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The *Candida albicans* exotoxin Candidalysin promotes alcohol-associated liver disease

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Abstract

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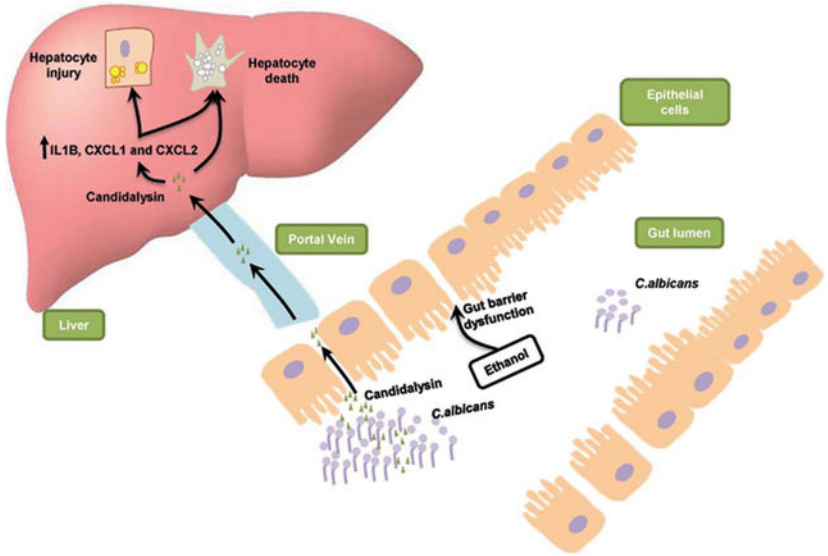
Background and Aims: Alcohol-associated liver disease is a leading indication for liver transplantation and leading cause of mortality. Alterations of the gut microbiota contribute to pathogenesis of alcohol-associated liver disease. Patients with alcohol-associated liver disease have increased proportions of *Candida* spp. in the fecal mycobiome. However, little is known about the effect of intestinal *Candida* on alcohol-associated liver disease. Here we evaluated the contributions of *Candida albicans* and its exotoxin Candidalysin on alcoholic liver disease.

Methods: *C. albicans* and *ECE1* were analyzed in fecal samples from controls, patients with alcohol use disorder and alcoholic hepatitis. Mice colonized with different and genetically manipulated *C. albicans* strains were subjected to the chronic-plus-binge ethanol diet model. Primary hepatocytes were isolated and incubated with Candidalysin.

Results: The percentages of subjects carrying *ECE1* are 0%, 4.76% and 30.77% in non-alcoholic controls, alcohol use disorder patients and alcoholic hepatitis patients, respectively. Candidalysin exacerbates ethanol-induced liver disease and is associated with increased mortality in mice. Candidalysin enhances ethanol-induced liver disease independent of the β-glucan receptor CLEC7A on bone-marrow derived cells, and Candidalysin does not alter gut barrier function. Candidalysin can damage primary hepatocytes in a dose-dependent manner *in vitro* and is associated with liver disease severity and mortality in patients with alcoholic hepatitis.

Conclusions: Candidalysin is associated with progression of ethanol-induced liver disease in preclinical models and worse clinical outcomes in patients with alcoholic hepatitis.

Graphical Abstract



Lay summary

Candidalysin is a peptide toxin secreted by the commensal gut fungus *Candida albicans*. Candidalysin enhances alcohol-associated liver disease independent of the β-glucan receptor CLEC7A on bone-marrow derived cells in mice without affecting intestinal permeability. Candidalysin is cytotoxic to primary hepatocytes, indicating a direct role of Candidalysin on ethanol-induced liver disease. Candidalysin might be an effective target for therapy in patients with alcohol-associated liver disease.

Keywords

mycobiome; microbiota; microbiome

Introduction

Alcohol-associated liver disease is one of the most prevalent liver diseases worldwide[1], and the leading cause of liver transplantation in the U.S.[2]. Alcohol-related liver disease is associated with changes in the intestinal microbiota. Gut dysbiosis induces intestinal inflammation and gut barrier dysfunction, which allows viable bacteria, bacterial (such as lipopolysaccharides (LPS)) and fungal products (such as β-glucan) to translocate to the liver. Bacteria and microbial products bind to pathogen recognition receptors causing an inflammatory response of resident Kupffer cells and an infiltration of macrophages. Although many efforts were made to evaluate the role of the bacterial microbiota in alcohol-associated liver disease, the interaction between fungi and their host, and especially their contribution to alcohol-associated liver disease remains poorly understood. We have shown that β-glucan, a cell wall component of many commensal fungi, binds to C-type lectin domain family 7 member A (CLEC7A; also known as DECTIN1) on hepatic macrophages to release interleukin 1 beta (IL-1β) and increase ethanol-induced liver disease in mice[3].

The yeast *Candida albicans* (*C. albicans*) normally resides as a harmless commensal in the human intestinal tract, but can translocate from the gut into the bloodstream in predisposed individuals such as immunocompromised patients[4]. Its virulence partly depends on the transition from yeast to hyphal growth forms[5]. Recent studies revealed that Ece1, the product of the gene *ECE1* (extent of cell elongation 1), is critical for mucosal pathogenesis of *C. albicans*[6]. Ece1 is a polyprotein consisting of eight distinct peptides processed by the sequential activities of the serine proteases Kex2 and Kex1[6] (Supplementary Fig. 1). Among them, Ece1-III 62–92K, also named Candidalysin, is a secreted cytolytic peptide (exotoxin) that is important for epithelial cell damage in the host[6]. Candidalysin can damage epithelial mucosa directly, but also induces a danger response signaling pathway via c-Fos and mitogen-activated protein kinase (MAPK) signaling and triggers inflammatory cytokine responses (including IL-1α, IL-1β, and IL-8)[6–8]. We have previously demonstrated that patients with alcohol use disorder had increased fecal proportions of *Candida* spp, including *C. albicans*, compared to non-alcoholic controls. Whether intestinal overgrowth of *C. albicans* contributes to alcohol-associated liver disease beyond increasing systemic β-glucan level is not known. In this study, we evaluate the effect of Candidalysin on promoting alcohol-associated liver disease.

Methods

Patients.

We evaluated 11 subjects without alcohol use disorder (controls; social drinkers consuming less than 20g/day), 42 patients with alcohol use disorder, and 91 patients with alcoholic hepatitis. Patients with alcohol use disorder fulfilling the DSM IV criteria[9] of alcohol dependence and with active alcohol consumption (self-reported > 60g/day) presented with

various stages of liver disease (15% had advanced F3/4 fibrosis based on fibrosis-4 index (FIB-4)[10]; Supplementary Table 1). Patients with alcohol use disorder were recruited from an alcohol withdrawal unit where they followed a detoxification and rehabilitation program. Controls or patients with alcohol use disorder did not take antibiotics or immunosuppressive medication during the two months preceding enrollment. Other exclusion criteria were diabetes, inflammatory bowel disease, known liver disease of any other etiology, and clinically significant cardio-vascular, pulmonary or renal co-morbidities. Alcoholic hepatitis patients were enrolled from the InTeam Consortium (ClinicalTrials.gov identifier number:) from 12 participating centers in the US, Mexico, Canada, United Kingdom, France and Spain. Inclusion criteria were: 1. Active alcohol abuse (> 50 g/day for men and > 40 g/day for women) in the last 3 months, 2. Aspartate aminotransferase (AST) > alanine aminotransferase (ALT), and total bilirubin > 3 mg/dl in the past 3 months, 3. Liver biopsy and/or clinical picture consistent with alcoholic hepatitis. Exclusion criteria were: 1. Autoimmune liver disease (ANA > 1/320), 2. Chronic viral hepatitis, 3. Hepatocellular carcinoma, 4. Complete portal vein thrombosis, 5. Extrahepatic terminal disease, 6. Pregnancy, and 7. Lack of signed informed consent[11]. In all patients, the clinical picture was consistent with alcoholic hepatitis and in patients who underwent liver biopsy, the histology was in line with the diagnosis of alcoholic hepatitis. Liver biopsies were only done if clinically indicated as part of routine clinical care for diagnostic purposes of alcoholic hepatitis. For 3 patients who underwent liver transplantation, the transplantation date was considered as date of death. Patients were censored at the time point they were last seen alive. Baseline characteristics are shown in Supplementary Table 1 and 2. Differences in BMI are likely explained by the presence of ascites in 68% (n=61) of alcoholic hepatitis patients. Fresh feces from healthy controls or patients were collected, immediately frozen and stored in −80°C freezer until experiments were performed. Fungal culture, quantitative PCR (qPCR) for total fungi, *C. albicans* and *ECE1* was performed from fecal samples. The model for end-stage liver disease (MELD) score was calculated from all alcoholic hepatitis patients from whom bilirubin level, international normalized ratio (INR), and creatinine level was available. The protocol was designed according to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Committee of each participating center and patients were enrolled after written informed consent was obtained from each patient.

