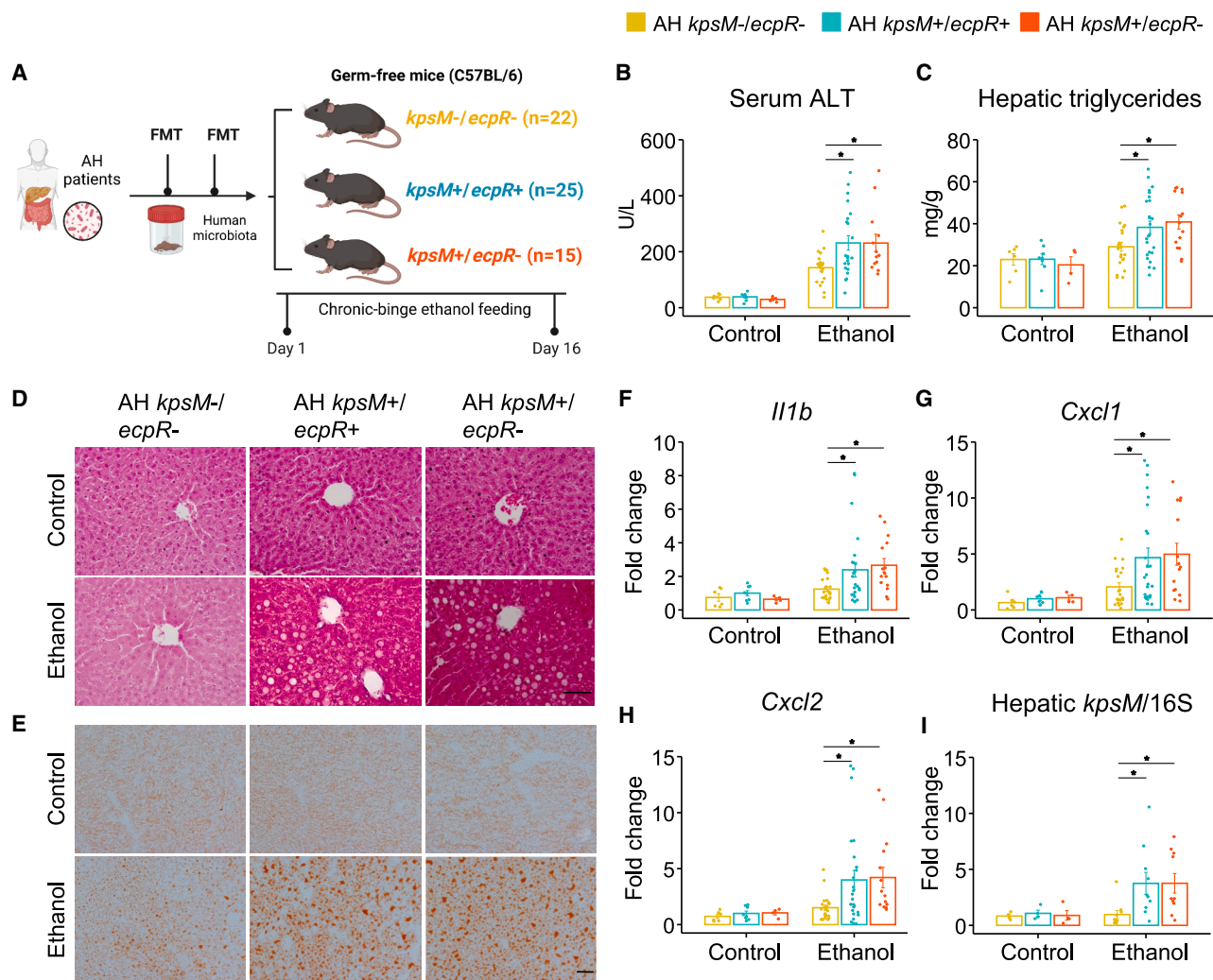


Figure 2. Fecal transplantation from *kpsM*-positive patients with alcohol-associated hepatitis exacerbates ethanol-induced liver disease in gnotobiotic mice

(A–I) Germ-free C57BL/6 mice were colonized with stool from one of three groups of patients with alcohol-associated hepatitis (AH): (1) *E. coli* *kpsM*-negative and *ecpR*-negative (AH *kpsM*-/*ecpR*-), (2) *E. coli* *kpsM*-positive and *ecpR*-positive (AH *kpsM*+/*ecpR*+), and (3) *E. coli* *kpsM*-positive and *ecpR*-negative (AH *kpsM*+/*ecpR*-). Colonized mice were fed oral isocaloric (control) or chronic-binge ethanol diets.

(A) Study overview.

(B) Serum levels of ALT.

(C) Hepatic triglyceride content.

(D) Representative images of liver sections stained with hematoxylin and eosin. (E) Representative images of liver sections stained with oil red O.

(F–H) Hepatic levels of *Il1b*, *Cxcl1*, and *Cxcl2* mRNA.

(I) Hepatic *kpsM* abundance normalized to bacterial 16S, as measured by qPCR.

The results are expressed as mean ± SEM and were generated from at least 2 technical replicates. *p* values were determined by one-way ANOVA with Tukey's post hoc test (B, C, and F–H) or Kruskal-Wallis test with Dunn's post hoc test followed by FDR procedures (I). **p* < 0.05. ALT, alanine aminotransferase. The exact group size (*n*) and *p* values for each comparison are listed in Table S7. Scale bars, 100 μm.

barrier are necessary for translocation of *kpsM*-positive *E. coli* from the intestine to the liver.

No significant differences were observed in serum level of ethanol or the mRNAs encoding the 2 primary enzymes that metabolize ethanol in the liver, alcohol dehydrogenase 1 (*Adh1*) and cytochrome P450 family 2 subfamily E polypeptide 1 (*Cyp2e1*) (Figures S4B–S4D). These observations indicate that bacterial colonization did not affect ethanol metabolism.

Fecal levels of *E. coli* were similar in all 3 groups following ethanol feeding (Figure S4E), while increased fecal *kpsM* and *ecpR* abundance was confirmed in microbiota-humanized mice (Figures S4F and S4G), indicating that *kpsM* or *ecpR* positivity was not due to a colonization advantage for *E. coli*. Together with the above results, our findings suggest that *E. coli* strains positive for *kpsM*, rather than *ecpR*, are associated with exacerbation of ethanol-induced liver disease.

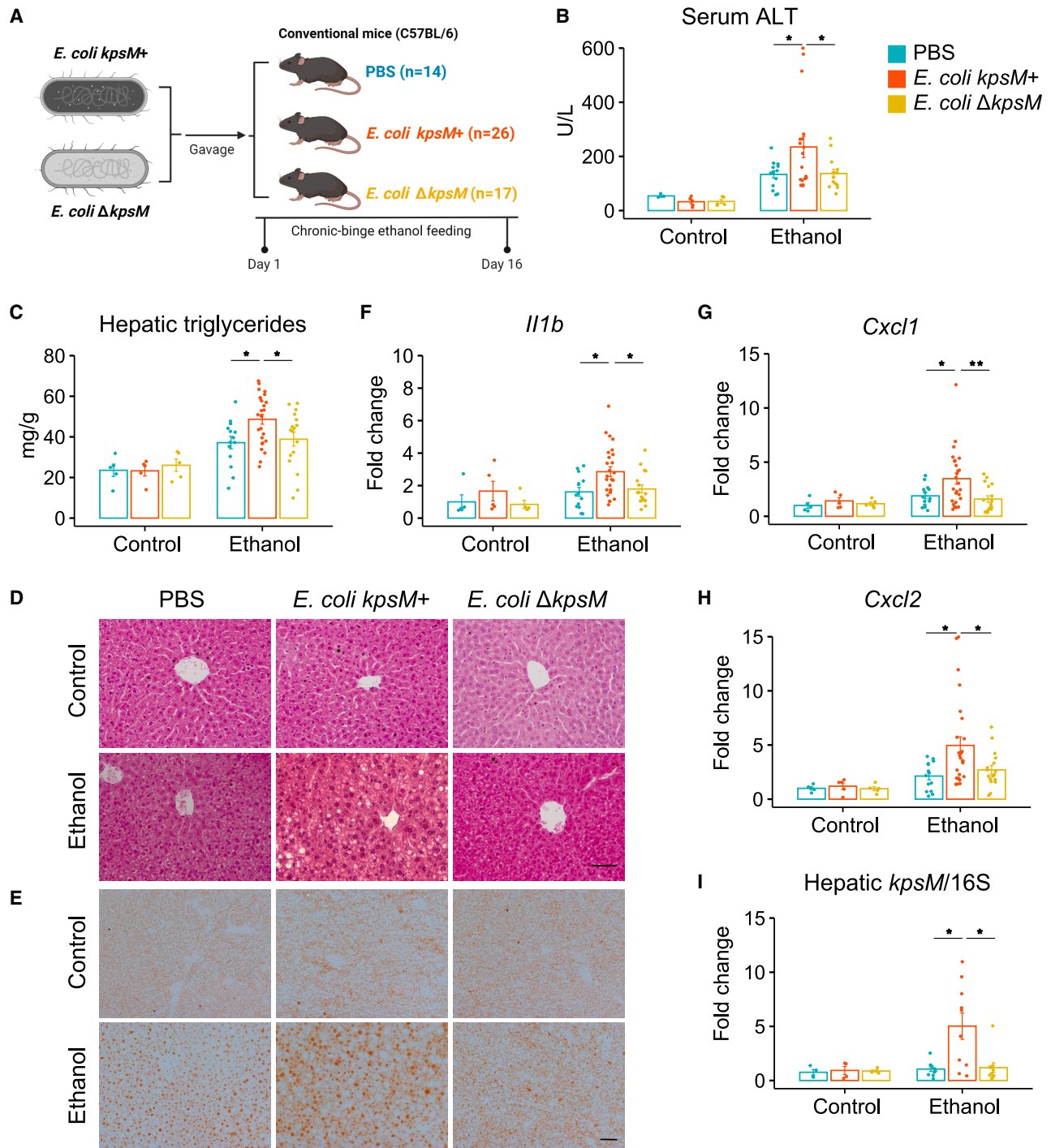


Figure 3. *KpsM*-positive *E. coli* causes progression of ethanol-induced liver disease in mice

(A–I) C57BL/6 mice were fed oral isocaloric (control) or chronic-binge ethanol diets and gavaged with vehicle (PBS), *kpsM*-positive (1×10^9 colony-forming units [CFUs]), or $\Delta kpsM$ *E. coli* (1×10^9 CFUs) every 3 days and 1 h before the binge.

