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Dysbiosis-Induced Intestinal Inflammation Activates Tumor Necrosis Factor Receptor I and Mediates Alcoholic Liver Disease in Mice

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Intestinal barrier dysfunction is an important contributor to alcoholic liver disease (ALD). Translocated microbial products trigger an inflammatory response in the liver and contribute to steatohepatitis. Our aim was to investigate mechanisms of barrier disruption after chronic alcohol feeding. A Lieber-DeCarli model was used to induce intestinal dysbiosis, increased intestinal permeability, and liver disease in mice. Alcohol feeding for 8 weeks induced intestinal inflammation in the jejunum, which is characterized by an increased number of tumor necrosis factor alpha (TNF-α)-producing monocytes and macrophages. These findings were confirmed in duodenal biopsies from patients with chronic alcohol abuse. Intestinal decontamination with nonabsorbable antibiotics restored eubiosis, decreased intestinal inflammation and permeability, and reduced ALD in mice. TNF-receptor I (TNFRI) mutant mice were protected from intestinal barrier dysfunction and ALD. To investigate whether TNFRI on intestinal epithelial cells mediates intestinal barrier dysfunction and ALD, we used TNFRI mutant mice carrying a conditional gain-of-function allele for this receptor. Reactivation of TNFRI on intestinal epithelial cells resulted in increased intestinal permeability and liver disease that is similar to wild-type mice after alcohol feeding, suggesting that enteric TNFRI promotes intestinal barrier dysfunction. Myosin light-chain kinase (MLCK) is a downstream target of TNF-α and was phosphorylated in intestinal epithelial cells after alcohol administration. Using MLCK-deficient mice, we further demonstrate a partial contribution of MLCK to intestinal barrier dysfunction and liver disease after chronic alcohol feeding. *Conclusion*: Dysbiosis-induced intestinal inflammation and TNFRI signaling in intestinal epithelial cells mediate a disruption of the intestinal barrier. Therefore, intestinal TNFRI is a crucial mediator of ALD. (HEPATOLOGY 2015;61:883-894)

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Alcohol abuse is one of the leading causes of chronic liver disease (CLD) and liver-related deaths worldwide. A prominent feature of alcohol abuse is disruption of intestinal barrier function.[1] Increased intestinal permeability is present in preclinical animal models and in patients with alcohol abuse.[2,3] Microbial products, such as lipopolysaccharide (LPS), translocate from the intestinal lumen to the extraintestinal space, blood, and liver.[4] In the liver, bacterial products induce inflammation and synergize with ethanol-induced hepatotoxicity to cause steatosis, steatohepatitis, and fibrosis.[5] Mice that express nonfunctional Toll-like receptor 4 (TLR4) or nonfunctional molecules of the LPS-signaling pathway are protected from experimental alcoholic liver disease

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; Ccl, chemokine (C-C motif) ligand; CLD, chronic liver disease; DCs, dendritic cells; FACS, fluorescence-activated cell sorting; IF, immunofluorescence; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MLCK, myosin light-chain kinase; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; TG, triglyceride; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TNFRI, tumor necrosis factor receptor I; VDAC1, voltage-dependent anion-selective channel protein 1; WT, wild type.

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(ALD).[6] Levels of LPS in the portal vein of mice with a nonfunctional TLR4 expression are similar to wild-type (WT) mice, indicating that TLR4 does not control intestinal permeability and that protection from disease occurs at the level of the liver.

The exact molecular mechanism of increased intestinal permeability during ALD is not known. Acetaldehyde, as the major oxidative metabolite of ethanol, induces Caco-2 cell monolayer disruption by tight-junction protein redistribution,[7] and several potential mediators, such as Snail, protein phosphatase 2A (PP2A), extracellular signal-regulated kinase (ERK), protein kinase C (PKC), and protein tyrosine phosphatase (PTPase), are involved.[8-12] In addition, increased inducible nitric oxide synthases (iNOSs) and myosin light-chain kinase (MLCK) expression correlated with barrier function disruption in differentiated Caco-2 cells.[13,14] Most studies are using cell-culture systems, and there are no *in vivo* data investigating the pathway that induces intestinal barrier disruption after chronic alcohol administration.

In the current study, we used an animal model of chronic ALD to demonstrate that intestinal dysbiosis induces tumor necrosis factor alpha (TNF-α) production in inflammatory cells of the intestinal lamina propria. TNF-receptor I (TNFRI) expressed on intestinal epithelial cells, mediates tight-junction disruption partially by activation of MLCK.

Materials and Methods

Animal Models of Alcohol Feeding. TNFRI$^{flxneo/flxneo}$, VillinCreTNFRI$^{flxneo/flxneo}$, and MLCK-deficient mice have been described and were all in a C57BL/6 genetic background. TNFRI$^{flxneo/flxneo}$ [15] and VillinCreTNFRI$^{flxneo/flxneo}$ [16] were kindly provided by Drs. Manolis Roulis and George Kollias (Biomedical Sciences Research Center 'Alexander Fleming', Vari, Greece), and littermates were used for the experiments. C57BL/6 WT mice were bred in the same room of our vivarium and used as controls for experiments involving TNFRI$^{flxneo/flxneo}$ and VillinCreTNFRI$^{flxneo/flxneo}$ mice. TNFR2-deficient mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were kindly provided by Dr. William McBride (University of California Los Angeles, Los Angeles, CA). Heterozygous long MLCK$^{-/-}$ mice[17] were crossed, and WT and knockout littermates were used for all experiments. The Lieber-DeCarli diet model of alcohol feeding was used for 8 weeks.