Mice.

C57BL/6 male mice were purchased from Charles River and used in all described experiments. *Clec7a*$^{-/-}$ mice on a C57BL/6 background have been described[3]. Heterozygous mice were used for breeding to generate wild type and *Clec7a* deficient littermates.

Male mice (8–12weeks) were subjected to either chronic-plus-binge ethanol diet model (NIAAA model)[12] or a chronic Lieber-DeCarli diet model for 8 weeks as previously described[3]. For the chronic-plus-binge ethanol diet model, mice were fed the Lieber-DeCarli diet for 15 days. The caloric intake from ethanol was 0% on days 1–5 and 36% from day 6 until the end. At day 16, mice were gavaged with one dose of ethanol (5g/kg bodyweight) in the morning and sacrificed 9 hours later. Pair-fed control mice received a diet with an isocaloric substitution of dextrose. For the chronic Lieber-DeCarli feeding model, the caloric

intake from ethanol was 0 on day 1, 10% of total calories on days 2 and 3, 20% on days 4 and 5, 30% from day 6 until the end of 6 weeks, and 36% for the last 2 weeks. Control mice received an isocaloric amount of iso-maltose instead of ethanol. Antibiotics treatment was started at day 1 of ethanol feeding, and mice were gavaged daily until harvesting. The composition of antibiotics mixture has been described (Polymyxin B (150mg/kg BW) and Neomycin (200mg/kg BW))[13].

To evaluate the effect of Candidalysin on liver disease, C57BL/6 male mice (Control diet: n=5–8/group; Ethanol diet: n=12–14/group) were gavaged with 10^8 colony forming units (CFUs) of four different *C. albicans* strains (wild type, M1477; *ece1*Δ/Δ, M2057; *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$, M2174; *ece1*Δ/Δ + *ECE1*, M2059)[6] or phosphate-buffered saline (PBS) as vehicle control every third day starting from day 6 to day 15 of the chronic-plus-binge ethanol diet model. As described[6], *C.albicans* was deleted for both copies of *ECE1* (*ece1Δ/Δ*) and restored with one full-length allele (*ece1Δ/Δ*+*ECE1*) or one mutant allele lacking the Candidalysin-encoding region of *ECE1* (*ece1Δ/Δ*+$ECE1_{\Delta 184-279}$).

Bone marrow chimeric mice were generated as previously described[3]. In brief, C57BL/6 recipient male mice were given lethal doses of radiation (650 rad) twice, using a ^{137}Cs source. *Clec7a*$^{-/-}$ male mice were used as bone marrow donors. Two weeks after bone marrow transplantation, mice were given intraperitoneal injections of 200 μl clodronate liposomes (5 mg/ml; Vrije Universiteit, Amsterdam, Netherlands) to deplete radio-resistant Kupffer cells. Chimeric mice (male, n=10–11/group) were subjected to the chronic-plus-binge ethanol diet model 4 weeks after bone marrow transplantation.

To determine the effect of Candidalysin on intestinal permeability, C57BL/6 male mice (Control diet: n=5–6/group; Ethanol diet: n=5–6/group) were gavaged with PBS as vehicle control, 10^8 CFUs of wild type *C. albicans* or *ece1*Δ/Δ *C. albicans* every third day starting from day 6 to day 15 using the chronic-plus-binge ethanol diet model. At day 16, fluorescein isothiocyanate (FITC)-dextran (4 kDa, Sigma) (200μl, 100mg/ml) was gavaged to all the mice 1 hour after binge and blood was harvested after 4 hours.

All mice were housed under specific pathogen-free (SPF) conditions in a standard environment with a 12-hour light–dark cycle at the animal facilities of University of California, San Diego under protocol S09042. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR).

For qPCR, fungal genomic DNA was extracted from human stool samples as described[3]. Published primer sequences for fungal 18S rRNA gene[14], *C. albicans* internal transcribed spacer (*ITS*) gene[15], *ECE1* gene[6] and *SEL1* gene[16] were used (Supplementary Table 3). For RT-qPCR to determine AML12 cellular and hepatic gene expression, AML12 cellular and mouse liver RNA was extracted using Trizol (Invitrogen) and cDNA was then synthesized using High Capacity cDNA Reverse Transcription kit (ABI). Primer sequences for mouse genes were originally obtained from NIH qPrimerDepot (Supplementary Table 3). qPCR and RT-qPCR were performed with iTaq Universal SYBR Green Supermix (Bio-Rad) using a

StepOnePlus thermocycler (ABI) real-time PCR system. The RT-qPCR values of cellular and mouse genes were normalized to 18S. Cellular gene expression data was expressed relative to the level of blank control-treated cells. Mouse gene expression data was expressed relative to the level of control-fed PBS-treated mice.

Fecal fungi.

To determine the number of fungal CFUs in human stool samples, 50 to 300 mg of feces was resuspended into 500 μl PBS and serial dilutions were then made, followed by plating 100 μl onto a YPD agar plate with antibiotics (1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar, 100 μg/ml gentamicin and 100 μg/ml chloramphenicol). Plates were incubated at 30°C for 48 hours. Colony numbers of each sample were then counted and CFUs were normalized to weight of feces. Each colony was assessed for the presence of fungus or *C. albicans* by qPCR using specific primers against fungal 18S rRNA gene or *C. albicans ITS* gene, respectively. Each colony was also assessed by qPCR to determine *ECE1* positivity using specific primers.

To determine the number of fungal CFUs in mouse feces, 10 to 30 mg of feces were resuspended into 500 μl PBS and serial dilutions were made, followed by plating 100 μl onto YPD agar plate with antibiotics. Plates were incubated at 30°C for 48 hours. Colony numbers of each sample were then counted and CFUs were normalized to weight of feces. Fungi, *C. albicans*,*ECE1* and *SEL1* presence were assessed by qPCR.

C. albicans culture.

C. albicans strains were grown in YPD broth with antibiotics (1% yeast extract, 2% peptone, 2% D-glucose, 100 μg/ml gentamicin and 100 μg/ml chloramphenicol) and incubated at 30°C with shaking at 150 rpm for 20 hours. Cell density was analyzed by measuring optical density at 600 nm in a microplate reader (Molecular Devices). Cells from YPD broth were collected by centrifugation, washed twice with PBS and adjusted to the required cell density.

Biochemical assays.

Serum levels of ALT were measured using the Infinity ALT kit (Thermo Scientific). Hepatic triglyceride levels were measured using the Triglyceride Liquid Reagents Kit (Pointe Scientific). Serum levels of ethanol were measured using the Ethanol Assay Kit (BioVision). Serum levels of intestinal fatty-acid binding protein (IFABP) were measured using the iFABP ELISA kit (MyBioSource). Serum levels of zonulin were measured using the Human Zonulin ELISA Kit (MyBioSource)[17].