(A) Study overview.

(B) Serum levels of ALT.

(C) Hepatic triglyceride content.

(D) Representative images of liver sections stained with hematoxylin and eosin.

(E) Representative images of liver sections stained with oil red O.

(F–H) Hepatic levels of *Il1b*, *Cxcl1*, and *Cxcl2* mRNAs.

(I) Hepatic *kpsM* normalized to 16S, measured by qPCR.

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To validate our findings from microbiota-humanized mice, we gavaged conventional C57BL/6 mice with *kpsM*-positive *E. coli* or isogenic *E. coli* with a *kpsM* mutation ($\Delta kpsM$ *E. coli*)²⁶ and subjected them to a chronic-binge ethanol diet (Figure 3A). Mice gavaged with *kpsM*-positive *E. coli* and fed ethanol showed greater liver injury, steatosis, and inflammation compared with mice given phosphate-buffered saline (PBS) (Figures 3B–3H). Mice fed ethanol after they were gavaged with $\Delta kpsM$ *E. coli* had significantly less ethanol-induced liver disease compared with mice fed ethanol given *kpsM*-positive *E. coli* (Figures 3B–3H). *kpsM* was detected in livers of ethanol-fed mice gavaged with *kpsM*-positive *E. coli* (Figure 3I). No significant differences were observed in ethanol metabolism (Figures S5A–S5C). Abundance of fecal *E. coli* was similar in mice gavaged with *kpsM*-positive or $\Delta kpsM$ *E. coli* (Figure S5D). Presence of fecal *kpsM* was confirmed in ethanol-fed mice gavaged with *kpsM*-positive *E. coli* (Figure S5E).

To further confirm our findings, conventional C57BL/6 mice were gavaged with clinical *kpsM*-positive or *kpsM*-negative *E. coli* isolates and subjected to a chronic-binge ethanol diet. Mice gavaged with *kpsM*-positive *E. coli* and fed ethanol showed greater liver injury and steatosis. *kpsM* was detected in livers of ethanol-fed mice gavaged with *kpsM*-positive *E. coli* (Figure S6). Together, these results confirm that *kpsM* promotes ethanol-induced liver disease.

Scavenger receptor Marco protects from *kpsM*-positive-*E. coli*-exacerbated ethanol-induced liver disease

KpsM is an ATP-binding cassette transporter to transport intracellularly synthesized capsular polysaccharides to the outer membrane.²⁷ Notably, *kpsM* is prevalent in *E. coli* strains whose capsular polysaccharides consist of polysialic acid.²⁸ Polysialic acid is commonly found on immune cells and can mimic the extracellular surface of mammalian cells,²⁹ therefore protecting the bacteria from elimination by phagocytosis.³⁰ When bacteria breach the intestinal barrier, they enter the portal circulation and reach the liver, which acts as a secondary barrier to curb bacterial dissemination.³¹ Kupffer cells, the resident macrophage population in the liver, capture bacteria that have translocated from the gut and phagocytose and kill them.³¹ We confirmed that isolated Kupffer cells phagocytosed *kpsM*-positive *E. coli* much less efficiently than $\Delta kpsM$ *E. coli* in cell culture (Figures 4A and 4B). To determine whether phagocytosis of translocated bacteria is also different *in vivo*, we gavaged ethanol-fed mice with *kpsM*-positive and $\Delta kpsM$ *E. coli*. The number of translocated *kpsM*-positive *E. coli* recovered in the liver was significantly lower 30 min after the gavage but significantly higher in the spleen, lungs, and heart compared with $\Delta kpsM$ *E. coli* (Figure 4C). The percentage of positive blood cultures was significantly higher in mice 30 min following gavage with *kpsM*-positive *E. coli* as compared with $\Delta kpsM$ *E. coli* ($p = 0.0267$, chi-squared test) but not after 9 h ($p = 0.2938$, chi-squared test) (Figure S7A). To confirm the role of Kupffer cells in bacterial capturing and phagocytosis *in vivo*, ethanol-adminis-

tered C57BL/6 mice were gavaged with *kpsM*-positive and $\Delta kpsM$ *E. coli*, and Kupffer cells were isolated 2 h later. The number of *E. coli* cultured from isolated Kupffer cells was significantly lower in mice gavaged with *kpsM*-positive *E. coli* as compared with mice receiving $\Delta kpsM$ *E. coli* (Figure S7B). These data indicate that translocated *kpsM*-positive *E. coli* can escape capture by Kupffer cells in the liver, resulting in increased systemic spread.

The scavenger receptor macrophage receptor with collagenous structure (Marco) is primarily expressed on periportal Kupffer cells.³² Marco-positive Kupffer cells exert anti-inflammatory functions through sequestration of bacteria such as *E. coli* and through production of anti-inflammatory cytokines.³² Following knockdown of Marco in Kupffer cells (Figure 4D), phagocytosis of $\Delta kpsM$ *E. coli* was reduced to the level of *kpsM*-positive *E. coli* (Figure 4E). Furthermore, recombinant Marco binds $\Delta kpsM$ *E. coli* more efficiently than *kpsM*-positive *E. coli* (Figure 4F). The Kupffer-cell-specific phagocytosis receptor V-set and immunoglobulin domain containing 4 (Vsig4; also called complement receptor of immunoglobulin superfamily or CRlg) binds and phagocytoses different Gram-positive bacteria.³³ We confirmed that Vsig4 does not mediate phagocytosis of *kpsM*-positive *E. coli* in ethanol-administered mice (Figure S7C). These results indicate that Marco is important for binding and uptake of *E. coli* by Kupffer cells, which is inhibited by *kpsM*.

To investigate the contribution of Marco-mediated phagocytosis of *kpsM*-positive *E. coli* to ethanol-induced liver injury, we performed *in vivo* knockdown of Marco selectively in Kupffer cells by virogenetic tools. Mice were subjected to a chronic-binge ethanol diet and gavaged with *kpsM*-positive and $\Delta kpsM$ *E. coli* (Figure 5A). Adeno-associated virus serotype 8 (AAV8)-mediated transgene expression of a double-floxed inverse open reading frame (ORF) (DIO) short hairpin RNA (shRNA) against Marco in *Clec4ef*-Cre mice efficiently reduced Marco expression compared with mice receiving the control vector expressing scrambled shRNA (Figure S7D). Hepatic Vsig4 expression was not affected by knockdown of Marco (Figure S7E). Ethanol-induced liver injury, steatosis, and inflammation were increased following knockdown of Marco in Kupffer cells in mice gavaged with *kpsM*-positive *E. coli* and fed ethanol compared with control mice (Figures 5B–5G). Mice with knockdown of Marco in Kupffer cells also showed increased liver disease when gavaged with $\Delta kpsM$ *E. coli* (Figures 5B–5G), indicating that *E. coli* lacking *kpsM* can also induce disease if they can evade phagocytosis. Hepatic *kpsM* levels were reduced after knockdown of Marco (Figure 5H). To confirm that phagocytosis in Kupffer cells is impaired after knockdown of Marco, ethanol-administered mice were gavaged with *kpsM*-positive *E. coli*, and Kupffer cells were isolated 2 h later. The number of translocated *kpsM*-positive *E. coli* recovered from Kupffer cells was significantly lower in mice with knockdown of Marco in Kupffer cells as compared with control mice (Figure S7F). There was no cardiac injury as a result of increased post-hepatic bacterial load (Figure S7G), and no mortality was observed in any of

The results are expressed as mean $\pm$ SEM and generated from at least 2 technical replicates. *p* values were determined by one-way ANOVA with Tukey's post hoc test (B, C, and F–I). **p* < 0.05, ***p* < 0.01. ALT, alanine transaminase. The exact group size (*n*) and *p* values for each comparison are listed in Table S7. Scale bars, 100 μ m.

