Intestinal fungi contribute to development of alcoholic liver disease

An-Ming Yang,1,2 Tatsuo Inamine,1,3 Katrin Hochrath,1 Peng Chen,1 Lirui Wang,1,4 Cristina Llorente,1,4 Sena Bluemel,1 Philipp Hartmann,1 Jun Xu,1 Yukinori Koyama,1 Tatiana Kisselueva,1 Manolito G. Torralba,1 Kelvin Moncera,6 Karen Beeri,6 Chien-Sheng Chen,7 Kim Freese,8 Claus Hellerbrand,9 Serene M.L. Lee,9 Hal M. Hoffman,10 Wajahat Z. Mehal,11,12 Guadalupe Garcia-Tsao,11,12 Ece A. Mutlu,13 Ali Keshavarzian,13 Gordon D. Brown,14 Samuel B. Ho,14 Ramon Bataller,15 Peter Stärkel,16 Derrick E. Fouts,17 and Bernd Schnabl1,4

1Department of Medicine, UCSD, La Jolla, California, USA. 2Department of Internal Medicine, En Chu Kong Hospital, New Taipei City, Taiwan. 3Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. 4Department of Medicine, VA San Diego Healthcare System, San Diego, California, USA. 5Department of Surgery, UCSD, La Jolla, California, USA. 6Institute of Systems Biology and Bioinformatics, National Central University, Taoyuan City, Taiwan. 7Institute of Biochemistry (Emil-Fischer Zentrum), Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany. 8Department of General, Visceral and Transplantation Surgery, Hospital of the LMU Munich, Munich, Germany. 9Department of Pediatrics, UCSD, La Jolla, California, USA. 10Section of Digestive Diseases, Yale University School of Medicine, New Haven, Connecticut, USA. 11Section of Digestive Diseases, VA Connecticut Healthcare System, West Haven, Connecticut, USA. 12Department of Medicine, Rush University Medical Center, Chicago, Illinois, USA. 13Aberdeen Fungal Group, Medical Research Council Centre for Medical Mycology, University of Aberdeen, Aberdeen, United Kingdom. 14Liver Center, Departments of Medicine and Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. 15Liver Center, Departments of Medicine and Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. 16Saint Luc University Hospital, Université Catholique de Louvain, Brussels, Belgium. 17J. Craig Venter Institute, Rockville, Maryland, USA.

Chronic liver disease with cirrhosis is the 12th leading cause of death in the United States, and alcoholic liver disease accounts for approximately half of all cirrhosis deaths. Chronic alcohol consumption is associated with intestinal bacterial dysbiosis, yet we understand little about the contribution of intestinal fungi, or mycobiota, to alcoholic liver disease. Here we have demonstrated that chronic alcohol administration increases mycobiota populations and translocation of fungal β-glucan into systemic circulation in mice. Treating mice with antifungal agents reduced intestinal fungal overgrowth, decreased β-glucan translocation, and ameliorated ethanol-induced liver disease. Using bone marrow chimeric mice, we found that β-glucan induces liver inflammation via the C-type lectin–like receptor CLEC7A on Kupffer cells and possibly other bone marrow–derived cells. Subsequent increases in IL-1β expression and secretion contributed to hepatocyte damage and promoted development of ethanol-induced liver disease. We observed that alcohol-dependent patients displayed reduced intestinal fungal diversity and Candida overgrowth. Compared with healthy individuals and patients with non–alcohol-related cirrhosis, alcoholic cirrhosis patients had increased systemic exposure and immune response to mycobiota. Moreover, the levels of extraintestinal exposure and immune response correlated with mortality. Thus, chronic alcohol consumption is associated with an altered mycobiota and translocation of fungal products. Manipulating the intestinal mycobiome might be an effective strategy for attenuating alcohol-related liver disease.

Introduction

Liver cirrhosis is the 12th leading cause of mortality worldwide (1). Approximately 50% of cirrhosis-related deaths are due to alcohol abuse (2). Chronic alcoholic liver disease can lead to intestinal bacterial overgrowth and dysbiosis, a leaky gut barrier, and increased systemic levels of bacterial products (3–7). Although the intestinal microbiome contains bacteria, fungi, and viruses, research in the field of alcoholic-associated disease has almost exclusively focused on the interaction between the host and bacteria. Changes in the composition of the commensal intestinal fungi, also called the mycobiome, and their interaction with human tissues have been associated with other diseases (8).

The predominant commensal fungal species in the human intestine are Candida species, Saccharomyces cerevisiae, and Malassezia species (9). Like commensal bacteria in the intestine, fungi interact with their host. Although the host immune system develops tolerance to colonization with commensal fungi, it must contain the spread, and, in particular, invasion, of fungi (10). The human intestine can serve as a source of systemic fungal products or fungal infection when the gut barrier is disrupted (11). Patients with cirrhosis frequently either are exposed to fungal products (12) or develop fungal infections, with high mortality (13–15). Spontaneous fungal peritonitis is mainly caused by Candida species — in particular by Candida albicans in patients with cirrhosis (16).

Authorship note: A.M. Yang and T. Inamine contributed equally to this work.

Note regarding evaluation of this manuscript: Manuscripts authored by scientists associated with Duke University, The University of North Carolina at Chapel Hill, Duke-NJUS, and the Sanford-Burnham Medical Research Institute are handled not by members of the editorial board but rather by the science editors, who consult with selected external editors and reviewers.

Conflict of interest: The authors have declared that no conflict of interest exists.