Staining procedures. To determine the accumulation of hepatic lipids, liver sections were embedded in OCT compound.

8 μm frozen sections were then cut and stained with Oil Red O (Sigma-Aldrich). Representative pictures from each group of mice are shown in each figure.

FITC-dextran permeability assay.

Intestinal permeability was assessed by oral gavage of FITC-dextran (4 kDa; Sigma), a non-metabolizable macromolecule that is used as a permeability probe[18, 19]. Serum concentration

of FITC-dextran was determined using a fluorimeter with an excitation wavelength at 490 nm and an emission wavelength at 530 nm, followed by calculations using serial dilutions of FITC-dextran as standards

Mouse cell culture studies.

Primary hepatocytes were isolated from C57BL/6 male mice (n=4–7/group) as described[20]. Cells were seeded on plates coated with rat collagen type I and cultured for 4 hours in DMEM-F12 (Thermo Fisher Scientific) with 1% (v/v) insulin-transferrin-selenium (Thermo Fisher Scientific), 40 ng/ml dexamethasone (MP Biomedicals), 10% (v/v) fetal bovine serum (FBS; Gemini Bio-Products), and an antibiotic-antimycotic mix (CoreBio). Following an overnight starvation in medium without FBS, hepatocytes were stimulated with different concentrations (0.6 μM and 3 μM) of Candidalysin for 24 hours[6]. Ece1-VII was used as negative control (0.6 μM and 3 μM). Culture medium was used as blank control. AML12 (immortalized mouse hepatocyte) cells were seeded and cultured in the same medium as primary hepatocytes (as described above) 24 hours prior to transfection. AML12 cells were transfected with siRNA for mouse *toll-like receptor* 2 (TLR2) and TLR4 (25 nM; set of 4 siRNAs, Dharmacon) or control siRNA (siGLO Green Transfection Indicator, Dharmacon), using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. 48 hours after transfection, cells were serum starved for 2 hours and treated with Candidalysin (15 μM), negative control (15 μM) or blank control for 24 hours.

Hepatocyte cytotoxicity was measured using the Pierce lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Thermo Fisher Scientific). Hepatocyte viability was determined by adding 3-(4,5-dimethylthiazol-2-yl)-2,5′-diphenyltetrazolium bromide solution (MTT; Sigma-Aldrich, final concentration 0.3 mg/ml) into cell culture plates and incubated in dark for 2 hours. The medium was then removed and dimethyl sulfoxide (Sigma-Aldrich) was added to dissolve the formazan. Formazan concentration was determined by absorbance at 550 nm and the survival percentage was calculated accordingly (the mean survival rate of hepatocytes incubated with culture medium alone was set as 100%).

Statistical analysis.

All data were expressed as mean ± SE unless otherwise specified. In mouse and cell culture experiments, for comparisons of > 2 groups within one experimental setting, one-way analysis of variance (ANOVA) was used followed by the Tukey's post-hoc test. For comparisons of >2 groups within two experimental settings, a two-way ANOVA followed by the Tukey's post-hoc test was used. For the human cohort, comparisons of baseline characteristics between the three groups were conducted using one-Way ANOVA with the Tukey's post-hoc test for multiple comparisons for continuous variables and Chi-squared tests for categorical variables. A Kruskal-Wallis test with the Dunn's post-hoc test was used to compare the mean number of fungal colonies in feces among different groups. A two-proportion z-test followed by FDR procedures was used to evaluate the percentage of individuals carrying fungi or *ECE1* among different groups. Multivariate Cox Proportional Hazard regression analysis was performed to detect associations of Candidalysin positivity with 90-day mortality in alcoholic hepatitis patients. Patients that were lost to follow-up

were censored at the day they were last seen alive. Univariate logistic regression analysis was performed to determine associations of Candidalysin and clinical parameters.

Statistical analyses were performed using the statistical software R (V.3.5.1, 2018 the R Foundation for Statistical Computing) and GraphPad Prism (V.6.01). A *P* value <0.05 was considered significant.

Results

Patients with alcoholic hepatitis have increased fecal numbers of Candidalysin positive *C. albicans*

C. albicans is a commensal fungus of the human gut flora. Using internal transcribed spacer (ITS) sequencing we showed that the relative proportions of *C. albicans* increased in patients with alcohol use disorder[3]. To determine quantitative differences of viable fungi in the fecal mycobiome, we performed fungal cultures of stool samples from non-alcoholic controls (controls, n=11), patients with alcohol use disorder (AUD, n=42) and patients with alcoholic hepatitis (AH, n=91). Each colony was further assessed for the presence of fungi or *C. albicans* by quantitative PCR (qPCR) using specific primers against fungi or *C. albicans*. Fecal samples from patients with alcoholic hepatitis grew significantly more colonies of fungi or *C. albicans* as compared with alcohol use disorder patients and controls (Fig. 1A and B). Furthermore, a significantly higher proportion of alcoholic hepatitis patients was culture positive for fungi or *C. albicans* than patients with alcohol use disorder (Fig. 1C and D).

We next sought to determine if *ECE1*, the gene encoding the *C. albicans* exotoxin Candidalysin, was also increased in alcoholic hepatitis patients. 30.77% of alcoholic hepatitis patients were tested positive for *ECE1*, while in patients with alcohol use disorder, this proportion was significantly lower (Fig. 1E). Taken together, *ECE1*, the gene encoding Candidalysin, was more abundant in patients with alcoholic hepatitis.

Candidalysin exacerbates ethanol-induced liver disease

We confirmed that chronic ethanol administration is associated with an increase of intestinal fungi in mice[3] (Supplementary Fig. 2). However, we could not detect *C. albicans* or *ECE1* in mice in the chronic-plus-binge ethanol diet model[12] or the chronic Lieber-DeCarli model for 8 weeks[3], consistent with a published report[21]. This indicates that *C. albicans* does not normally colonize the gastrointestinal mucosa of mice, or that the level of *C. albicans* cells or *ECE1* genes was below the detection limit of fungal cultures or nucleic acid amplification.

To study the role of Candidalysin positive *C. albicans* for alcohol-associated liver disease, we colonized mice by repeated oral gavage of *C. albicans* (10^8 CFUs every third day) during ethanol feeding. The average amount of *C. albicans* in mouse feces is 1.5×10^5 CFU/g feces (24 hours following gavage) (Supplementary Fig. 3), which is similar to the *C. albicans* amount in feces from alcoholic hepatitis patients (4.7×10^5 CFU/g feces) (Fig. 1B). Compared to vehicle (PBS) treated mice, mice colonized with wild type *C. albicans* displayed more severe ethanol-associated liver injury, indicated by elevated level of serum

alanine amino-transferase (ALT) (Fig. 2A) and increased hepatic steatosis (Fig. 2B and 2C). Those animals also showed more liver inflammation, with higher expression of genes *Il1b, chemokine (C-X-C motif) ligand 1* (*Cxcl1*) and *Cxcl2*, which encode inflammatory cytokines and chemokines (Fig. 2D – 2F). To determine if this disease exacerbation effect depends on *ECE1*, we colonized mice with *C. albicans* strain carrying an *ECE1* gene deletion (*ece1*Δ/Δ). These mice had less severe ethanol-associated liver injury, steatosis and inflammation (Fig. 2A – 2F). Re-introducing *ECE1* back into the *ece1*Δ/Δ strain (*ece1*Δ/Δ + *ECE1*) and colonizing mice with this strain, resulted in comparable levels of liver injury, steatosis and inflammation compared to mice colonized with wild type *C. albicans* following ethanol administration (Fig. 2A – 2F). These results indicate that the gene product of *ECE1* is important for *C. albicans* to enhance ethanol-induced liver disease.