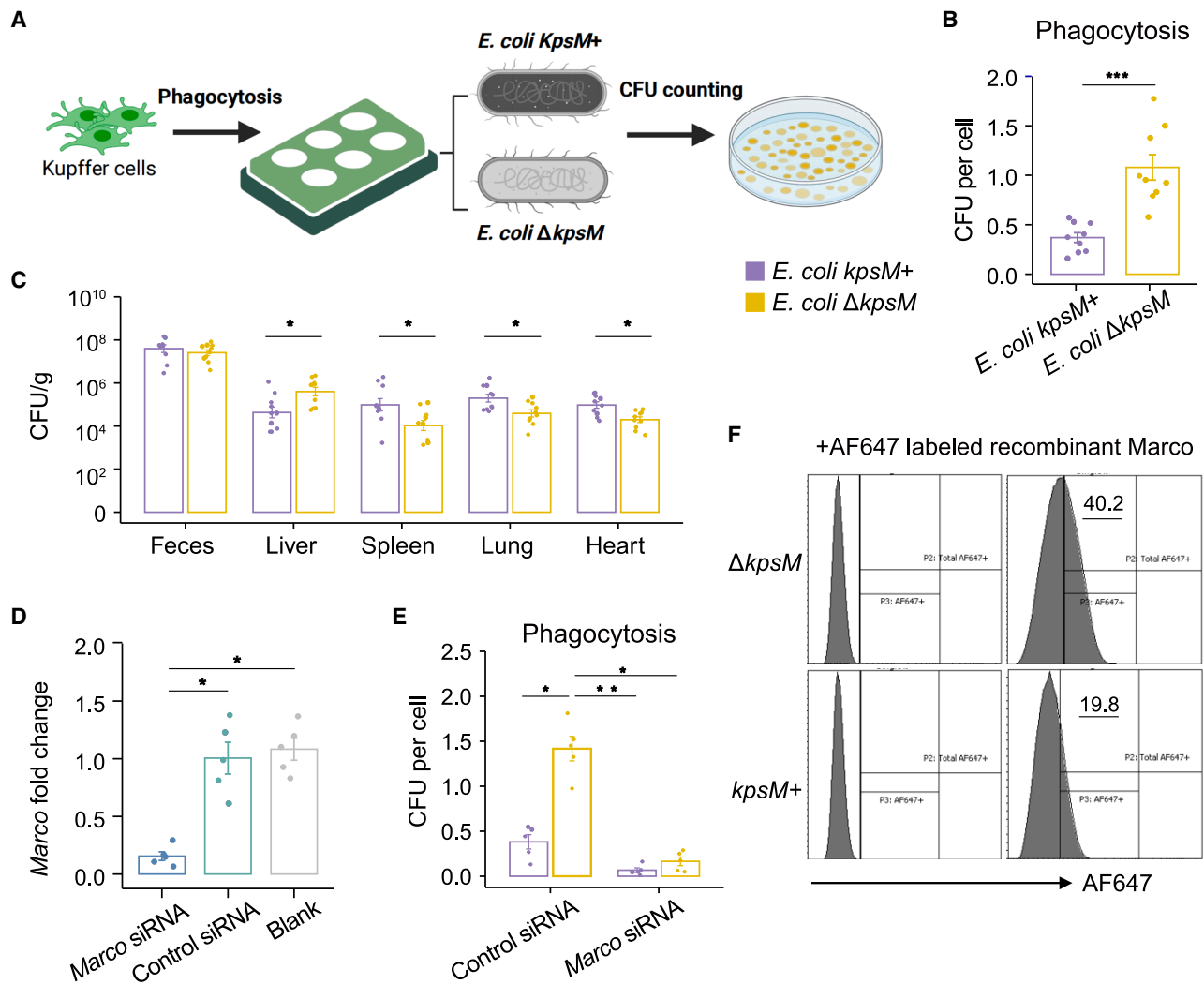


Figure 4. Marco in Kupffer cells eliminates translocated *kpsM*-positive *E. coli*

(A) Primary mouse Kupffer cells were incubated with *E. coli* strains for 2 h. Phagocytosis of *E. coli* was assessed by intracellular survival of *E. coli*.

(B) Primary mouse Kupffer cells were incubated with the indicated *E. coli* strains for 2 h. Bacterial phagocytosis and intracellular survival were assessed by CFU assay after elimination of extracellular bacteria. CFUs were normalized per Kupffer cell.

(C) C57BL/6 mice were fed a chronic-binge ethanol diet, and *E. coli* (1×10^9 CFUs) were gavaged 1 h after ethanol binge. *E. coli* load in different tissues was determined by culture of homogenates of the indicated organs 30 min after gavage.

(D) Primary mouse Kupffer cells were transfected with control small interfering RNA (siRNA) or siRNA against *Marco*. After 24 h, *Marco* mRNA was measured by qPCR.

(E) Primary mouse Kupffer cells were transfected with control siRNA or siRNA against *Marco*. After 24 h, cells were incubated with *E. coli* strains for 2 h. Phagocytosis of *E. coli* was assessed by intracellular survival of *E. coli*, with bacterial CFUs normalized per Kupffer cell. (F) AF 647-labeled recombinant mouse Marco protein (20 μ g/mL) was incubated with *E. coli* (1×10^6 CFUs) for 2 h. Binding of Marco with *E. coli* was analyzed by flow cytometry. Translocation experiments in (C) were done once, and all other experiments were generated from at least two technical replicates. *p* values were determined by Mann-Whitney-Wilcoxon rank-sum test (B and C) or Kruskal-Wallis test with Dunn's post hoc test, followed by FDR procedures (D and E). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. The exact group size (*n*) and *p* values for each comparison are listed in Table S7.

the groups (not shown). No significant differences were observed in ethanol metabolism (Figures S7H–S7J). Taken together, these results indicate that Marco on Kupffer cells limits ethanol-induced liver disease by elimination of translocated *E. coli*. *kpsM*-positive *E. coli* evade phagocytosis mediated by Marco and thus exacerbate ethanol-associated liver disease. By contrast, *E. coli* lacking *kpsM* do not exacerbate ethanol-induced liver disease because they are normally phagocytosed

and killed. Consequently, KpsM is an important virulence factor in *E. coli*, crucial for evading phagocytosis and promoting liver disease.

Targeted inhibition of *kpsM* attenuates ethanol-induced liver disease

To further demonstrate a causative role of *kpsM* in the development of ethanol-induced steatohepatitis, we investigated the

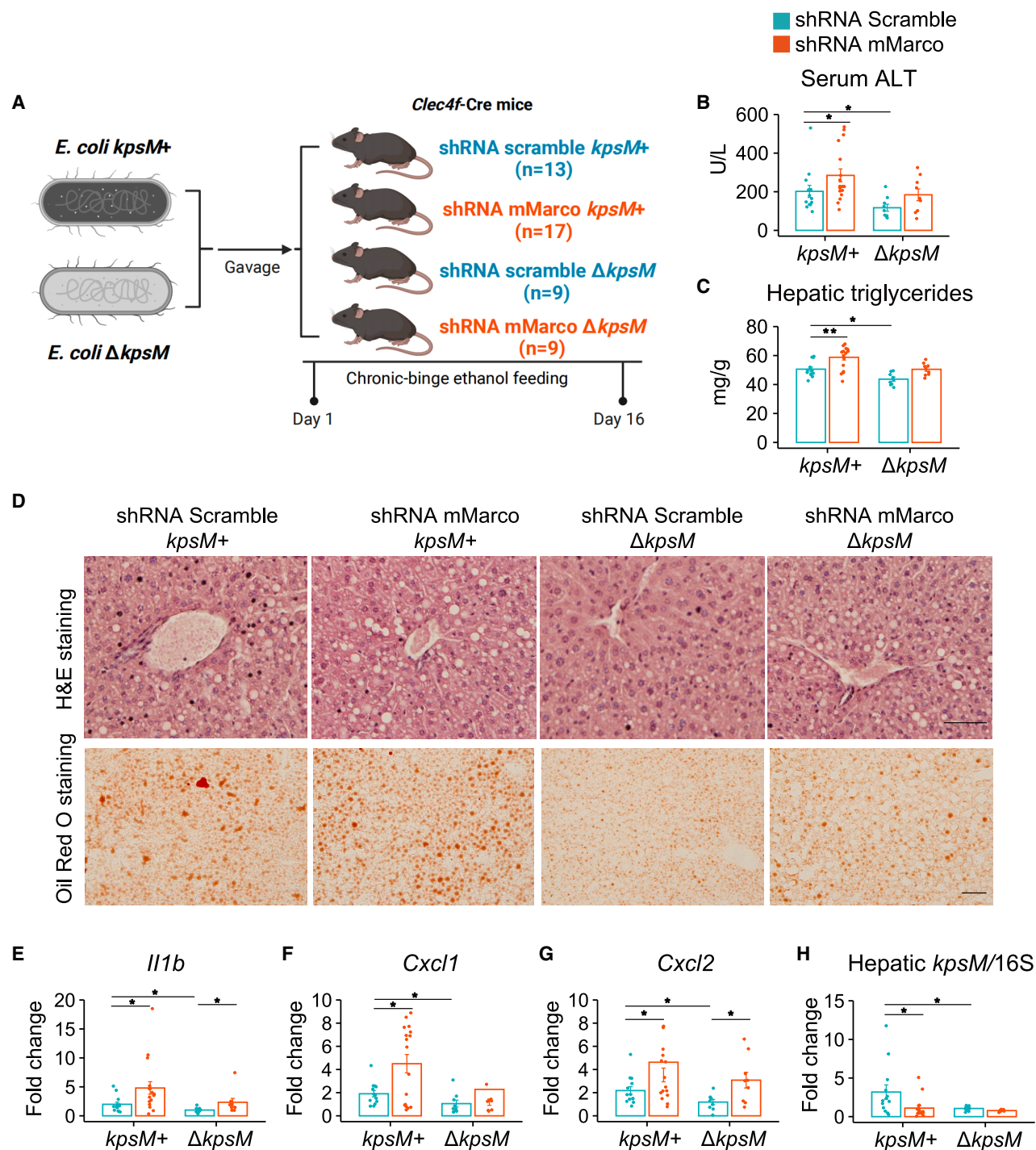


Figure 5. Marco protects against *kpsM*-positive-*E. coli*-exacerbated ethanol-induced liver disease

Clec4f-Cre mice were injected with AAV8-CAG-DIO-eGFP-mMarco-shRNAmir (1×10^{11} Gc/mouse) or AAV8-CAG-DIO-eGFP-scramb-shRNAmir as control (1×10^{11} Gc/mouse) into the tail vein. After 2 weeks, mice were fed oral chronic-binge ethanol diets and gavaged with 1×10^9 CFUs of *kpsM*-positive or $\Delta kpsM$ *E. coli* every 3 days and 1 h before the binge.

(A) Study overview.

(B) Serum levels of ALT.

(C) Hepatic triglyceride content.

(D) Representative images of liver sections stained with hematoxylin and eosin (upper) or with oil red O (lower).

(E–G) Hepatic levels of *Il1b*, *Cxcl1*, and *Cxcl2* mRNA.

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effects of the small molecule 2-(4-phenylphenyl)benzo[g]quinoline-4-carboxylic acid (C7), which blocks the assembly and translocation of the polysialic acid capsule in *kpsM*-positive *E. coli*.³⁴ C7 increased phagocytosis of *kpsM*-positive *E. coli* by Kupffer cells in culture and eliminated the difference in phagocytosis rate between *kpsM*-positive and $\Delta kpsM$ *E. coli* (Figures 6A and 6B). C7 also inhibited *kpsM*-positive *E. coli*-induced expression of inflammatory genes in Kupffer cells (Figures 6C–6E), likely through increased phagocytosis and subsequent elimination of *E. coli*, and decreased binding to pathogen recognition receptors on Kupffer cells. C7 did not affect the growth rate of *E. coli* in the absence of Kupffer cells (Figure 6F). Furthermore, C7 inhibited capsule formation in *kpsM*-positive *E. coli*, so both $\Delta kpsM$ *E. coli* and C7-treated *kpsM*-positive *E. coli* lacked an intact capsule structure (a capsule-deficient phenotype) (Figure 6G).