C-type lectin domain family 7 member A (CLEC7A; also known as DECTIN1) is a pattern recognition receptor. CLEC7A recognizes a variety of 1,3-β-glucans (10) — cell wall polysaccharides found in and released from most fungi, including species of *Candida*, *Aspergillus*, and *Pneumocystis* (17). Upon ligand binding, CLEC7A activates caspase recruitment domain family member 9 (CARD9) and NF-κB, leading to transcription of several cytokines, including pro-interleukin-1β (pro-IL-1β) (18). The CLEC7A signaling pathway results in cleavage of pro-IL-1β and production of mature IL-1β by activating the NLR family pyrin domain–containing 3 (NLRP3) and caspase-1 inflammasome pathways (19).

We investigated the role of the mycobacteria and CLEC7A signaling in development of alcoholic liver disease.

**Results**

Chronic ethanol administration results in intestinal fungal overgrowth and increased plasma levels of β-glucan in mice. Intestinal bacterial overgrowth is common in alcohol-dependent patients (3). To determine whether chronic ethanol administration increases commensal fungi in mice, fecal numbers of fungi were measured by quantitative PCR (qPCR). Significantly increased fungal populations were observed after 8 weeks of ethanol administration in C57BL/6 WT mice (Figure 1A); a nonsignificant trend toward increased fungal species richness (average of 189.2 ± 94.7 operational taxonomic units [OTUs] for ethanol vs. 74.7 ± 17.5 OTUs for control diet) and diversity (average Simpson index of 0.256 ± 0.097 for ethanol vs. 0.664 ± 0.224 for control diet). Compositional changes in the intestinal mycobiome were characterized by significantly increased proportions of *Humicola* species (*P = 0.0017), *Fusarium* (*P = 0.0190), and *Aspergillus* (*P = 0.0258), while proportions of *Candida* species decreased (*P = 0.0007) with ethanol consumption (Supplemental Figure 1B). *Candida* species constitute between 40% and nearly 90% of all fungal reads, with the majority being *Candida parapsilosis* and *Candida albicans* (Supplemental Figure 1B). There was a significant decrease in relative abundance of *C. parapsilosis* (*P = 0.0007) with ethanol consumption. The apparent increase in *C. albicans* relative abundance was not significantly different.

Development of alcoholic liver disease involves increased translocation of microbial products from the intestinal lumen to the systemic circulation, facilitated by dysfunctional intestinal epithelial tight junctions, and increased paracellular permeability (20). The mean plasma level of 1,3-β-d-glucan was slightly higher in WT mice fed an ethanol diet than in WT mice fed an isocaloric control diet for 8 weeks (Figure 1B). A shorter ethanol feeding period of 5 weeks did not increase plasma 1,3-β-d-glucan (Supplemental Figure 1C). These results indicate that chronic ethanol feeding promotes expansion of intestinal fungi and translocation of fungal cell products to the systemic circulation. Future studies are required to assess not only the quantity of intestinal fungi, but also compositional changes at different time points after ethanol feeding.

Reducing an alcohol-associated increase in intestinal fungi attenuates features of ethanol-induced liver disease in mice. To determine whether reducing intestinal fungi protects against ethanol-induced liver disease, the commensal mycobacteria was decreased during ethanol feeding with the nonabsorbable antifungal agent amphotericin B. Intestinal fungal overgrowth was observed in mice fed ethanol, but not in mice given amphotericin B with the ethanol diet (Figure 2A). Reducing the alcohol-associated increase in intestinal fungi significantly lowered the mean plasma level of 1,3-β-d-glucan produced with chronic ethanol feeding (Figure 2B).

Ratios of liver to body weight were similar in mice given vehicle versus amphotericin B and fed ethanol for 8 weeks (Supplemental Figure 2A). Mice receiving amphotericin B developed less severe ethanol-associated liver disease; these mice had lower levels of liver injury (based on plasma level of alanine aminotransferase) and hepatic steatosis than mice receiving vehicle (Figure 2, C and D). This was confirmed by measurement of hepatic triglycerides and staining for hepatic steatosis (Figure 2, E and F). Amphotericin B also prevented a hepatic increase of the macrophage marker F4/80, indicating a lower number of inflammatory Kupffer cells (Figure 2, G and H).

To determine whether treatment with amphotericin B affects intestinal absorption and hepatic metabolism of ethanol, we measured plasma levels of ethanol and analyzed hepatic ethanol metabolism. Plasma levels of ethanol were comparable between mice receiving vehicle versus amphotericin B following chronic ethanol administration (Supplemental Figure 2B). Alcohol dehydrogenase (ADH) and cytochrome P450 enzyme 2E1 (CYP2E1) are main hepatic enzymes that metabolize ethanol and convert it to acetaldehyde (5). Hepatic ADH activity did not differ significantly between groups given vehicle versus amphotericin B while fed ethanol (Supplemental Figure 2C). Hepatic levels of CYP2E1 protein increased to a similar extent following ethanol administration to mice given vehicle versus amphotericin B (Supplemental Figure 2D). These findings suggest that amphotericin B prevents liver disease without affecting intestinal absorption or hepatic metabolism of ethanol.
To determine whether a reduction of fungi in the intestine alters the bacterial composition, the fecal microbiota was analyzed by 16S rRNA gene sequencing. Chronic ethanol administration induced bacterial dysbiosis in mice given vehicle (4, 21, 22) (Supplemental Figure 2E). Antifungal treatment did not produce major changes in the bacterial composition in isocaloric or ethanol diet-fed mice by comparison of bacterial 16S rRNA data sets (Supplemental Figure 2E). In addition, total numbers of bacteria did not differ significantly between mice given vehicle versus amphotericin B while fed ethanol (Supplemental Figure 2F).