ECE1 encodes for a polyprotein composed of eight different peptides[6] (Supplementary Fig. 1). Candidalysin is among them and is a secreted cytolytic peptide exotoxin[6]. To test whether Candidalysin is responsible for increased ethanol-induced disease mediated by *ECE1*, we colonized mice with a *C. albicans* strain carrying a deletion of the Ece1-III$_{62–93}$ Candidalysin-encoding region (*ece1*Δ/Δ + *ECE1*$_{Δ184–279}$), thus not producing Candidalysin. These mice showed similar ethanol-induced liver disease as compared with mice colonized with the *ece1*Δ/Δ *C. albicans* strain (Fig. 2A – 2F), indicating that Candidalysin is necessary for exacerbation of ethanol-induced liver disease by *C. albicans*.

In addition, Candidalysin is associated with increased mortality. Mice colonized with Candidalysin positive strains had a significant higher mortality rate as compared with mice colonized with Candidalysin negative strains (Supplementary Figure 4). Importantly, all *C. albicans* strains did not affect intestinal absorption or hepatic metabolism of ethanol, as serum levels of ethanol as well as expression of hepatic *alcohol dehydrogenase 1* (*Adh1*) and *cytochrome P450 family 2 subfamily E polypeptide 1* (*Cyp2e1*) (two genes encoding main hepatic enzymes that metabolize ethanol and convert it to acetaldehyde) did not differ significantly between mouse groups (Fig. 2G – 2I).

Bacteria and fungi actively interact with each other in the gut[22]. To test whether the effects of *C. albicans* on liver disease are affected by its interaction with bacteria, mice were treated with antibiotics to reduce the amount of intestinal bacteria, and gavaged wild type *C. albicans* or vehicle (PBS). Compared with antibiotics plus vehicle treated mice, mice receiving antibiotics plus wild type *C. albicans* showed more severe ethanol-induced liver injury (Supplementary Fig. 5A) and steatosis (Supplementary Fig. 5B and 5C), indicating that bacteria-fungi interaction is not the major contributor to liver damage caused by *C. albicans*.

Taken together, these results indicate that *C. albicans* producing Candidalysin can enhance ethanol-associated liver disease and increase mortality in mice.

Candidalysin exacerbates ethanol-induced steatohepatitis independent of the receptor CLEC7A on bone marrow derived cells

β-glucan is the most abundant cell wall polysaccharide in most fungal species including *C. albicans*[23]. CLEC7A is a pattern recognition receptor that recognizes a variety of β-glucans

and thus activates host immune response[24]. CLEC7A is primarily expressed on myeloid cells[25, 26] and only expressed on Kupffer cells and macrophages in the liver[3]. We have previously shown that loss of CLEC7A from Kupffer cells decreases ethanol-induced steatohepatitis[3]. To investigate whether the disease promoting effect of *C. albicans* is mediated by CLEC7A signaling, we generated CLEC7A bone marrow chimeric mice using a combination of clodronate-mediated Kupffer cell depletion, irradiation, and bone marrow transplantation[3]. Wild type mice were transplanted with bone marrow cells from CLEC7A-deficient mice, which results in full reconstitution of Kupffer cells[3, 27]. Chimeric mice were colonized with wild type *C. albicans*, *ece1*Δ/Δ *C. albicans* (*ece1*Δ/Δ) or vehicle (PBS) and subjected to the chronic-plus-binge ethanol diet model. Chimeric mice with bone-marrow derived cells that lacked CLEC7A and were colonized with wild type *C. albicans* showed more severe ethanol-induced liver injury (Fig. 3A), steatosis (Fig. 3B and 3C) and inflammation (Fig. 3D – 3F) as compared with PBS treated mice and mice colonized with *C. albicans* strain carrying an *ECE1* gene deletion. No significant difference in serum ethanol level and hepatic expression of *Adh1* and *Cyp2e1* was observed among all groups (Fig. 3G – 3I). These results indicate that *C. albicans* and Candidalysin induce liver disease independent of CLEC7A signaling on Kupffer cells and other bone-marrow derived cells following ethanol administration.

Candidalysin does not affect intestinal permeability

In a cell culture system, Candidalysin causes necrosis of intestinal epithelial cells, which is a key factor in subsequent fungal translocation of *C. albicans* through intestinal layers[28]. To determine if Candidalysin induces gut barrier dysfunction *in vivo*, we determined intestinal permeability by measuring recovery of indigested dextran. In line with our previous report[29], intestinal permeability increased in mice fed with ethanol diet (Fig. 4A). However, no significant difference was observed among mice colonized with wild type *C. albicans*, *ece1*Δ/Δ *C. albicans* (*ece1*Δ/Δ) and vehicle (PBS) following ethanol administration (Fig. 4A). Intestinal fatty-acid binding protein (IFABP) is specifically expressed in intestinal epithelial cells and released to the systemic circulation upon enterocyte damage[30]. Intestinal epithelial damage was not different between groups as measured by ELISA for serum IFABP (Fig. 4B). These results indicate that Candidalysin does not cause measurable intestinal barrier disruption or enterocyte death in ethanol-fed mice.

Candidalysin is cytotoxic to primary hepatocytes

To further define a mechanism by which Candidalysin enhances ethanol-induced liver disease, primary hepatocytes were isolated from mice fed an isocaloric control diet or subjected to the chronic-plus-binge ethanol feeding model. Cultured hepatocytes were incubated with pure bioactive Candidalysin. Candidalysin dose-dependently caused death of cultured hepatocytes as compared with hepatocytes incubated with control peptide (Fig. 5). Hepatocytes from ethanol-fed mice showed similar cytotoxicity as compared to hepatocytes isolated from control diet fed mice. However, incubation with ethanol increased the Candidalysin-induced cytotoxicity of hepatocytes (Figure 5A and 5B). These results indicate that Candidalysin can damage hepatocyte directly and additional ethanol stimulation *in vitro* can increase the cytotoxic effect.

Sel1, a small secreted cysteine-rich protein of *C. albicans*, is critical in mediating the effects of *C. albicans* on innate immune cells via activation of NF kappa B and mitogen-activated protein kinases (MAPK) signaling[16]. Since Candidalysin can also activate MAPK/c-Fos/MAP kinase phosphatase 1 (MKP1) signaling pathway in epithelial cells[8], we tested whether the effects of Candidalysin on liver injury and hepatocyte damage are modulated by Sel1. We first tested the presence of *SEL1* in the four strains of *C. albicans* used in this study. All strains (wild type, *ece1*Δ/Δ, *ece1*Δ/Δ + $ECE1_{\Delta 184–279}$, and *ece1*Δ/Δ + *ECE1*) carried *SEL1* (Supplementary Fig. 6A). We further assessed the presence of *SEL1* in mouse feces after *C. albicans* gavage; *SEL1* is detected in both groups of mice (treated with either wild type or *ece1*Δ/Δ + $ECE1_{\Delta 184–279}$ *C. albicans*) (Supplementary Fig. 6B). A recent study reported that *SEL1* can trigger inflammatory response through TLR2/4[16]. To determine whether Candidalysin causes hepatocyte damage via Sel1-TLR2/4 pathway, we performed TLR2/4 knockdown using siRNA in a mouse hepatocyte cell line (AML12 cells) (Supplementary Fig. 7A), and incubated these cells with pure bioactive Candidalysin. Candidalysin caused a cytotoxic effect on AML12 cells independent of TLR2/4 (Supplementary Fig. 7B and 7C). Our in vivo and in vitro studies indicate that Candidalysin exerts its effects on hepatocytes and liver independent of Sel1.