We next tested the efficacy of C7 as a therapeutic approach *in vivo*. Gnotobiotic mice were colonized with feces from *kpsM*-positive or *kpsM*-negative *E. coli* patients with alcohol-associated hepatitis and then orally gavaged with C7 (Figure 7A). C7 attenuated ethanol-induced steatohepatitis in mice colonized with feces from the *kpsM*-positive *E. coli* patients, as shown by decreased levels of ALT, reduced hepatic triglycerides, lower hepatic expression of *Ii1b* and *Cxcl1* mRNAs, and reduced hepatic levels of *kpsM*, compared with mice given vehicle (Figures 7B–7H). C7 did not ameliorate ethanol-induced steatohepatitis in mice colonized with feces from *kpsM*-negative *E. coli* patients (Figures 7B–7H). Hepatic levels of *F4/80* and *Marco* mRNA did not differ significantly between the different groups of ethanol-fed mice (Figures S8A and S8B). Intestinal absorption of ethanol and hepatic metabolism of ethanol were similar in all groups (Figures S8C–S8E). Abundance of fecal *E. coli* was similar in all mice, and the presence of fecal *kpsM* was confirmed in ethanol-fed mice colonized with *kpsM*-positive stool from patients with alcohol-associated hepatitis (Figures S8F and S8G). Ethanol-administered and C7-treated mice were gavaged with *kpsM*-positive *E. coli*, and Kupffer cells were isolated 2 h later. The number of translocated *kpsM*-positive *E. coli* recovered from Kupffer cells was significantly higher in mice treated with C7 as compared with control mice (Figure S8H). Our findings indicate that a small molecule inhibitor can selectively attenuate ethanol-induced liver disease caused by *kpsM*-positive *E. coli* in microbiota-humanized mice.

DISCUSSION

Our results provide insights into the pathogenesis and potential treatment of chronic alcohol-associated liver disease by precisely targeting a gut pathobiont. We show that the presence of virulence-related genes in the gut metagenome, and more specifically of the virulence factor KpsM encoded in the genome of *E. coli*, is independently associated with mortality in patients with alcohol-associated hepatitis, a non-communicable disease. The broad phylogenetic distribution of *kpsM* indicates that the

gene presence is a variable trait among *E. coli* isolates and that *kpsM*-positive *E. coli* are largely non-epidemic and capsule-dependent strains. This is also consistent with the low frequency of *kpsM* detection in fecal samples of patients with alcohol-associated hepatitis, as epidemic isolates have typically higher frequencies of virulence genes.³⁵ Liver failure as the main cause of death in our cohort, rather than classical infections by pathobionts, contributes to the association between mortality and the presence of these virulence factors in patients with alcohol-associated hepatitis.

The *kpsM* gene is predominantly found in *E. coli* strains with polysialic acid-rich capsules²⁸ and facilitates the transport of capsular polysaccharides to the cell surface.²⁷ Polysialic acid mimics the extracellular surface of mammalian cells²⁹ and enables translocated *kpsM*-positive *E. coli* to evade phagocytosis by Marco on Kupffer cells. Escaped bacteria might then activate pattern recognition receptor pathways in various cell types in the liver and thereby induce inflammatory mediators contributing to hepatocyte death and progression of liver disease. This mechanism leads to additional harm (second hit) to the liver beyond the damage caused by ethanol alone and could be mediated by toll-like receptor 4 recognizing lipopolysaccharides from Gram-negative *E. coli*. However, this notion needs to be confirmed in future experiments. We previously showed that *Vsig4*-deficient mice were less efficient than wild-type mice at clearing Gram-positive *E. faecalis* that translocated from gut to liver.³⁶ *Vsig4*-deficient mice developed more severe ethanol-induced liver disease than wild-type mice. Liver disease severity was reduced with loss of the pathogen recognition receptor toll-like receptor 2 (recognizing peptidoglycan in Gram-positive *E. faecalis*) in *Vsig4*-deficient mice.³⁶ Although *Vsig4* was not involved in eliminating Gram-negative *kpsM*-positive *E. coli*, we cannot rule out that other phagocytosis receptors are able to phagocytose *kpsM*-positive *E. coli*.

Several studies have identified Marco as a major receptor for the binding and uptake of unopsonized environmental particles and *E. coli*.^{37,38} However, *in vitro* studies have also demonstrated that Marco-positive macrophages are capable of mediating bacterial phagocytosis both in the presence of complement³² and in its absence.³⁹ The specific role of complement in Marco-mediated phagocytosis of *kpsM*-positive *E. coli* remains unclear and requires future studies.

The discovery that small molecules, such as C7, that inhibit *kpsM*-dependent transport of capsular polysaccharides to the capsule³⁴ can provide therapeutic benefits in models of alcohol-associated liver disease highlights an approach to treat a non-communicable condition. This precision-targeting strategy is significant given the limited and often inadequate treatment options for alcohol-associated liver disease, which frequently fail to address the underlying causes of disease progression.⁴⁰ Agents that target specific virulence-related microbial functions offer distinct advantages over conventional antibiotics, since they preserve the commensal microbiota and can avoid the selective

(H) Hepatic *kpsM* abundance normalized to bacterial 16S, as measured by qPCR.

The results are expressed as mean $\pm$ SEM. All experiments were generated from at least two technical replicates. *p* values were determined by Kruskal-Wallis test with Dunn's post hoc test followed by FDR procedures. **p* < 0.05, ***p* < 0.01. ALT, alanine aminotransferase. The exact group size (*n*) and *p* values for each comparison are listed in Table S7. Scale bars, 100 μ m.

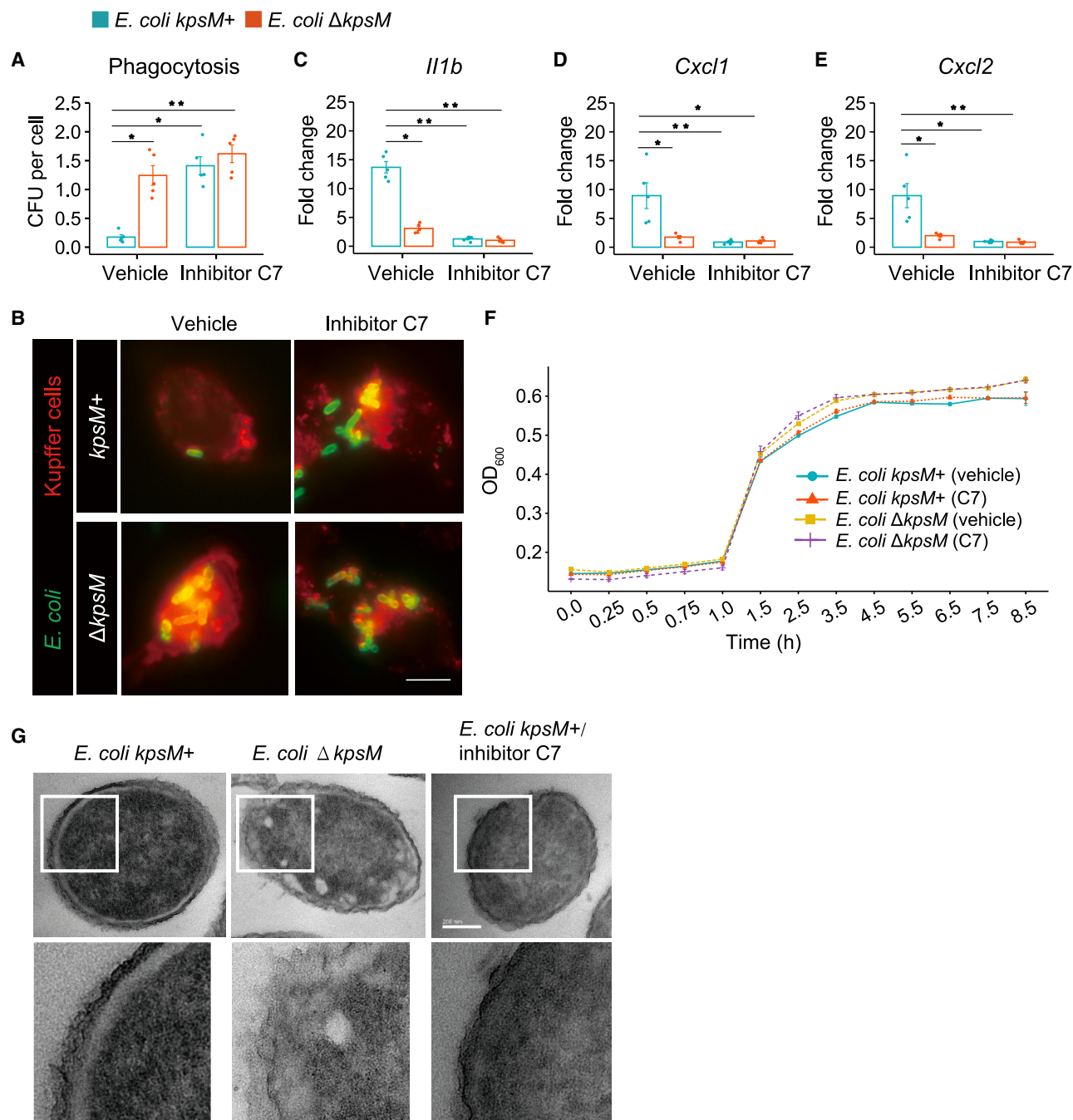


Figure 6. Small molecule C7 increases phagocytosis of *kpsM*-positive *E. coli* by Kupffer cells

(A) Primary mouse Kupffer cells were incubated with *kpsM*-positive or $\Delta kpsM$ *E. coli* in the presence or absence of C7 (25 μM) for 2 h. Phagocytosis of *E. coli* was assessed by intracellular survival of *E. coli*.