Chronic ethanol administration is associated with a breach in the gut barrier and lower expression of intestinal tight junction proteins (23). We confirmed that occludin expression decreased in the jejunum after chronic ethanol feeding of mice given vehicle, similar to the reduced expression in mice receiving amphotericin B (Supplemental Figure 2G). Consistent with occludin expression data, paracellular permeability, based on systemic levels of translocated lipopolysaccharide (LPS), did not differ between vehicle- and amphotericin B–treated mice after ethanol feeding (Supplemental Figure 2H). These findings indicate that a reduction of ethanol-associated fungal overgrowth does not alter bacterial dysbiosis, increased intestinal permeability, and translocation of LPS. Interestingly, hepatic IL-1R–associated kinase 3 (Irak3, also called IRAK-M) expression was significantly higher in control and ethanol-fed mice after amphotericin B treatment (Supplemental Figure 2I). IRAK3 is a negative regulator of Toll-like receptor (TLR) signaling through inhibition of MYD88 signaling (24), which might decrease LPS/TLR4 signaling during chronic ethanol feeding.

Loss of CLEC7A from bone marrow–derived cells decreases ethanol-induced steatohepatitis. CLEC7A is primarily expressed on myeloid cells (18, 25). To determine the expression pattern of CLEC7A in liver, parenchymal and nonparenchymal hepatic cells were isolated from WT mice. Clec7a mRNA was highly expressed on Kupffer cells but not on hepatocytes or hepatic stellate cells in mice (Figure 3A). Using immunofluorescence analyses, we found that in livers of mice, CLEC7A colocalized with F4/80 (Supplemental Figure 3, A and B). These results were confirmed in human liver cells and tissue sections (Supplemental Figure 3, B and C). CLEC7A gene expression was not significantly different in liver and duodenal biopsies of alcohol-dependent patients as compared with controls (Supplemental Figure 3, D and E). To
investigate whether innate immune signaling through CLEC7A on bone marrow–derived cells and Kupffer cells mediates the effect of translocated fungal products following chronic ethanol administration, we generated CLEC7A bone marrow chimeric mice using a combination of clodronate-mediated Kupffer cell depletion, irradiation, and bone marrow transplantation; WT or CLEC7A-deficient bone marrow was transplanted into WT recipient mice. This protocol achieves full reconstitution of Kupffer cells (26).

To confirm successful bone marrow transplantation, we measured whole-liver expression of Clec7a mRNA; we detected low levels of Clec7a mRNA in livers of WT mice that received Clec7a–/– bone marrow (Figure 3B). Chimeric mice with bone marrow–derived cells that lacked Clec7a expression had slightly lower ratios of liver to body weight (Supplemental Figure 4A) and developed less severe ethanol-induced liver injury (Figure 3, C and D) and steatosis (Figure 3, E and F) than chimeric mice with bone marrow–derived cells that expressed CLEC7A.

Chimeric mice lacking CLEC7A on bone marrow–derived cells also had fewer F4/80-positive cells in the liver following ethanol feeding than mice with WT bone marrow (Figure 3, G and H). Plasma levels of ethanol and hepatic ethanol metabolism did not differ significantly with ethanol feeding in chimeric mice (Supplemental Figure 4, B–D). Following ethanol administration, paracellular intestinal permeability (as quantified by plasma level of LPS and 1,3-β-d-glucan) did not differ between mice with WT versus Clec7a–/– bone marrow (Supplemental Figure 4, E and F). These findings indicate that CLEC7A expression on Kupffer cells and possibly other bone marrow–derived cells contributes to liver disease following chronic ethanol administration without altering ethanol metabolism or affecting ethanol-induced gut barrier dysfunction.

In mice fed ethanol, fungal products induce secretion of IL-1β from Kupffer cells via CLEC7A signaling. CLEC7A signaling leads to upregulation of the inflammatory cytokine IL-1β (19). Development of alcoholic liver disease requires IL-1β (27). Blocking IL-1β prevents alcoholic steatohepatitis in mice (27). We confirmed
that levels of $\text{Il}1\beta$ mRNA and IL-1$\beta$ protein significantly increased after chronic administration of ethanol to WT mice (Figure 4, A and B). Amphotericin B prevented increases in hepatic $\text{Il}1\beta$ mRNA and IL-1$\beta$ protein following ethanol feeding (Figure 4, A and B). Inflammation, mediated by Kupffer cells, is required for ethanol-induced liver disease (28). It is therefore possible that CLEC7A-mediated signals in Kupffer cells are required for induction of IL-1$\beta$ and liver inflammation.

Using immunofluorescence analysis we found that in livers of ethanol-fed mice, F4/80 colocalized with IL-1$\beta$ (Figure 4C), indicating that Kupffer cells are the main liver cell type to express and secrete IL-1$\beta$ during ethanol-induced liver disease. This colocalization disappeared from livers of mice given amphotericin B. Consistent with our data from mice given amphotericin B, chimeric mice lacking Clec7a on Kupffer cells expressed lower levels of $\text{Il}1\beta$ mRNA and IL-1$\beta$ protein (Figure 4F). Plasma IL-1$\beta$ did not significantly differ between mice lacking CLEC7A on bone marrow-derived cells following ethanol feeding and mice with WT bone marrow (data not shown). To demonstrate that soluble β-glucan can activate CLEC7A in vivo, carboxymethyl-β-1,3-d-glucan (CM-curdlan) was injected into WT and Clec7a-deficient mice. CM-curdlan significantly induced hepatic $\text{Il}1\beta$ in WT but not in Clec7a$^{-/-}$ mice (Supplemental Figure 4G).