Candidalysin is related with the severity and mortality of alcoholic hepatitis

Alcohol-associated liver disease can progress to alcoholic hepatitis, a severe form of liver disease with considerable morbidity and mortality. We therefore analyzed clinical parameters and outcomes in patients with alcoholic hepatitis, who were positive for the Candidalysin coding gene *ECE1* in feces. Patients with fecal samples containing genetic material for Candidalysin had higher serum levels of zonulin (a surrogate marker for gut barrier dysfunction) compared with *ECE1* negative patients (Supplementary Fig. 8). Compared to *ECE1* negative patients, fecal patient samples containing genetic material for Candidalysin had a higher model for end-stage liver disease (MELD) score (Fig. 6A). In the univariate logistic regression analysis, the MELD score was significantly associated with detection of *ECE1* (Table 1). The detection of *ECE1* was further associated with an increased 90 day mortality in alcoholic hepatitis patients with an adjusted hazard ratio of 3.01 (1.02–8.87, P=0.046) (Table 2). Importantly, 28.42% of *ECE1* – positive patients with alcoholic hepatitis died within 90 days after admission compared with 11.71% of *ECE1* – negative patients (Log-rank P=0.044) (Fig. 6B).

Discussion

Our previous study showed that patients with alcohol use disorder have higher intestinal proportions of *C. albicans*. Whether this leads to more severe alcohol-associated liver disease and the precise mechanism of *C. albicans* promoting alcohol-associated liver disease remains poorly understood. Our current results link a specific fungal exotoxin with the progression of alcohol-associated liver disease. Candidalysin, a cytotoxic peptide secreted by *C. albicans*, causes direct hepatocyte damage and exacerbates ethanol-induced liver disease in mice. Fecal positivity for the Candidalysin coding gene *ECE1* is associated with more severe disease and mortality in alcoholic hepatitis patients.

Different forms of cell death contribute to the pathogenesis of alcoholic liver disease[31]. Apoptosis of hepatocytes can be induced by organelle stress and cytokine receptor activation[31, 32]. It has also been shown that receptor-interacting protein 1 (RIP1), RIP3 and mixed lineage kinase domain-like protein (MLKL) trigger necroptosis of hepatocytes in alcohol-associated liver disease[31, 33]. Furthermore, hepatocytes undergo gasdermin-D mediated pyroptosis in alcoholic hepatitis patients[34], indicating that different forms of hepatocellular death may exist/coexist based on the different microenvironment of cells. Candidalysin was found to activate MAPK/c-Fos/MAP kinase phosphatase 1 (MKP1) signaling pathway resulting in the production of pro-inflammatory cytokines such as IL-1α, IL-1β and IL-6 in epithelial cells[6–8]. Our study confirmed that Candidalysin increases hepatic levels of *Il1b*, *Cxcl1* and *Cxcl2* mRNAs in mice following ethanol administration. These pro-inflammatory cytokines may further recruit immune cells and hepatocytes injury. This might contribute to direct Candidalysin-induced hepatocyte death. Taken together, Candidalysin might be able to induce multiple forms of hepatocellular death through different mechanisms, which deserve further investigation in the future.

Our data indicate that Candidalysin does not increase intestinal permeability or intestinal epithelial cell damage in mice fed ethanol. *C. albicans* is larger in size than bacteria[35] and seems to translocate predominantly *via* a transcellular route, and maybe to a lesser extent through the paracellular space of intestinal epithelial cells[28]. Chronic ethanol diet is associated with increased intestinal permeability[36]. Unlike what has been shown with bacteria, we have not been able to detect *C. albicans* in the liver of ethanol-fed mice by culture or by amplification of genetic material via qPCR. We have confirmed that Candidalysin positive *C. albicans* could increase liver injury, steatosis and inflammation in mice fed ethanol diet, but this injurious effect is absent in mice fed control diet. Our clinical data showed that patients with fecal samples containing genetic material for Candidalysin had increased gut permeability, higher MELD score and 90 day mortality compared with *ECE1* negative patients. Thus, most likely Candidalysin produced in the intestinal lumen reaches the liver via increased intestinal permeability and exerts its effects on the liver. A direct interaction between Candidalysin and hepatocytes needs to be confirmed in future in vivo studies.

Importantly, we have discovered a link between the abundance of the Candidalysin gene *ECE1* and severity of disease in alcoholic hepatitis patients. There is a positive correlation between *ECE1* detection and MELD, and between *ECE1* detection and patient mortality. Although an independent and larger patient cohort is required to confirm our findings, fecal *ECE1* detection might be useful as non-invasive biomarker for predicting mortality in alcoholic hepatitis patients. Personalized diagnostics could be applied to identify patients colonized with Candidalysin-positive *C. albicans*.

In summary, our study shows that Candidalysin produced by *C. albicans* ethanol-induced liver disease in mice, is associated with severity and mortality in alcoholic hepatitis patients. Effective methods such as antifungal drugs targeting Candidalysin expressing *C. albicans* should be explored and considered as therapeutic approach for patients with alcoholic hepatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Adh1	alcohol dehydrogenase 1
AH	alcoholic hepatitis
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
AUD	alcohol use disorder
C. albicans	Candida albicans
CFUs	colony forming units
Clec7a	C-type lectin–like receptor 7a
Cxcl	chemokine (C-X-C motif) ligand
Cyp2e1	cytochrome P450 family 2 subfamily E polypeptide 1
ECE1	extent of cell elongation 1
FBS	fetal bovine serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
HR	hazard ratio
I-FABP	intestinal fatty-acid binding protein
IL-1β	interleukin 1 beta
INR	international normalized ratio

ITS	internal transcribed spacer
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MELD	model for end-stage liver disease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5′-diphenyltetrazolium bromide
OR	odds ratio
PBS	phosphate-buffered saline
qPCR	quantitative PCR
RIP1	receptor-interacting protein 1
RT-qPCR	reverse transcription quantitative PCR
TLR	Toll-like receptor; YPD broth, yeast extract, peptone and D-glucose broth

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Highlights

- Fecal levels of *C. albicans* and *ECE1* are increased in patients with alcoholic hepatitis.
- Candidalysin enhances ethanol-induced liver disease and is associated with higher mortality in mice.
- Candidalysin damages hepatocytes in a dose–dependent manner.
- Candidalysin is associated with the severity of liver disease and mortality of patients with alcoholic hepatitis.