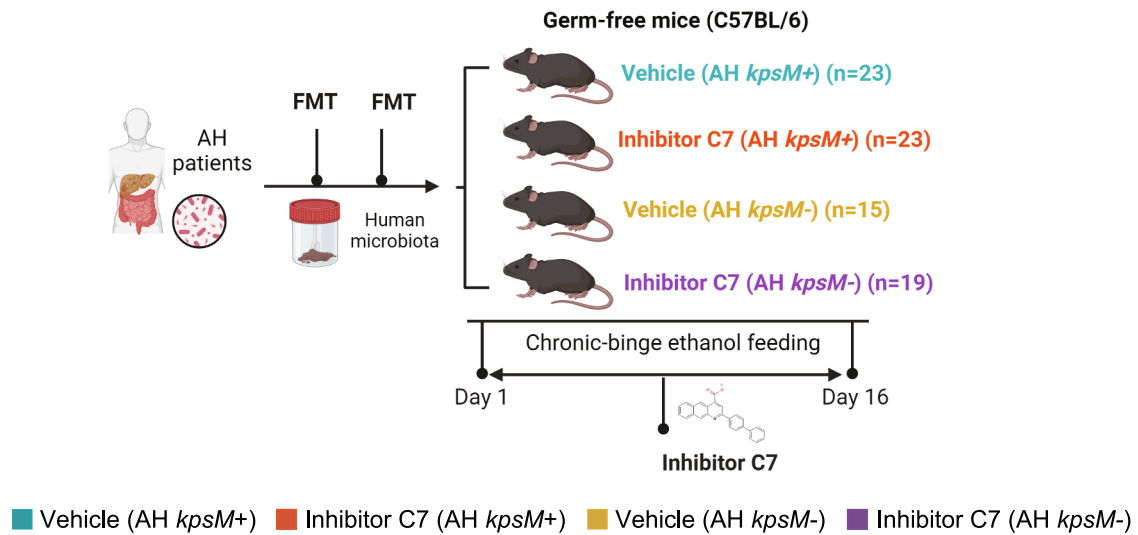
(B) Primary mouse Kupffer cells were incubated with *kpsM*-positive or $\Delta kpsM$ *E. coli* in the presence of an inhibitor of capsule formation, C7 (25 μM), or vehicle for 2 h. Confocal microscopy images of Kupffer cell phagocytosis of *E. coli*. Kupffer cells were stained with CellBrite cytoplasmic membrane dye (red), while *E. coli* were stained with CellBrite Fix Membrane Dye (green). Scale bar, 5 μm.

(C–E) Primary mouse Kupffer cells were incubated with *kpsM*-positive or $\Delta kpsM$ *E. coli* in the presence or absence of C7 (25 μM) for 12 h. Levels of *Il1b*, *Cxcl1*, and *Cxcl2* mRNAs in cultured Kupffer cells were assessed by qPCR.

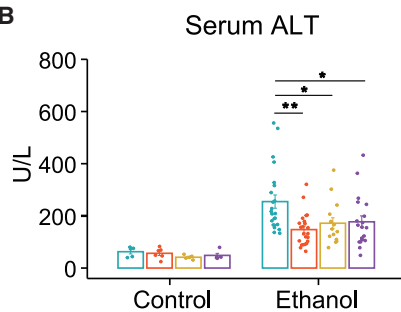
(F) Growth curve of *E. coli* strains in the presence or absence of C7 (25 μM).

(G) Transmission electron microscopy images of *E. coli* in the presence of C7 (25 μM) or vehicle. Scale bar, 200 nm. The results are expressed as mean ± SEM and generated from at least 2 technical replicates. *p* values were determined by Kruskal-Wallis test with Dunn's post hoc test followed by FDR procedures. **p* < 0.05, ***p* < 0.01. The exact group size (*n*) and *p* values for each comparison are listed in Table S7.

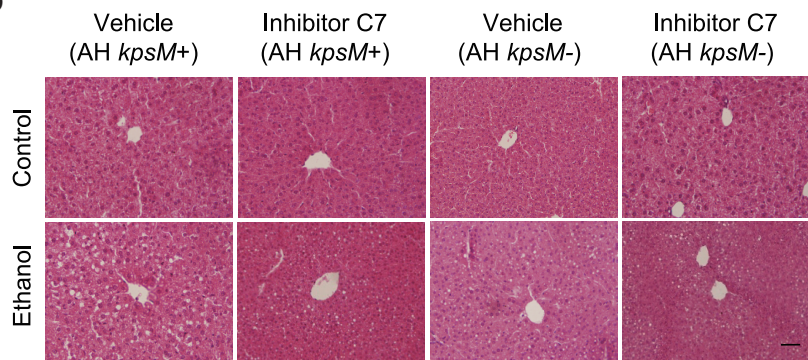
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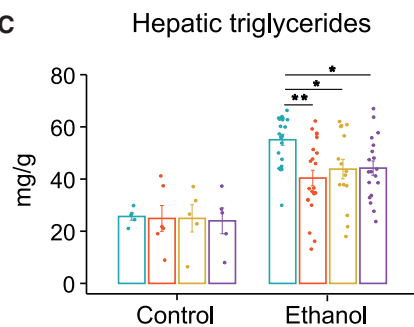
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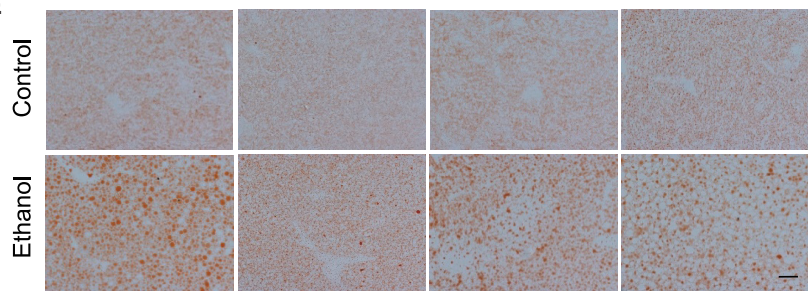
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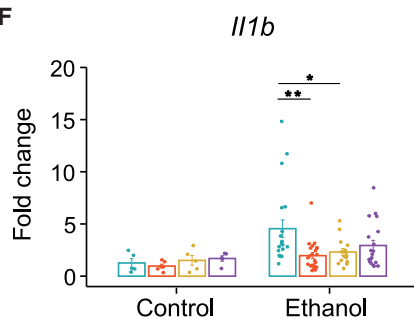
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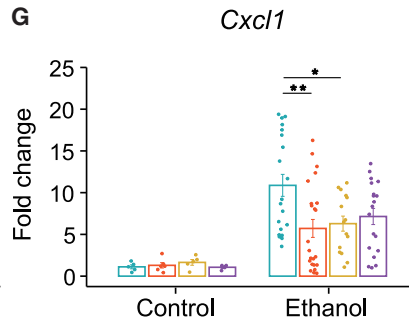
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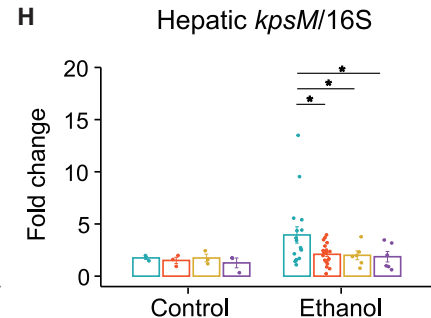
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pressures that lead to multidrug resistance and the emergence of virulence factors.⁴¹

A strength of this study is the inclusion of patients from an international multicenter observational trial, which reduced the chances that the virulence-related genes studied are region specific. However, translating these findings into clinical practice will require prospective clinical trials to evaluate the safety and efficacy of small-molecule inhibitors targeting *kpsM* in patients with alcohol-associated hepatitis. Given the strong association between *kpsM*-positive *E. coli* and increased mortality, our data support a precision medicine approach in which patients are screened for intestinal carriage of *kpsM*-positive *E. coli* prior to therapy. This targeted strategy could maximize therapeutic benefit while minimizing unnecessary exposure to novel agents in patients unlikely to respond. Nevertheless, it is important to note that a subset of *kpsM*-negative patients also experience poor outcomes, highlighting the heterogeneity of disease mechanisms in alcohol-associated hepatitis. As such, *kpsM* inhibition would be most appropriate as an adjunct or stratified therapy for those with confirmed *kpsM*-positive status, rather than a universal intervention.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bernd Schnabl (bschnabl@ucsd.edu).

Materials availability

Bacterial strains used in this study will be shared by the [lead contact](#) upon request.

Data and code availability

- Shotgun metagenomic sequencing data were deposited in the European Nucleotide Archive under accession numbers ERP106878. Culture-based whole-genome sequencing data were deposited in the NCBI database under BioProject accession number PRJNA1127827.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Y.Y. and Y.D. were responsible for animal studies, *in vitro* experiments, human fecal DNA extraction, interpretation of data, and writing of the manuscript. Y.Y. was responsible for metagenomic and culture-based whole-genome sequencing analysis. S.L. was responsible for human data analysis, interpretation of data, and writing of the manuscript. M.F.F., D.S., A.H., N.C., and S. C. provided assistance with experiments and were responsible for critical revision of the manuscript. Y.M. and L.E. provided assistance with gnotobiotic experiments and were responsible for critical revision of the manuscript. X.Z. provided assistance with statistical analysis and was responsible for critical revision of the manuscript. H.C., L.Y., and C.T. provided important resources for the study and were responsible for critical revision of the manuscript. F. B.-P., E.C.V., J.G.A., R.S.B., V.V., J.A., J.C., D.L.S., A.L., M.R.L., P.M., G. G.-T., R.B., and P.S. were responsible for the collection of human samples and critical revision of the manuscript. Y.S., K.V., and T.D.L. coordinated metagenomics sequencing data generation and processing and provided feedback on the manuscript. B.S. was responsible for the concept, design, and supervision of the study and writing of the manuscript.