Plasma IL-1$\beta$ was undetectable in vehicle-treated WT mice and in WT mice 2, 8, and 24 hours after CM-curdlan injection (data not shown). These findings suggest that alcohol-induced translocation of fungal products, such as β-glucans, induces liver rather than systemic inflammation because these molecules bind to and activate CLEC7A on Kupffer cells. The subsequent increase in expression and secretion of IL-1$\beta$ promotes ethanol-induced liver disease.

1,3-β-Glucan-induced secretion of IL-1$\beta$ from Kupffer cells contributes to hepatocyte damage. To further define a mechanism by which β-glucan induces an inflammatory response that is mediated by Kupffer cells, we isolated Kupffer cells from WT and Clec7a−/− mice and stimulated them with curdlan, a linear form of 1,3-β-d-glucan. Curdlan induced $\text{Il}1\beta$ mRNA expression and IL-1$\beta$ secretion in WT Kupffer cells, and at much lower levels in Clec7a$^{-/-}$ Kupffer cells (Figure 5, A and B). Curdlan-stimulated Kupffer cells that lacked CLEC7A still expressed higher levels of $\text{Il}1\beta$ mRNA and IL-1$\beta$ protein than unstimulated Clec7a$^{-/-}$ Kupffer cells. This could be because β-glucan induces caspase-1 activation and IL-1$\beta$ production through other receptors, such as complement receptor 3 (CR3) (29). Curdlan-induced expression of chemokine (C-X-C motif) ligand 1 (Cxcl1), Cxcl2, and tumor necrosis factor (Tnf) was independent of CLEC7A (Supplemental Figure 5A).

Together, our results suggest that curdlan activates the transcription of $\text{Il}1\beta$ and processing of pro–IL-1$\beta$ into mature IL-1$\beta$ in cultured Kupffer cells. The CLEC7A signaling pathway results in cleavage of pro–IL-1$\beta$ and production of mature IL-1$\beta$ by activating canonical NLRP3 and caspase-1 inflammasome pathways (19). We confirmed that curdlan leads to increased levels of cellular NLRP3 and cleaved caspase-1 in supernatant of Kupffer cells (see immunoblot, Figure 5C). Caspase-1-deficient Kupffer cells exposed to curdlan secrete lower levels of IL-1$\beta$ than WT Kupffer cells (Figure 5D). Consistent with these findings, cleaved caspase-1 was lower
in the liver of chimeric mice that received Clec7a–/– bone marrow versus mice with WT bone marrow following ethanol administration (Supplemental Figure 5B).

We transferred conditioned medium from Kupffer cells stimulated with curdlan to cultured primary mouse hepatocytes. The supernatant from Kupffer cells stimulated with curdlan significantly increased death of the primary hepatocytes. This cytotoxic effect was reduced with a neutralizing antibody against IL-1β (Figure 5E).

Oil Red O staining showed marked lipid accumulation in cultured hepatocytes following incubation with conditioned medium from Kupffer cells (stimulated or not stimulated with curdlan) was transferred to primary mouse hepatocytes in the presence of control (IgG) or IL-1β neutralizing antibody. Hepatocyte cytotoxicity (E) and lipid accumulation, determined by Oil Red O staining (F) (n = 3 independent experiments performed in triplicate). Unpaired Student’s t test (A and B); Mann-Whitney U-statistic test (D); 1-way ANOVA with Newman-Keuls post-test (E). *P < 0.05.

Figure 5. Curdlan induction of IL-1β by Kupffer cells contributes to hepatocyte injury and steatosis. (A and B) Primary mouse WT and Clec7a–/– Kupffer cells were stimulated with curdlan; graphs show expression of Il1b mRNA (A) and secretion of IL-1β (B) (n = 3–6 independent experiments). (C) Levels of cellular NLRP3 and caspase-1 and cleaved caspase-1 in supernatants of Kupffer cells with and without curdlan stimulation. (D) IL-1β secretion by WT or caspase-1–deficient Kupffer cells following curdlan stimulation (n = 4–8 independent experiments). (E and F) Conditioned medium from Kupffer cells (stimulated or not stimulated with curdlan) was transferred to primary mouse hepatocytes in the presence of control (IgG) or IL-1β neutralizing antibody. Hepatocyte cytotoxicity (E) and lipid accumulation, determined by Oil Red O staining (F) (n = 3 independent experiments performed in triplicate). Unpaired Student’s t test (A and B); Mann-Whitney U-statistic test (D); 1-way ANOVA with Newman-Keuls post-test (E). *P < 0.05.

Immune response to fungi correlates with survival of patients with chronic alcohol abuse. We investigated whether patients who abuse alcohol have disturbances in their intestinal mycobiome and extraintestinal exposure to fungal products. Using ITS sequencing, we observed changes in the abundance and composition of the fecal mycobiome between alcohol-dependent patients and healthy individuals (controls). Fungal species richness (average number of OTUs, 5 ± 3 vs. 9 ± 6) and diversity (average Simpson index, 0.74 ± 0.25 vs. 0.42 ± 0.24) were lower in alcoholics compared with controls. There was also a dramatic overgrowth of Candida, with concomitant decreases in Epicoccum, unclassified fungi, Galactomyces, and Debaryomyces (Figure 6, A and B, and Supplemental Figure 6, A and B). Interestingly, overgrowth of Candida was independent of the stage of liver disease; patients with nonprogressive alcoholic liver disease, alcoholic hepatitis, or alcoholic cirrhosis had similar levels of fungal dysbiosis in the intestine. The opportunistic pathogen Candida albicans was among the most abundant Candida species in feces of patients abusing alcohol; however, its average relative abundance diminished with increasing severity of liver disease. Instead, there was an increase in the average relative abundance of Candida dubliniensis (Supplemental Figure 6C).