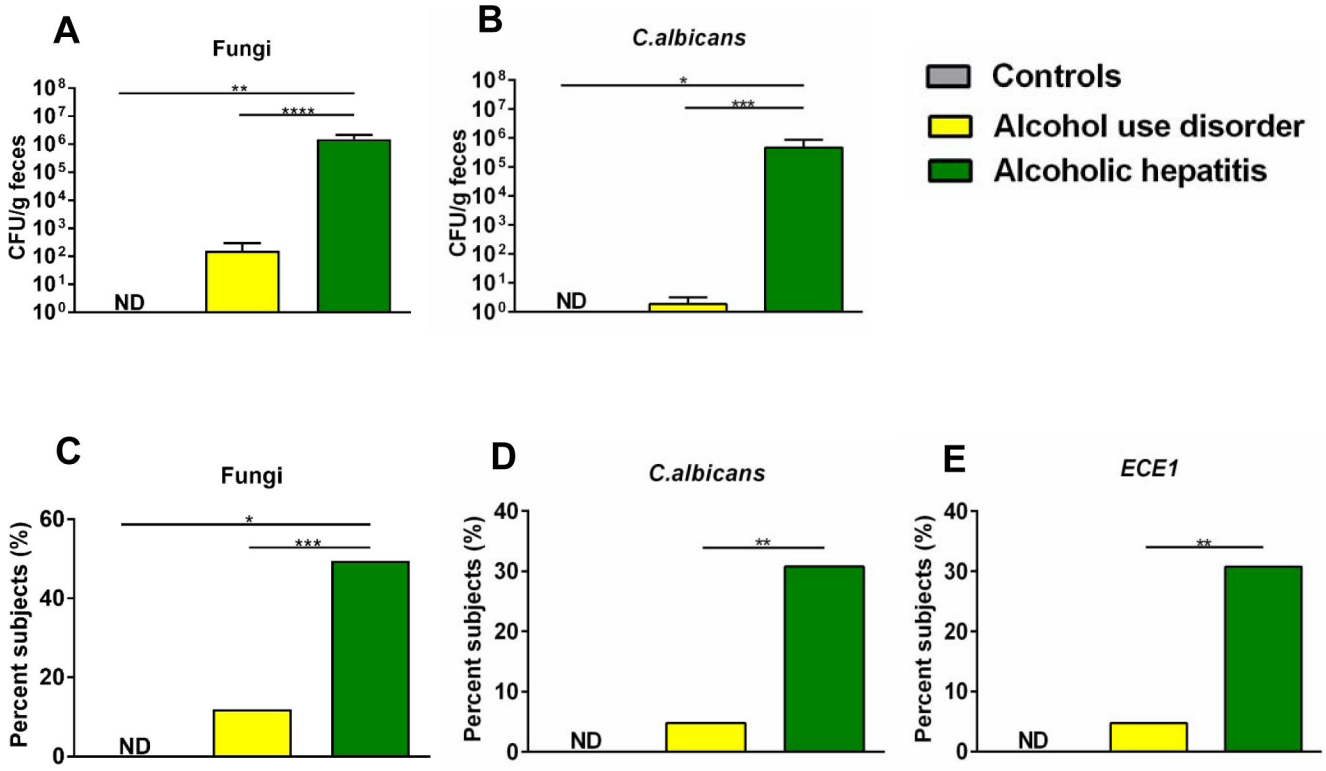

Fig. 1. Candidalysin is related with the development of alcoholic liver disease.

Fecal samples from controls (n=11), patients with alcohol use disorder (AUD) (n=42) and alcoholic hepatitis (n=91) were cultured on YPD agar plates with antibiotics. Each colony was then assessed by qPCR to confirm as fungus, and to determine positivity for *C. albicans* or *ECE1*. (**A**) Colony forming units (CFUs) of total fungi in fecal samples. (**B**) Colony forming units (CFUs) of *C. albicans* in fecal samples. (**C**) Percentage of subjects with fecal samples positive for fungi. (**D**) Percentage of subjects with fecal samples positive for *C. albicans*. (**E**) Percentage of subjects with fecal samples positive for *ECE1*. Results are expressed as mean ± s.e (**A** and **B**). *P* values were determined by Kruskal-Wallis test with Dunn's post-hoc test (**A** and **B**), and Z test followed by false discovery rate (FDR) procedures (**C** - **E**). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

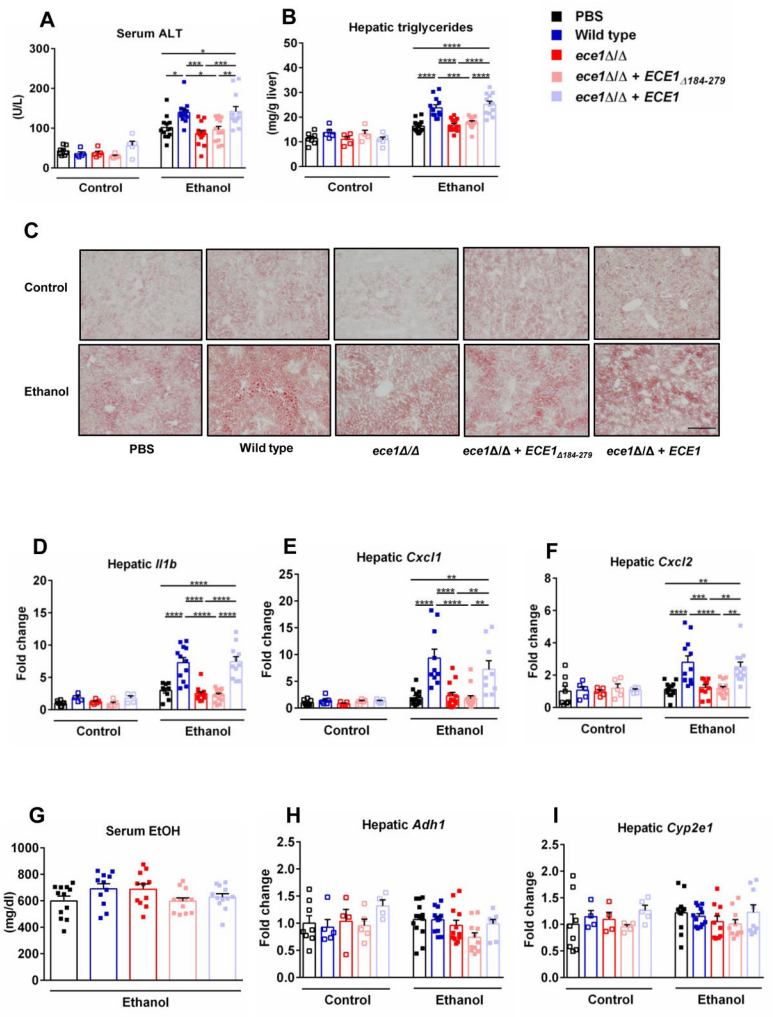

Fig. 2. Effects of Candidalysin on ethanol-induced liver disease.

C57BL/6 mice were fed an oral isocaloric (control) diet (1–2 technical replicates) or chronic-plus-binge ethanol diet (3–4 technical replicates), and gavaged with vehicle (PBS), wild type *C. albicans* (Wild type), *ECE1* deleted *C. albicans* (*ece1*Δ/Δ), only Ece1-III$_{62–93}$ deleted *C. albicans* (*ece1*Δ/Δ + *ECE1*$_{Δ184–279}$) or *ECE1* re-integrant *C. albicans* (*ece1*Δ/Δ + *ECE1*) with an amount of 10^8 colony forming units (CFUs) every third day. (**A**) Serum levels of alanine aminotransferase (ALT). (**B**) Hepatic triglycerides levels. (**C**) Representative images of Oil Red O stained liver tissue. (**D**) Hepatic levels of *Il1b* mRNA. (**E**) Hepatic levels of *Cxcl1* mRNA. (**F**) Hepatic levels of *Cxcl2* mRNA. (**G**) Serum levels of ethanol in mice fed chronic-plus-binge ethanol diet. (**H**) Hepatic levels of *Adh1* mRNA. (**I**) Hepatic levels of *Cyp2e1* mRNA. (Control diet: PBS, n=8; Wild type, n=5; *ece1*Δ/Δ, n=5; *ece1*Δ/Δ + *ECE1*$_{Δ184–279}$, n=5; *ece1*Δ/Δ + *ECE1*, n=5; Ethanol diet: PBS, n=14; Wild type, n=14; *ece1*Δ/Δ, n=14; *ece1*Δ/Δ + *ECE1*$_{Δ184–279}$, n=14; *ece1*Δ/Δ + *ECE1*, n=12). Scale bars = 100 μm. Images were taken at ×100 magnification. Results are expressed as mean ± s.e (**A**, **B**, **D** - **I**). *P* values were determined by one-way ANOVA with Tukey's post-hoc test (**A**, **B**, **D** - **I**). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