DECLARATION OF INTERESTS

K.V. is currently an employee of the company Novartis. V.V. is on the advisory board for Ipsen and receives speaker fees from Orphan. B.S. has been consulting for Ambys Medicines, Boehringer Ingelheim, Ferring Research Institute, Gelesis, HOST Therapeutics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals, Surrozen, and Takeda. B.S.'s institution, UC San Diego, has received research support from Axial Biotherapeutics, BiomX, ChromoLogic, CymaBay Therapeutics, Intercept, NGM Biopharmaceuticals, Prodigy Biotech, and Synlogic Operating Company. B.S. is the founder of Nterica Bio. UC San Diego has filed several patents with Y.D., S.L., and B.S. as inventors related to this work.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)

Figure 7. Small-molecule-C7 targeting of *kpsM*-positive *E. coli* reduces ethanol-induced liver disease in gnotobiotic mice

Germ-free C57BL/6 mice were colonized with stool from *E. coli kpsM*-positive (AH *kpsM*+) or *E. coli kpsM*-negative (AH *kpsM*-) patients with alcohol-associated hepatitis (AH) and fed oral isocaloric (control) or chronic-binge ethanol diets. Mice were gavaged with C7 (1 mg/kg body weight) every third day and 1 h before the binge.

(A) Study overview.

(B) Serum levels of ALT.

(C) Hepatic triglyceride content.

(D) Representative images of liver sections stained with hematoxylin and eosin.

(E) Representative images of liver sections stained with oil red O.

(F and G) Hepatic levels of *Irf1b* and *Cxcl1* mRNAs.

(H) Hepatic *kpsM* abundance normalized to bacterial 16S, as measured by qPCR.

The results are expressed as mean ± SEM and generated from at least 2 technical replicates. *p* values were determined by one-way ANOVA with Tukey's post hoc test. **p* < 0.05, ***p* < 0.01. ALT, alanine aminotransferase. AH, alcohol-associated hepatitis. FMT, fecal microbiota transplantation. The exact group size (*n*) and *p* values for each comparison are listed in [Table S7](#). Scale bars, 100 μm.

- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
 - Human subjects
 - Mice
- **METHOD DETAILS**
 - Shotgun metagenomic sequencing and analysis
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 - Statistical analysis

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>kpsM</i> -positive <i>Escherichia coli</i>	Zong et al. ²⁶	PCN033
$\Delta kpsM$ <i>E. coli</i>	Zong et al. ²⁶	PCN033
<i>E. coli</i> clinical isolates	Schnabl lab	This study
AAV8-CAG-DIO-eGFP-mMarco-shRNAmir	Vector Biolabs	NA
AAV8-CAG-DIO-eGFP-scrmb-shRNAmir	Vector Biolabs	NA
Biological samples		
Stool samples from human cohorts	Schnabl lab	This study
Chemicals, peptides, and recombinant proteins		
Luria-Bertani broth	Sigma	Cat# L3397-250G
Agar	BD	Cat# 214010
RPMI-1640	Thermo Fisher Scientific	Cat# 11875093
FBS (heat inactivated)	CoreBio	Cat# FB-02
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# 15140122
Gentamicin solution	Sigma	Cat# G1397-10
DPBS, no calcium, no magnesium	Gibco™	Cat# 14190144
CHROMagar™ <i>E. coli</i>	Thermo Fisher Scientific	Cat# NC9755621
Ethanol	VWR	Cat# 89125-188
DNase I	Sigma	Cat# 10104159001
Proteinase K	Thermo Fisher Scientific	Cat# DB0451
RNaseA	Qiagen	Cat# 19101
UltraPure Buffer-Saturated Phenol	Invitrogen	Cat# 15513039
UltraPure Phenol:Chloroform:Isoamyl alcohol	Thermo Fisher Scientific	Cat# 15593031
Collagenase D	Sigma	Cat# 11088882001
Pronase	Roche	Cat# 10165921001
Nycodenz	Thermo Fisher Scientific	Cat# NC1229914
Small molecule C7	NIH/NCI	NSC136469
Recombinant mouse Marco	Sino Biological	Cat# 50283-M07H
Critical commercial assays		
TRIZOL	Thermo Fisher Scientific	Cat# 15596-018
ALT (SGPT) Kinetic	TECO Diagnostics	Cat# A524-150
Ethanol Colorimetric/Fluorometric Assay Kit	Abcam	Cat# ab65343
Mouse Troponin T Type 2 (Cardiac) ELISA Kit	Biomatik	Cat# EKF58659-96T
Triglycerides Liquid, Reagent Set, 1x120 ml	Pointe Scientific Inc.	Cat# T7532-120
iTaq UniverSYBR Green SMX 1000	Core Bio service	Cat# 1725124
High Capacity cDNA reverse Transcription Kit	Invitrogen (ABI)	Cat# 4368814
Lipofectamine 3000 Transfection Reagent	Thermo Fisher Scientific	Cat# L3000008
CellBrite Fix Membrane Dye	Biotium	Cat# 30090-T
CellBrite cytoplasmic membrane dye	Biotium	Cat# 30023
Alexa Fluor 647 labeling kit	Thermo Fisher Scientific	Cat# A20186
FastDNA Spin Kit for Soil	MP Biomedicals	Cat# 116560200
QIAamp DNA microbiome Kit	Qiagen	Cat# 51704
QIAamp Fast DNA Stool Mini Kit	Qiagen	Cat# 51604

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Shotgun metagenomic sequencing data	European Nucleotide Archive	ERP106878
Whole-genome sequencing data	NCBI	PRJNA1127827
Experimental models: Organisms/strains		
Mouse: C57BL/6	Charles River	N/A
Mouse: germ-free C57BL/6	UC San Diego	N/A
Mouse: <i>Clec4f</i> -Cre mice	Sakai et al. ⁴²	N/A
Mouse: <i>Vsig4</i> mice	Helmy et al. ³³	N/A
Oligonucleotides		
ON-TARGETplus Mouse Marco	Horizon	Cat# L-043847-00-0005
ON-TARGETplus Non-targeting Pool	Horizon	Cat# D-001810-10-05
Software and algorithms		
MetaPhlAn v3.1 ⁴³	Github	https://github.com/biobakery/MetaPhlAn
Kraken2 v2.1.2 ⁴⁴	Github	https://github.com/DerrickWood/kraken2
KneadData v0.7.5	Github	https://github.com/biobakery/kneaddata
RAxML v8.2.10 ⁴⁵	Github	https://github.com/stamatak/standard-RAxML
StrainPhlAn ⁴⁶	Github	https://github.com/biobakery/MetaPhlAn
ARIBA ⁴⁷	Github	https://github.com/sanger-pathogens/ariba
FASTQC	Github	https://github.com/s-andrews/FastQC
Trimmomatic ⁴⁸	Github	https://github.com/usadellab/Trimmomatic
ABRicate v0.5	Github	https://github.com/tseemann/abricate
SPAdes v3.15.3 ⁴⁹	Github	https://github.com/ablab/spades
Prodigal v2.6 ⁵⁰	Github	https://github.com/hyatt/Prodigal
Prokka v1.13.3 ⁵¹	Github	https://github.com/tseemann/prokka
Roary v3.12 ⁵²	Github	https://github.com/sanger-pathogens/Roary
SNP-sites ⁵³	Github	https://github.com/sanger-pathogens/snp-sites
ITOL ⁵⁴	NA	https://itol.embl.de/
R	CRAN	https://www.R-project.org
FlowJo	Treestar	RRID:SCR_000410
GraphPad Prism v8.2	GraphPad Software, LLC	https://www.graphpad.com/scientific-software/prism/
BioRender	BioRender	https://www.biorender.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**Human subjects**

Our study comprised a total of 180 subjects: 130 patients with alcohol-associated hepatitis (81 for metagenomics analysis, 49 for qPCR validation), 41 patients with alcohol use disorder, and 9 individuals who do not misuse alcohol (controls). These cohorts have been described in detail.^{55–57} Demographic information is provided in [Tables S1](#) and [S5](#).

Patients with alcohol-associated hepatitis were hospitalized in 12 centers from 8 regions (France, n=24; Mexico, n=7; Spain, n=14; United Kingdom, n=19; US East, n=27; US Midwest, n=18; US West, n=15; Canada n=6) that were part of the InTeam Consortium ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier number: NCT02075918). For patients with alcohol-associated hepatitis, samples were collected during the hospital stay, mainly within the first days of admission (median number of days between admission and sampling was 3 days, [Tables S2](#) and [S5](#)). Inclusion criteria for patients with alcohol-associated hepatitis were active alcohol abuse (>50 g/day for men and >40 g/day for women) in the past 3 months; level of aspartate aminotransferase (AST) greater than level of alanine aminotransferase (ALT), and total bilirubin >3 mg/dl in the past 3 months; liver biopsy and/or clinical picture consistent with alcohol-associated hepatitis. Exclusion criteria were autoimmune liver disease (ANA >1/320), chronic viral hepatitis, hepatocellular carcinoma, complete portal vein thrombosis, extra-hepatic terminal disease, pregnancy, and lack of signed informed consent. In all patients, the clinical picture was consistent with alcohol-associated hepatitis and in patients who underwent liver biopsy, histology findings supported the

diagnosis of alcohol-associated hepatitis. Liver biopsies were only collected if indicated, as part of routine clinical care for diagnostic purposes of alcohol-associated hepatitis. The number of patients treated with antibiotics is indicated in [Tables S2](#) and [S5](#).

Patients with alcohol use disorder were recruited from an alcohol treatment program in San Diego, US (n=10) and Brussels, Belgium (n=31). Fecal samples were collected at the time point of the admission. Non-alcoholic controls were social drinkers who consumed alcohol less than 20 g/day as one inclusion criteria and were enrolled in Brussels, Belgium. Patients with alcohol use disorder fulfilled the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV criteria)⁵⁸ of alcohol dependence with active alcohol consumption (self-reported > 60 g/day for more than one year). Patients with alcohol use disorder and non-alcoholic controls did not take antibiotics or immunosuppressive medications in the 2 months preceding enrollment. A complete medication and medical history were collected at admission. Biospecimens, basic demographic data (such as age, gender, weight, and height), and self-reported daily alcohol consumption were collected and patients underwent a physical examination. Other exclusion criteria were diabetes, inflammatory bowel diseases, liver diseases of any other etiology, and clinically significant cardiovascular, pulmonary, or renal comorbidities. The protocol was approved by the ethics committee of each participating center, and patients were enrolled after written informed consent was obtained.