We next determined systemic exposure and immune response to intestinal fungi by measuring serum anti–Saccharomyces cerevisiae IgG antibodies (ASCA). Although S. cerevisiae mannan is used to detect ASCA, C. albicans is an important immunogen for ASCA and is likely the origin of an aberrant immune response in humans (30). Serum samples from patients with cirrhosis due to alcohol abuse had significantly higher levels of ASCA compared with controls or patients with cirrhosis due to chronic hepatitis B virus (HBV) infection (Figure 6C). These results indicate fungal dysbiosis and...
increased systemic exposure and immune response to intestinal fungi in patients with alcohol abuse. Moreover, levels of ASCA correlated with mortality in patients with alcoholic cirrhosis. A significantly smaller proportion of patients with high levels of ASCA survived for 5.5 years than patients with low levels (Figure 6D). The 2 groups were similar with respect to model for end-stage liver disease (MELD) score at inclusion (12.9 ± 2 for patients with low ASCA levels vs. 11.9 ± 1.2 for patients with high ASCA levels).

Discussion
Commensal fungi have important functions in health, but alterations in the intestinal mycobiota have been associated with disease (8, 31). Our study links compositional changes of the intestinal mycobiota to progression of alcoholic liver disease. Chronic alcohol abuse is associated with fungal dysbiosis in mice and humans. Fungal cell wall components, mainly β-glucan, translocate from the intestinal lumen to extraintestinal spaces that include the liver. Binding of β-glucan to CLEC7A on Kupffer cells and possibly other bone marrow–derived cells promotes alcoholic liver disease by inducing hepatic inflammation, ultimately leading to steatosis and cell death of hepatocytes. Preventing fungal overgrowth protects mice from ethanol-induced liver disease (Figure 7). In our human cohort, the degree of exposure to fungal products correlates with mortality of patients with cirrhosis from alcohol abuse but not viral hepatitis. Although a larger, prospective study is required to confirm data from our retrospective analysis of a human cohort with a relatively small number of patients, our findings support the importance of the mycobiota for patients with alcoholic liver disease.

We confirmed that ethanol-induced liver disease is associated with an increase in hepatic F4/80-positive cells (22). CLEC7A is primarily expressed on myeloid cells (18). Due to technical experimental limitations, there is a possibility that CLEC7A-positive...
bone marrow-derived cells other than Kupffer cells mediate the effects of β-glucan in the liver. However, CLEC7A on bone marrow-derived cells does not alter gut barrier function and systemic inflammation. Elimination of resident Kupffer cells and infiltrating macrophages prevents ethanol-induced liver disease in rodents (28), suggesting that these myeloid cells are required for progression of alcoholic liver disease. Activation of pathogen recognition receptors of the innate immune system induces the appropriate host defense response to pathogens. Receptors such as CLEC7A detect fungal infections and activate a strong innate immune response. Once the infection is cleared, the inflammatory response is turned off. However, chronic exposure of CLEC7A to fungal products continuously activates the cellular inflammasome pathway, causing a continuous inflammatory response and tissue damage. Similar processes might contribute to fungus allergies following chronic exposure in mice (32).

Alcoholic liver disease is associated with a dysfunctional gut barrier that allows translocation of microbial products from the intestine to the liver (20, 33, 34). We have previously demonstrated that dysbiosis-induced intestinal inflammation increases intestinal permeability (23, 35). Reducing bacteria with nonabsorbable antibiotics and restoration of bacterial eubiosis abolishes the onset of an intestinal barrier dysfunction (23, 36). Interestingly, decreasing intestinal fungal overgrowth with nonabsorbable antifungals did not alter gut barrier function, and intestinal permeability remained increased. These findings suggest that a main pathogenic mechanism for mycobacteria-associated progression of liver disease is an increase in intestinal fungal populations. Overgrowth of fungi produces more fungal products such as β-glucan, which can escape from the intestinal lumen through already dysfunctional enteric tight junctions to the liver. Thus, fungal dysbiosis and overgrowth do not seem to modulate the intestinal barrier function.

The mycobiome of mice is dominated by Candida parapsilosis; following alcohol feeding its abundance decreased, while other fungal populations increased. In humans, Candida albicans is the most abundant species in nonalcoholics, and its abundance is further increasing in alcoholics with nonprogressive alcoholic liver disease. Given these differences between alcohol-associated changes in the human and mouse mycobiome, our data indicate that compositional changes might not be causatively linked to progression of alcoholic liver disease. Rather, overgrowth of intestinal fungi in combination with a dysfunctional gut barrier results in increased systemic levels of β-glucan, eliciting a chronic inflammatory response in the liver. Future studies are required to determine whether single fungal species contribute to liver disease progression more than others by possibly generating larger amounts of β-glucan. Candida dubliniensis is increasing in patients with alcoholic hepatitis and is the most abundant Candida species in patients with end-stage liver disease due to alcohol abuse. C. dubliniensis is an opportunistic fungal pathogen recently shown to cause meningitis in a patient with hepatitis C virus–related cirrhosis (37). Chronic liver disease is an independent risk factor for C. dubliniensis bloodstream infections (38).

We have identified a cross-talk between bacterial and fungal pathogen-associated molecular patterns (PAMPs) during alcoholic liver disease. Hepatic IRAK3, a negative regulator of TLR signaling (24), was increased after reduction of an alcohol-associated increase in intestinal fungi. IRAK3-deficient mice are more susceptible to alcohol-induced liver injury (39). This indicates that in the absence of fungal PAMPs, bacterial signaling through TLRs and MYD88 is inhibited. A synergism between bacterial and fungal PAMPs is necessary for development of ethanol-induced liver disease.