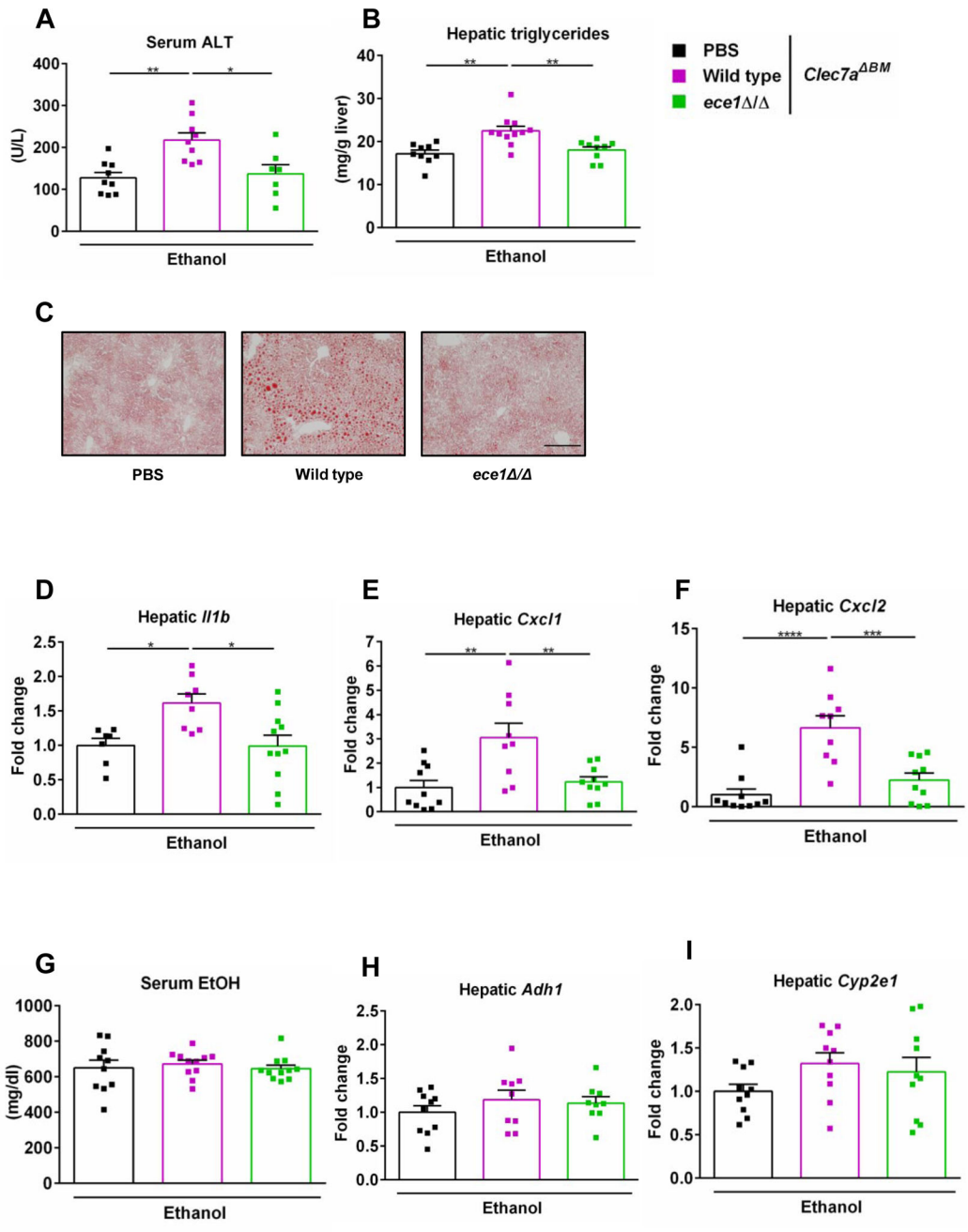

Fig. 3. Effects of Candidalysin on ethanol-induced liver disease in mice lacking CLEC7A in bone marrow derived cells.

C57BL/6 mice underwent transplantation of *Clec7a*$^{-/-}$ bone marrow (*Clec7a*$^{\Delta BM}$) and were fed an oral chronic-plus-binge ethanol diet (3 technical replicates). Mice were gavaged with vehicle (PBS), wild type *C. albicans* (Wild type) or *ECE1* deleted *C. albicans* (*ece1*Δ/Δ) with an amount of 10^8 CFUs every third day. (**A**) Serum levels of ALT. (**B**) Hepatic triglycerides levels. (**C**) Representative images of Oil Red O stained liver tissue. (**D**) Hepatic levels of *Il1b* mRNA. (**E**) Hepatic levels of *Cxcl1* mRNA. (**F**) Hepatic levels of *Cxcl2* mRNA. (**G**) Serum levels of ethanol. (**H**) Hepatic levels of *Adh1* mRNA. (**I**) Hepatic levels of *Cyp2e1* mRNA. (PBS, n=10; Wild type, n=11; *ece1*Δ/Δ, n=11). Scale bars = 100 μm. Images were

taken at ×100 magnification. Results are expressed as mean ± s.e. (**A**, **B**, **D - I**). *P* values were determined by one-way ANOVA with Tukey's post-hoc test (**A**, **B**, **D - I**). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

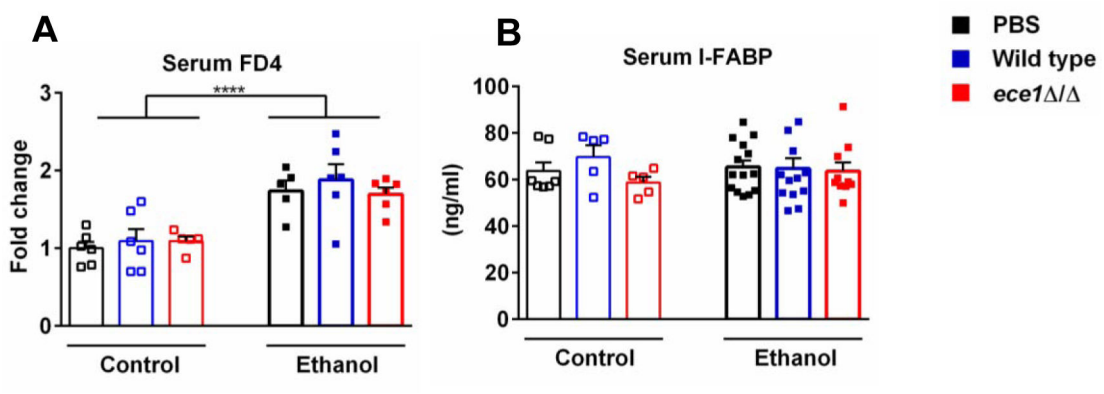

Fig. 4. Candidalysin does not affect intestinal permeability in mice fed ethanol.

C57BL/6 mice were fed an oral isocaloric (control) diet (1–2 technical replicates) or chronic-plus-binge ethanol diet (3–4 technical replicates) and gavaged with vehicle (PBS), wild type *C. albicans* (Wild type) or *ECE1* deleted *C. albicans* (*ece1*Δ/Δ) with an amount of 10^8 CFUs every third day. (**A**) Serum levels of FD4. Mice were gavage-fed FITC labeled dextran (200μl, 100mg/ml) 1 hour after binge on the last day and then sacrificed 4 hours later, and fluorescence was measured in the serum. (Control diet: PBS, n=6; Wild type, n=6; *ece1*Δ/Δ, n=5; Ethanol diet: PBS, n=5; Wild type, n=6; *ece1*Δ/Δ, n=6). (**B**) Serum levels of intestinal fatty-acid binding protein (IFABP) (Control diet: PBS, n=7; Wild type, n=5; *ece1*Δ/Δ, n=5; Ethanol diet: PBS, n=14; Wild type, n=13; *ece1*Δ/Δ, n=10). Results are expressed as mean ± s.e (**A** and **B**). *P* values were determined by two-way ANOVA with Tukey's post-hoc test (**A** and **B**). **** $P<0.0001$.