Mice

Germ-free, female C57BL/6 mice, were bred at UC San Diego as described before.⁵⁹ Germ-free status of the animals was routinely confirmed by sampling feces on blood agars and incubation for up to 2 days at 37°C under both aerobic (room air) and anaerobic (AnaeroPack System, Mitsubishi) conditions. Germ-free mice were colonized twice with fecal homogenates from patients diagnosed with alcohol-associated hepatitis. In brief, stool samples (1 g) were suspended in 30 mL LB broth containing 15% glycerol under anaerobic conditions. Subsequently, mice were orally gavaged with 100 μ L of the stool suspensions, initiating at 4–5 weeks of age and repeating the process two weeks later. After the second gavage, mice were subjected to a chronic–binge ethanol diet (NIAAA model)⁶⁰ or an isocaloric control diet. Mice were fed the Lieber DeCarli diet, in which the caloric intake from ethanol was 0% on days 1–5 and 36% (v/v) from day 6 until the end of the study period. At day 16, mice were gavaged with a single dose of ethanol (5 g/kg body weight) in the early morning, followed by sacrifice 9 hrs later. Pair-fed control mice received a diet with an isocaloric substitution with maltodextrin. For inhibiting *kpsM* in *E. coli*, mice were gavaged with the small molecule C7 (2-(4-phenylphenyl)-benzo[g]quinoline-4-carboxylic acid, NSC136469, 1 mg/kg body weight; kindly provided by the NIH/NCI Developmental Therapeutic Program)³⁴ every third day and 1 h before the binge.

To colonize conventional mice with $\Delta kpsM$ or isogenic *kpsM*-positive *E. coli*, clinical *kpsM*-positive and *kpsM*-negative *E. coli* isolates, wild-type C57BL/6 mice (female, age 8–10 weeks, Charles River) were subjected to the chronic–binge ethanol diet (NIAAA model)⁶⁰ or an isocaloric control diet, and gavaged with *E. coli* (1×10^9 CFUs) every third day and 1 hr before the binge.

To knockdown Marco in Kupffer cells *in vivo*, mice with Cre expression under the Kupffer cell specific promoter *Clec4f* (*Clec4f*-Cre mice⁴²; female, age 8–10 weeks) were injected with AAV8-CAG-DIO-eGFP-mMarco-shRNAir (Vector Biolabs; 1×10^{11} Gc/mouse in 100 μ L) or AAV8-CAG-DIO-eGFP-scrmb-shRNAir as control (Vector Biolabs; 1×10^{11} Gc/mouse in 100 μ L) into the tail vein. Injection in the lateral vein of the tail in mice is an efficient strategy to administer viral vectors to the liver.⁶¹ Serotype 8 of AAV has a high hepatic tropism (90–95% of infection rate following tail vein injection).⁶² AAV8 expresses double-floxed inverse ORF (DIO)-eGFP-shRNA driven by an ubiquitous CAG promoter. In the DIO scenario, scrambled shRNA or shRNA for silencing mouse *Marco*, is expressed in the presence of Cre in Kupffer cells. The transgene is not expressed in the absence of Cre. After 2 weeks, mice were subjected to the chronic–binge ethanol diet described above. Mice were gavaged with *E. coli* (1×10^9 CFUs) every 3 days and 1 h before the binge.

To study bacterial translocation from the gut to other organs in the short term before the bacteria are cleared *in vivo*, mice were subjected to the chronic–binge ethanol diet (NIAAA model).⁶⁰ One hour after the ethanol binge, mice were gavaged with either $\Delta kpsM$ or *kpsM*-positive *E. coli* (1×10^9 CFUs). Mice were euthanized after 30 min. Organs were collected, homogenized with PBS, plated on CHROMagar *E. coli*-selective agar plates and incubated for 24 h at 37°C.

To study phagocytosis of *E. coli* by Kupffer cells *in vivo*, wild-type C57BL/6, C57BL/6 mice treated with C7, and *Vsig4*^{-/-} mice³³ (male and female) received a single dose of ethanol (5 g/kg body weight). One hour after the ethanol binge, mice were gavaged with *E. coli* (1×10^9 CFUs). Mice were euthanized after 2 h and Kupffer cells isolated. Cell lysate was collected, and cultured on CHROMagar *E. coli*-selective plates and incubated for 24 h at 37°C.

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

METHOD DETAILS

Shotgun metagenomic sequencing and analysis

DNA was extracted from human stool samples using FastDNA Spin Kit for Soil (MP Biomedicals). Whole-genome shotgun metagenomic sequencing was performed on Illumina HiSeq 4000 generating 150 bp pair-end reads. Quality control of shotgun metagenomics reads was performed as described previously⁶³ using a combination of KneadData v0.7.5. Taxonomic classification of processed metagenomics reads were performed using two methods of MetaPhlAn v3.1,⁴³ and Kraken2 v2.1.2⁴⁴ against genomes of the Human gastrointestinal Bacteria Genome Collection.⁶⁴ The maximum-likelihood phylogenetic trees of *E. coli* shown in [Figures S2A](#) and [2B](#) were built by applying RAxML v8.2.10⁴⁵ (internally in StrainPhlAn⁴⁶) on the alignment of *E. coli* marker genes, using default

parameters and with the options ‘-alignment_program mafft’ and ‘-relaxed_parameters3’. The major lineages were taxonomically assigned to GTDB⁶⁵ species clusters using GTDB r89 representative genomes. Quality-filtered metagenomic reads were screened for the presence of genes encoding for bacterial virulence factors using ARIBA⁴⁷ with “-min_scaff_depth 1”, and otherwise default parameters. As previously described,⁶³ VFDB database⁶⁶ core virulence genes were grouped into clusters based on $\geq 90\%$ nucleotide identity, and then included to build a BLAST database for virulence-factor screening. The detected virulence genes were grouped by their host genera as found in VFDB. All potential host genera are shown (e.g. *Klebsiella/Yersinia* in Figure 1C) if the virulence factor gene cluster was found in multiple genera.

Whole-genome sequencing and genotyping

E. coli strains were isolated from patients’ stool samples using CHROMagar *E. coli* selective medium (CHROMagar) and cultured in LB broth medium (Sigma). Genomic DNA was extracted using the QIAamp DNA microbiome Kit (Qiagen). DNA libraries were constructed with 100-bp paired-end fragments and sequenced using an Illumina NovaSeq S4 platform. The quality of the original reads was evaluated using FASTQC. FASTX-trimmer and Trimmomatic were used for trimming sequencing reads.⁴⁸ Short-read sequence data were *de novo* assembled using SPAdes v3.15.3.⁴⁹ Acquired antimicrobial resistance genes and virulence genes were identified using ABRicate v0.5 (<https://github.com/tseemann/abricate>) by aligning genome sequences to the ResFinder database⁶⁷ and VFDB database⁶⁶ with 80% coverage and 80% identity. For each *de novo* assembly, coding sequences were predicted using Prodigal v2.6⁵⁰ and annotated using Prokka v1.13.3.⁵¹ The core genes were identified and used to build the core genome using Roary v3.12⁵² with the -e -mafft setting to create a concatenated alignment of core genomic CDS. SNP-sites was used to extract the core genomic SNPs.⁵³ To construct a maximum likelihood phylogeny of the sequencing isolates, RAXML v8.2.10 was used with the generalized time-reversible model and a GTRGAMMA distribution to model site-specific rate variation.⁴⁵ Support for the ML phylogeny was assessed by 1,000 bootstrap pseudo-analyses of the alignment data. iTOL⁵⁴ was used to visualize and edit the phylogenetic tree. To explore the prevalence of virulence factors in *E. coli*, available *E. coli* assembled genomes were retrieved from RefSeq databases as of January 1, 2022. Exclusion criteria for all the data were as follows: sequencing performed on non-Illumina platforms; single-end reads; sequencing depth of <30 x, >300 assembled contigs, or abnormal genome lengths.

Fecal qPCR for *kpsM*

For DNA extraction, 250 mg stool were placed in 2 mL screw-cap tubes. 500 μ L of InhibitEX Buffer (Qiagen) were added to each stool sample and maintained on ice. Samples were homogenized using a bead beater at 2,000 rpm for 2 cycles of 30 s each. Subsequently, genomic DNA from human stool was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen) following the provided instructions.⁶⁸ *kpsM*-positive *E. coli* and $\Delta kpsM$ *E. coli* strains served as positive and negative controls, respectively, while nuclease-free water served as a no-template control. The qPCR reaction mix consisted of 10 μ L SYBR Green master mix, 1 μ L of a 10 μ M primer mix (forward and reverse), and 9 μ L of DNA at a concentration of 10 ng/ μ L. The qPCR was performed as previously described.⁶⁸ Once amplification was complete, Ct (cycle threshold) values were calculated for each reaction. A cut-off Ct value of <30 was applied to indicate the presence of *kpsM*. Primer sequences are provided in Table S6.

Blood cultures

Wild-type C57BL/6 mice received a single dose of ethanol (5 g/kg body weight). One hour after the ethanol binge, mice were gavaged with either $\Delta kpsM$ or *kpsM*-positive *E. coli* (1×10^9 CFUs). The mice were sacrificed, and whole blood was collected 30 min after gavage. A total of 400 μ L of blood was cultured in 20 mL of LB broth for 24 h, followed by plating on CHROMagar *E. coli*-selective plates and incubation at 37°C for an additional 24 h.

Biochemical and histological analyses

Blood was collected from mice in 2 mL collection tubes (Greiner Bio-One, Germany) and serum was obtained through centrifugation at 3,000 $\times$ g for 20 min. Serum levels of alanine transaminase (ALT) were determined using ALT liquid reagent kit (Teco Diagnostics) to assess hepatic injury. Lipids were extracted from the liver as described.⁶⁹ Hepatic triglyceride levels were measured according to manufacturer’s instructions using Triglyceride Liquid Reagents kit (Pointe Scientific). For analyses of alcohol absorption and hepatic metabolism in mice, serum ethanol levels were determined using the Ethanol Colorimetric Assay kit (Abcam) according to the manufacturer’s protocol. Cardiac troponin T in serum was measured by ELISA (Biomatik).