Our study demonstrates an important role of fungal dysbiosis in the development of alcoholic liver disease. We found that antifungal drugs prevented ethanol-induced steatohepatitis in mice. This therapeutic strategy can easily be translated into clinical practice, because oral amphotericin B has no systemic side effects and is well tolerated by patients. Alcohol-associated fungal dysbiosis in humans is characterized by a large increase in Candida, which is susceptible to amphotericin B. The effects of this drug should be tested in patients with alcohol-related liver disease, who are in urgent need of new therapeutics. Manipulation of the intestinal mycobiome might be an effective strategy for attenuation of alcohol-related liver disease.
Methods

Mice. Clec7a−/− mice have been described (40). Heterozygous (Clec7a+−) mice on a C57BL/6 genetic background were bred to generate mice without disruption of Clec7a (WT) and mice with homozygous disruption (Clec7a−/− mice). Female mice (age, 8 weeks) were fed ethanol for 8 weeks (the Lieber-DeCarli diet model of chronic alcohol consumption), as previously described (23). The Lieber-DeCarli diet comprised 60% alcohol-containing and control diets. Female C57BL/6 mice (60 mg/kg/d) (41) (Medisca) was added to the liquid Lieber-DeCarli diet began 4 weeks after bone marrow transplantation. clodronate liposomes (5 mg/ml; Vrije Universiteit, Amsterdam, Netherlands) of radiation (600 rad) twice, using a 137Cs source. Two weeks after clodronate treatment, mice were injected i.p. with 200 μl of clodronate liposomes (5 mg/ml; Vrije Universiteit, Amsterdam, Netherlands) to deplete radiosensitive Kupffer cells. The Lieber-DeCarli diet began 4 weeks after bone marrow transplantation.

To reduce intestinal commensal fungi, nonabsorbable amphotericin B (60 mg/kg/d) (41) (Medisca) was added to the liquid Lieber-DeCarli alcohol-containing and control diets. Female C57BL/6 mice were purchased from Charles River Laboratories for this experiment.

To prove whether soluble β-glucan can activate CLEC7A in vivo, carboxymethyl-β-1,3,β-glucan (2 mg; CM-curdian, Wako) was injected i.p. into WT and Clec7a−/− mice. Control animals received a comparable vehicle (0.01 M NaOH, saline; pH 7) treatment. Mice were sacrificed 2, 8, and 24 hours after CM-curdian injection and investigated for plasma IL-1β levels and hepatic Il1b expression.

Real-time qPCR. RNA of mouse tissues was extracted using Trizol (Invitrogen). DNase treatment of RNA was performed using the DNA-free Kit (Ambion), and RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (ABI). Real-time qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) using primer sequences obtained from NIH qPrimerDepot (mouse and human) and a StepOnePlus thermocycler (ABI). The qPCR value was normalized to mouse 18S. Gene expression results are expressed relative to the levels of control (WT) mice or cells. DNA was isolated from feces as previously described (4, 21, 42). Published microbial primer sequences for 16S rRNA gene (43) and fungal-specific 18S rDNA gene were used (44). To quantify the total bacterial or fungal load present in feces, the qPCR value of fungal 18S rRNA gene or the bacterial 16S rRNA gene, respectively, was multiplied by the total amount of DNA (micrograms) per milligram of feces for each sample.

Sequencing and analysis. We performed deep DNA pyrosequencing of fecal DNA, targeting the hypervariable V4 region of prokaryotic 16S rRNA loci, using a dual indexing strategy on the Illumina MiSeq platform, as previously described (45). Microbial community profiles were generated using species-level (97% similarity) operational taxonomic unit-based (OTU-based) classification and analysis, as previously described by us (22). Sequence data were registered at NCBI under BioProject PRJNA317649. Sequence reads are available at NCBI under the following consecutive BioSample IDs: SAMN04874715–SAMN04874755.

To evaluate the human intestinal mycobiome, which has a lower abundance of fungi than that of humans, we optimized RNA extraction, quantitation, and amplification methods. Samples were vortexed in Qiogene lysing matrix B tubes using a Mini-Beadbeater instrument (Biospec Products). We then followed a published RNA isolation protocol (8). DNA samples were quantified using PicoGreen dsDNA assay (Life Technologies); 100 ng DNA was used for PCR with Q5 High Fidelity Polymerase (NEB) with BITS and B58S3 primers specific for ITS1 containing partial dual-indexed Illumina adapters as for 16S rRNA (47) under the following conditions: 98°C denaturing step followed by 39 cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, followed by a final extension step of 72°C for 5 minutes. Amplicons were purified using the Genejet PCR purification system (Thermo Fisher Scientific). Purified amplicons were then quantified using SYBR Gold (Thermo Fisher Scientific), normalized, and pooled to generate a library of equimolar DNA molecules per sample.

To evaluate the mouse intestinal mycobiome, which has a lower abundance of fungi than that of humans, we optimized RNA extraction, quantitation, and amplification methods. Samples were vortexed in Qiogene lysing matrix B tubes using a Mini-Beadbeater instrument (Biospec Products). We then followed a published RNA isolation protocol (8). DNA samples were quantified using PicoGreen dsDNA assay (Life Technologies); 100 ng DNA was used for PCR with Q5 High Fidelity Polymerase (NEB) with BITS and B58S3 primers specific for ITS1 containing partial dual-indexed Illumina adapters as for 16S rRNA (47) under the following conditions: 98°C denaturing step followed by 39 cycles of 98°C for 10 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, followed by a final extension step of 72°C for 2 minutes. Amplicons were purified twice with Agencourt AMPure beads (Beckman Coulter) at 0.9 beads to DNA ratio. To assess whether amplicons were free of adapter dimers and to verify insert size, amplicons were analyzed and quantified on the Bioanalyzer, using the high-sensitivity dsDNA chip (Agilent), and pooled. The final pool was quantified with Library Quant kit (Kapa Biosystems). Sequences were generated using the MiSeq 2*150 V2 kit (Illumina) with 35% PhiX library added and custom primers spiked in. The ITS and index primer sequences were the same as those used for the human mycobiome.