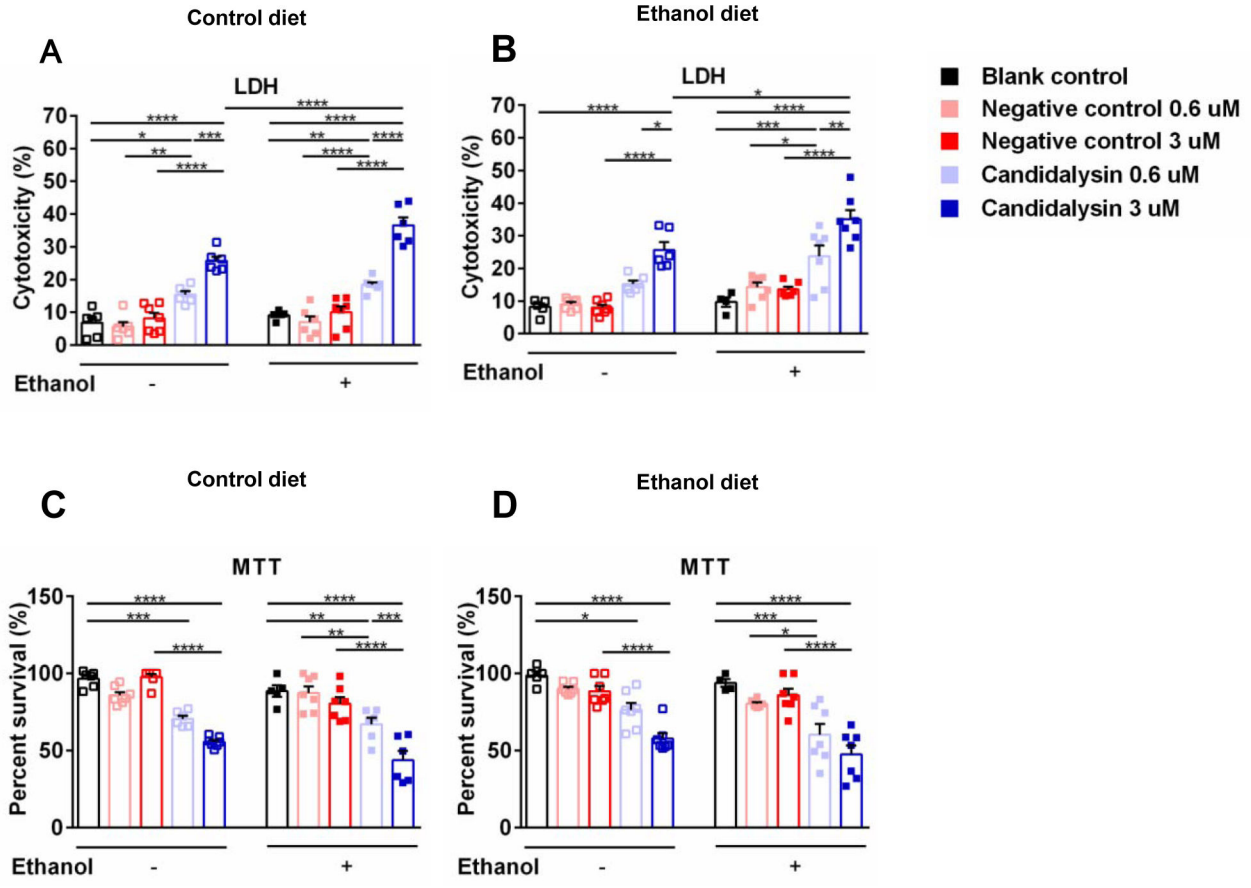

Fig. 5. Candidalysin induces death of cultured primary hepatocytes.

Primary hepatocytes were isolated from mice fed an oral isocaloric (control) diet (**A** and **C**) or from mice subjected to the chronic-plus-binge ethanol diet model (**B** and **D**). Hepatocytes were incubated with different concentrations (0.6 μM and 3 μM) of Candidalysin or negative control peptide (Ece1-VII), as well as blank control (equal volume of culture medium), without (−) or with (+) ethanol (25 mM) for 24 hours (3 independent experiments performed in 4–7 replicates). (**A** and **B**) Cytotoxicity was determined by measuring LDH release in the supernatant. (**C** and **D**) Hepatocyte survival was measured using the MTT assay. Results are expressed as mean ± s.e. *P* values were determined by two-way ANOVA with Tukey's post-hoc test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

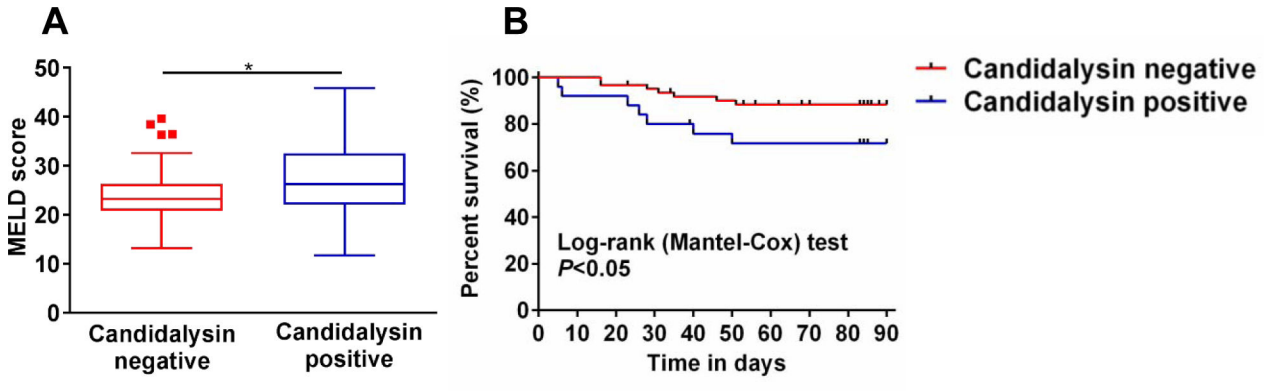

Fig. 6. Presence of Candidalysin associates with disease severity and mortality in patients with alcoholic hepatitis.

(**A**) MELD score for patients with alcoholic hepatitis. (Candidalysin positive, n=27; Candidalysin negative, n=62). (**B**) Kaplan-Meier curve of 90-day mortality for patients with alcoholic hepatitis. (Candidalysin positive, n=25; Candidalysin negative, n=61). Patients were grouped according to their *ECE1* status in stool. Patients lost to follow-up were censored at the time they were last seen alive. Results are expressed as median with range. *P* value was determined by Mann-Whitney-Wilcoxon test (**A**) or Log-rank test (**B**).

Table 1:

Logistic regression analysis of clinical parameters associated with Candidalysin

Candidalysin	Negative	Positive	OR, (95% CI, *P* value)
MELD	24 (5.5)	27.8 (7.7)	1.09 (1.02–1.18, ***P*=0.017**)
Antibiotic use	14 (23.0)	10 (35.7)	1.87 (0.69–4.96, *P*=0.211)
Steroids	23 (37.7)	10 (35.7)	0.92 (0.35–2.30, *P*=0.857)

Data in second and third column presented as mean (SD) or n (%). Bold font indicates significance (*P* value <0.05). All variables shown in Supplementary Table S1 and S2 were included in the logistic regression analysis. Shown are only the most relevant.

OR, odds ratio; CI, confidence interval; MELD, Model for End-stage Liver Disease.

Table 2:

Cox regression analysis of Candidalysin and MELD associated with mortality

Dependent: 90 Day Mortality	HR (multivariable), (95% CI, *P* value*)
Candidalysin positive	3.01 (1.02–8.87, ***P*=0.046**)
MELD	1.08 (1.01–1.15, ***P*=0.035**)

**P*-value adjusted for steroid and antibiotic use. Bold font indicates significance (*P* value <0.05).

HR, hazard ratio; CI, confidence interval; MELD, Model for End-stage Liver Disease.