Formalin-fixed liver samples were embedded in paraffin (Paraplast plus, McCornick) and 5 μ m sections were stained with hematoxylin-eosin (H&E) (Surgipath) for liver morphology. For frozen sections, liver samples were embedded in OCT compound, and 5 μ m sections were stained with Oil Red O (Sigma-Aldrich) to visualize lipid accumulation.

RNA extraction and RT-qPCR

To isolate liver RNA, approximately 50 mg tissue pieces from the left lateral liver lobes were snap frozen in liquid nitrogen. Frozen tissue was homogenized mechanically using stainless steel beads in a Tissue Lyser II (Qiagen). RNA extraction was performed using Trizol reagent (Life Technologies). For RNA isolation from cultured Kupffer cells, cells were harvested by adding 1 mL Trizol reagent to each well and employing the same procedure as for liver RNA isolation. RNA content and quality were assessed using Nanodrop (Peglab). For quantitative real-time PCR, up to 1 μ g of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) to generate cDNA. Real-time PCR was performed on an ABI StepOnePlus real-time PCR system

(Bio-Rad Laboratories) using the SYBR green master mix (Bio-Rad Laboratories). Gene expression was normalized to mouse 18S rRNA. Expression of target genes in relation to reference gene was determined using the $2^{-\Delta\Delta CT}$ method. Primer sequences for mouse genes were originally obtained from the NIH qPrimerDepot. Primer sequences are listed in Table S6.

DNA extraction from sterile mouse liver and qPCR

For bacterial DNA extraction from sterile liver, right lobes of the mouse liver were dissected under sterile conditions. Genomic DNA was isolated to quantify bacterial 16S as previously described with slight modifications.⁷⁰ Briefly, the tissue was weighed and homogenized using a bead beater at 2,000 rpm for 2 cycles of 30 s each, with 1.0 mm Zirconia/Silica beads in PBST (PBS with 0.05% Tween 20) supplemented with Proteinase K (20 mg/mL), and digested with RNaseA (100 mg/mL) and 10% SDS. The suspensions were then transferred to Qbiogene lysing matrix B tubes and homogenized (2,000 rpm for 2 cycles of 30 s each) with UltraPure Buffer-Saturated Phenol (Thermo Fisher Scientific). Lysate was extracted twice with UltraPure Phenol:Chloroform:Isoamyl alcohol (Thermo Fisher Scientific) and once with chloroform and sodium acetate buffer. DNA was then precipitated, washed with ethanol, and resuspended in sterile water. Relative expression of *kpsM* was normalized to 16S rRNA. Primer sequences including 16S rRNA⁷¹ and *kpsM*⁷² are provided in Table S6.

Isolation of primary mouse Kupffer cells

Kupffer cells were isolated as described.³⁶ In brief, the liver of C57BL/6 mice (age 12 weeks; Charles River) was perfused in situ for 5 min at 10 mL/min with perfusion buffer SC1, followed by a 2-step collagenase-pronase perfusion (Roche Diagnostics) at 10 mL/min for 5 min in anesthetized mice. The liver was subsequently dissected from the mouse and ruptured with forceps in buffer D containing collagenase D and pronase, followed by 20 min incubation at 37 °C. The cells were filtered through a 70 μ m cell strainer and centrifuged at 800 x g for 7 min. Supernatant was resuspended with GBSS-B. Kupffer cells were obtained by a 3-layer discontinuous density gradient centrifugation with 8.2% (w/v) and 14.5% (w/v) Nycodenz (Accurate Chemical and Scientific Corporation) centrifugation at 2,000 x g for 20 min without brake. Isolated cells were counted and plated in 6-well or 12-well plates in RPMI 1640 (Thermo Fisher Scientific) containing 10% (v/v) FBS and 1% penicillin-streptomycin. After 30 min attachment, the cell medium was changed and cultured cells were incubated at 37 °C.

Phagocytosis and intracellular survival of *E. coli* within Kupffer cells

The intracellular survival of bacteria within macrophages was assessed following a previously described method with slight modifications.⁷³ Kupffer cells were plated at a density of 5×10^5 cells per well (12-well plate) in RPMI 1640 medium. On the day of co-culture, the medium was replaced with antibiotic-free RPMI 1640, and cells were infected with freshly cultured *E. coli* strains at a multiplicity of infection (MOI) of 30:1 for 2 h at 37 °C. Then a mixture of gentamicin (200 μ g/mL), streptomycin (500 μ g/mL), and chloramphenicol (100 μ g/mL) were added to the medium to eliminate extracellular bacteria for 1 hr, followed by 3 PBS washes. Cells were then lysed with 0.1% Triton X-100 (Sigma) and cell lysates were diluted and plated on LB agar plates. Bacterial colonies were counted on the next day.

siRNA mediated *in vitro* knockdown of Marco

Three hours after isolation, primary mouse Kupffer cells were transfected with either control siRNA (ON-TARGETplus Non-targeting Control Pool, Horizon) or mouse Marco siRNA (ON-TARGETplus siRNA, Horizon) using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.⁷⁴ After 24 h, cells were harvested for RNA extraction and qPCR to detect gene expression of Marco.

Flow cytometry of *E. coli* binding to Marco

Freshly cultured and pelleted *E. coli* were resuspended in 1 mL of PBS with 1x 488 CellBrite Fix Membrane Dye (Biotium; green, Ex/Em: 480/513 nm) to stain bacterial outer membrane and incubated for 30 min at room temperature. After centrifugation at 12,000 x g for 5 min, the pellet was washed thrice with PBS. Recombinant mouse Marco (Sino Biological) was labeled with AF 647 using a protein labeling kit according to the manufacturer's instructions (Thermo Fisher Scientific, Ex/Em: 650/668 nm). AF 647-labeled recombinant Marco protein (20 μ g/mL) was incubated with *E. coli* (1×10^6 CFUs) at room temperature for 2 hrs. Bacterial cells were then washed three times, resuspended in fixation buffer (1% paraformaldehyde) for 30 min, and subjected to flow cytometry⁷⁵ and analyzed with FlowJo.

Transmission electron microscopy

E. coli strains were cultured overnight in LB broth with or without adding the small molecule C7.³⁴ Cultures were centrifuged at 12,000 x g for 5 min and bacterial pellets were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), followed by post-fixation with 1% osmium tetroxide in cacodylate buffer (0.1 M) for 1 hr on ice. The cells were stained with 2% uranyl acetate for 1 hr and dehydrated in ethanol (50–100%) on ice. Cells were washed with 100% ethanol for 10 min, followed by two times with acetone, and embedded with Durcupan. Sections were cut at 60 nm using a Leica UCT ultramicrotome and placed on 300 mesh copper grids. The sections were post-stained with 2% uranyl acetate for 5 min and Sato's lead stain for 1 min. Images were obtained using a Jeol 1400 plus transmission electron microscope equipped with a bottom-mount Gatan digital camera at the Cellular and Molecular Medicine Electron Microscopy Core at the University of California San Diego.

Confocal and super-resolution imaging

Approximately 5×10^4 primary Kupffer cells were plated in μ -slide 8 well dishes with glass bottom (Ibidi). Kupffer cells were labeled 30 min at 37 °C with a CellBrite cytoplasmic membrane dye (Biotium, red, Ex/Em: 644/665 nm), a lipophilic carbocyanine dye based on 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD). Cells were washed 3 times with PBS. *E. coli* strains were freshly cultured overnight, centrifuged, and resuspended in PBS. Bacteria were stained with 488 CellBrite Fix Membrane Dye (Biotium, green) for 30 min at room temperature. After staining, the bacteria were washed 3 times with PBS and co-cultured with Kupffer cells for 1 h. Extracellular bacteria were removed by washing 3 times with PBS. Live or fixed (with 4% para-formaldehyde) cells were imaged using a Leica SP8 confocal microscope and analyzed using ImageJ.⁷⁶ For super-resolution imaging, images were collected with a Zeiss Elyra 7 Lattice SIM super-resolution instrument using a Plan Apochromatic 63x (1.40 NA) oil objective lens. Z-stack images were collected in Leap-mode with 10 phase positions of the lattice SIM pattern. Images were processed with Zeiss Zen Black SIM2 processing software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

For comparison of three or more groups, the Kruskal-Wallis test was used with Dunn's post-hoc test for continuous and Fisher's exact test for categorical variables, each followed by false discovery rate (FDR) procedures to correct for multiple comparisons. We used univariate and multivariate Cox regression analysis to assess associations of virulence factors with 180-day mortality. The multivariate model was adjusted for treatment with antibiotics and the sequence batch. The 2-sided Wald test was used to determine the *P* value. Kaplan-Meier curves were used to compare survival between virulence factor-positive and virulence factor-negative patients with alcohol-associated hepatitis. The 2-sided Log-rank (Mantel-Cox) test was used to compare the two groups. For one patient who underwent liver transplantation, the transplantation date was considered as the date of death. Patients were censored at the time point they were last seen alive. Spearman's rank correlation coefficient was used to investigate associations between 2 continuous variables. To investigate gut microbiota profiles among patients with alcohol-associated hepatitis that were either positive or negative for specific virulence factors, the logarithmic relative abundance of all intestinal bacteria was calculated at the genus level. Jaccard dissimilarity matrices were determined for the principal component analysis (PCA) and *P* values were determined by permutational multivariate analysis of variance (PERMANOVA). Statistical analyses were performed using R statistical software, R v3.5.1, 2018 the R Foundation for Statistical Computing. *P* < 0.05 was considered to be statistically significant. All statistical tests were 2-sided.

For mouse or cell culture experiments, results are expressed as mean $\pm$ SEM unless stated otherwise. Two groups were compared using the Mann-Whitney-Wilcoxon rank-sum test for continuous and Fisher's exact test or Chi-square test for categorical variables. For comparison of three or more groups, 1-way Analysis of Variance (ANOVA) with Tukey's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test followed by FDR procedures was used. Statistical analyses were performed in GraphPad Prism v8.2.