Sequence processing and analysis steps were performed by our open-access, mothur-based (49) workflow software, YAP (46, 50). Mothur version 1.34 was used in this study. To achieve acceptable memory and run-time scalability with large MiSeq data sets, the pre-clustering and clustering stages of the pipeline were implemented with CD-HIT (51, 52) sequence clustering software instead of the corresponding built-in mothur commands. Taxonomic classification of assembled forward and reverse reads was performed by mothur’s implementation of the RDP naïve Bayesian Classifier (53) using version 1 of the manually curated targeted host-associated fungi (THF) database (54), trimmed to include only the ITS1 region amplified by the BITS/B58S3 primers, removal of non-ITS sequences, and trimmed sequences less than 45 nucleotides in length, and then formatted for use with mothur. Fungal species-level OTUs were defined at 97% sequence identity (47). Candida species-level classification of ITS sequences was performed using STIRRUPS (55) with a custom sequence library of 31 non-redundant representative sequences pulled from the trimmed THF database. Sequence data were registered at NCBI under BioProject PRJNA317653. Sequence reads are available from the NCBI under the following consecutive BioSample IDs: SAMN04874830–SAMN04874861.
Detection Kit (Thermo Fisher Scientific). Oil Red O staining was used to identify lipid accumulation in cultured hepatocytes.

Hepatocyte death was quantified using the Pierce LDH Cytotoxicity ELISA. Plasma was diluted 1:4 for 1,3-β-d-glucan measurements. IL-1β neutralization experiments, Kupffer cells were cultured with RPMI 1640 medium containing 10% FBS for 4 hours and serum starved in RPMI 1640 and Medium 199 (50% vol/vol) overnight. Kupffer cells were then stimulated with curdlan for 8 hours. Cell supernatants were transferred to hepatocytes, and incubated for 24 hours. A neutralizing antibody against IL-1β (10 ng/ml; ab9722; Abcam) or isotype IgG (10 ng/ml, control) was added to the conditioned medium after the transfer. Hepatocyte cytotoxicity and intracellular lipid staining experiments were performed.

Isolation of human liver cells. Primary human hepatocytes, Kupffer cells, and hepatic stellate cells were isolated as previously described (59, 60). RNA extraction and quantitative reverse transcriptase PCR were performed as previously described (60) using specific primers for human CLEC7A (NIH human qPrimerDepot) and 18S RNA (forward: 5′-AACCGGCTTACCACATCCAAG-3′; reverse: 5′-CCTCAATGGATCCTCGTTA-3′). 18S was used for normalization. Liver tissues used in this study were provided anonymized by the Biobank under the Administration of the Human Tissue and Cell Research (HTCR) Foundation at the Hospital of the LMU Munich (http://www.klinikum.uni-muenchen.de/Chirurgische-Klinik-und-Poliklinik-Grosshadern/de/0800-gewebebank/index.html). The framework of HTCR Foundation (http://www.htcr.org) (61) includes obtaining written informed consent from all donors after the nature and possible consequences of the donation were explained. This framework has been approved by the ethics commission of the Faculty of Medicine in the University of Munich (no. 025-12) as well as the Bavarian State Medical Association (no. 11142).

Human serum samples. We analyzed data collected from 28 patients with alcoholic liver cirrhosis from July 1, 2010, through October 31, 2010, as previously described (62). In brief, our analysis excluded patients who tested positive for hepatitis C antibody or hepatitis B surface antigen or had any other liver diseases. Liver cirrhosis was detected by ultrasonography (based on presence of cirrhotic liver parenchyma), presence of esophageal or gastric varices (based on upper endoscopy examination), or splenomegaly (based on imaging studies). Continuous alcohol drinking was defined as ingestion of more than 60 g/d of alcohol for more than 10 years in men and more than 20 g/d for more than 10 years in women. We also analyzed data from 2 control cohorts of similar age and sex distribution: 14 healthy individuals and 43 patients with HBV-related liver cirrhosis who did not consume alcohol. Patient characteristics are presented in Supplemental Table 1.

Patients did not take antibiotics or immunosuppressive agents during the 2 months preceding enrollment. Other exclusion criteria were diabetes, inflammatory bowel disease, known liver disease of any other etiology, and clinically significant cardiovascular, pulmonary, or renal comorbidities. The study protocol was approved by the Ethics Committee of En Chu Kong Hospital, and a written informed consent was signed by each participant after the nature and possible consequences of the donation were explained. The ethics commission of the Faculty of Medicine in the University of Munich (no. 025-12) as well as the Bavarian State Medical Association (no. 11142).
Human fecal samples. Healthy individuals without chronic disease (controls, n = 8), alcohol-dependent patients without evidence of progressive liver disease (nonprogressive liver disease, n = 10), patients with alcoholic hepatitis (alcoholic hepatitis, n = 6), and patients with alcoholic liver cirrhosis (alcoholic cirrhosis, n = 4) were included in the study. Diagnosis of alcoholic hepatitis was made by biopsy in 3 of 6 patients. The remaining patients with alcoholic hepatitis and all patients with alcoholic cirrhosis were diagnosed based on a combination of clinical, biochemical, and imaging parameters. All patients included were actively drinking. Baseline features of this cohort are provided in Supplemental Table 2. Stool samples were collected, and DNA was extracted from fecal samples (21). Written informed consent was signed by each participant after the nature and possible consequences of the studies were explained. The study protocol was approved by the IRB of each institution involved.

Human liver and duodenal biopsies. Patients with alcohol dependence and with active alcohol consumption were compared with individuals without alcohol dependency (controls). Duodenal biopsies were taken and immediately snap-frozen from otherwise normal subjects who underwent outpatient upper gastrointestinal endoscopy for gastroesophageal reflux symptoms. Only biopsies from subjects with endoscopically and histologically normal duodenum were finally retained as true controls. Alcohol-dependent patients who followed an inpatient alcohol withdrawal program underwent routine upper gastrointestinal endoscopy with duodenal biopsy on the second day of admission. Patients with disturbed liver function tests and suspicion of significant fibrosis determined noninvasively by Fibroscan were proposed for a liver biopsy. Liver biopsy was performed on the third day of admission in patients. Liver specimens from control patients were obtained from surgical resections or from size-reduced donor livers before transplantation. Written informed consent was signed by each participant after the nature and possible consequences of the studies were explained. The study protocol was approved by the Ethics Committee of the Université Catholique de Louvain.

Statistics. Results are expressed as mean ± SEM. Significance was evaluated using the unpaired Student’s t test except when stated otherwise. For comparison of 3 groups, 1-way ANOVA with Newman-Keuls post-test was used. Not normally distributed (Shapiro-Wilk test) serum levels of ASCA were transformed by taking the logarithm to the base of 10. Significance of mouse microbiome data was determined using the Kruskal-Wallis rank-sum statistical test with YAP. Kaplan-Meier curves were used to compare survival between groups; significance was assessed using the log-rank test. A P value less than 0.05 was considered to be statistically significant.

Study approval. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of UCSD. Studies using human materials were reviewed and approved by an appropriate IRB: (a) ethics commission of the Faculty of Medicine in the University of Munich (no. 025-12) as well as the Bavarian State Medical Association (no. 11142) (human liver cell isolation); (b) Ethics Committee of En Chu Kong Hospital (human serum samples, human cohort study); (c) Human Research Protections Program of UCSD and of the VA San Diego Healthcare System; IRB at Rush University Medical Center; Yale University IRB; University of Wisconsin–Madison Health Sciences IRBs (Madison, Wisconsin, USA); University of North Carolina at Chapel Hill IRB; Ethics Committee of the Université Catholique de Louvain (human fecal samples); (d) Ethics Committee of the Université Catholique de Louvain (human duodenal and liver biopsies). All subjects provided informed consent prior to their participation in the study.

Author contributions
AMY and TI were responsible for acquisition of data, analysis and interpretation of data, and drafting of the manuscript. PG, LW, CL, SB, KH, PH, JX, and YK assisted with experiments and data analysis. MGT, KM, KB, and DEF were responsible for deep sequencing and analysis. TK, CSC, HMH, and GDB provided experimental resources. KF, CH, and SMLL analyzed human liver cells. WZM, GGT, EAM, AK, SBH, RB, and PS were responsible for collection of human samples. BS was responsible for the study concept and design, writing of the manuscript, and study supervision.

Acknowledgments
This study was supported in part by NIH grants R01 AA020703, U01 AA24726 and U01 AA021856 and by Award 101BX002213 from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development (to BS). KH was supported by a DFG fellowship (HO/5690/1-1). SB was supported by a grant from the Swiss National Science Foundation (P2SKP3_158649). GGT received funding from the Yale Liver Center NIH P30 DK34989 and RB from National Institute on Alcohol Abuse and Alcoholism grant U01 AA021908. AK received support from NIH grants RC2 AA019405, R01 AA020216, and R01 AA023417. GDB is supported by funds from the Wellcome Trust. We acknowledge the Human Tissue and Cell Research (HTCR) Foundation for making human tissue available for research, and Hepacult GmbH (Munich, Germany) for providing primary human hepatocytes for in vitro analyses. We thank Chien-Yu Lin, Department of Medicine, Fu-Jen Catholic University, Taiwan, for statistical analysis.

Address correspondence to: Bernd Schnabl, Department of Medicine, University of California, San Diego, MCO063, 9500 Gilman Drive, La Jolla, California 92093, USA. Phone: 858.822.5311; Email: beschnabl@ucsd.edu.

6. Keshavarzian A, et al. Evidence that chronic alco-
Research Article


7. Mutlu EA, et al. Colonic microbiome is altered in alcoholism. Am J Physiol Gastrointest Liver Physi-


19. Kankunpen P, Teirilä L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)-β-glucans activate both dectin-1 and NLRP3 inflamma-


24. Kobayashi K, Hernandez LD, Galán JE, Janeway CA, Medzhivor R, Flavell RA. IRAK-M is a nega-


2016-310970.


34. Kirpich IA, et al. Saturated and unsaturated dietary fats differentially modulate ethanol-induced changes in gut microbiome and metabo-


36. Wang Y, et al. Laetoctobacillus rhamnosus GG treat-

37. Yamahiro A, Lau KH, Peaper DR, Villanueva MT, Wolff H, Matikainen S. (1,3)-β-glucan recognition and control of fungal infec-

38. Wagner M, Srivastava KK. Decontamination of gnotobiotic mice experimentally monos-associ-


43. Bokulich NA, Thornage JH, Richardson PM, Mills DA. Microbial biogeography of wine grapes is conditioned by cultivar, vint-

44. Schloss PD, et al. Introducing mothur: open-source, platform-independent, community-sup-


46. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-gen-

47. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of pro-

48. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assign-