Other materials and methods are described in the Materials and Methods section in the Supporting Information.

Results

Chronic Alcohol Feeding Enhances TNF-α Expression in the Jejunum of Mice. TNF-α disrupts intestinal tight junctions and is a well-characterized mediator of intestinal barrier dysfunction.[18] Therefore, we assessed whether intestinal TNF-α is increased in an animal model of chronic alcohol feeding for 8 weeks. TNF-α gene expression was significantly induced in the jejunum of alcohol-fed mice, compared to isocaloric controls (Fig. 1A). Because intestinal inflammation caused by inflammatory cells in the lamina propria is involved in increasing intestinal permeability,[19] lamina propria cells were isolated and separated from epithelial cells. Increased jejunal TNF-α was the result of an induction of gene expression in isolated lamina propria cells of mice subjected to alcohol feeding (Fig. 1B). Fluorescence-activated cell sorting (FACS) analysis was used to further characterize the innate immune cell infiltrate producing TNF-α. The number of TNF-α$^+$ monocytes and macrophages, but not dendritic cells (DCs), was significantly increased in the jejunum after 8 weeks of alcohol feeding (Fig. 1C). TNF-α$^+$ innate immune cells were not elevated in the ileum and colon after ethanol administration in mice (Supporting Fig. 1). Moreover, the absolute number of monocytes, macrophages, or DCs was not significantly elevated in the small or large intestine after alcohol administration (Supporting Fig. 2). These results indicate that the induction of TNF-α expression does not result from an increased infiltration of innate immune cells, but is rather a consequence of innate immune cell activation.

Alcohol Abuse Increases TNF-α Production of Intestinal Monocytes and Macrophages in Humans. To confirm these results in humans, duodenal biopsies from healthy individuals and patients with chronic

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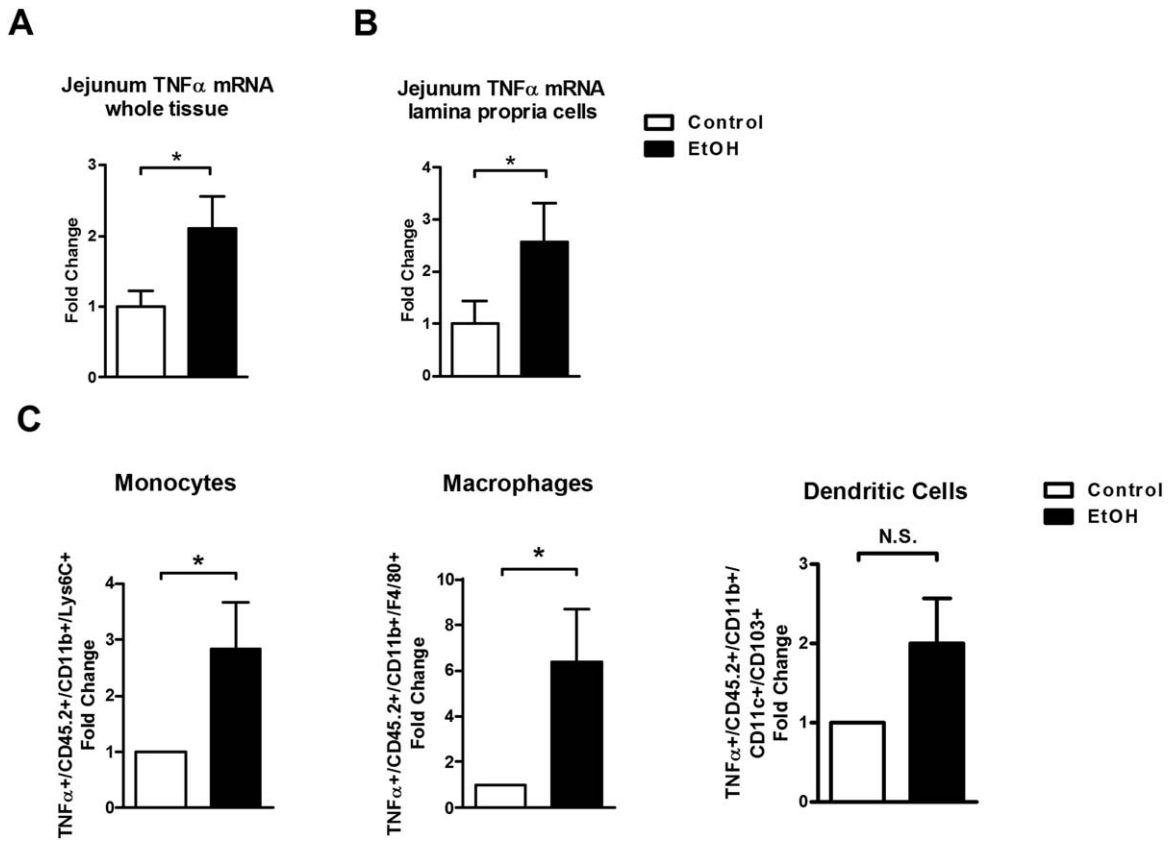

Fig. 1. Chronic ethanol administration elevates intestinal TNF-α production in mice. C57BL/6 mice were orally fed a control or alcohol diet for 8 weeks. (A) TNF-α mRNA level in jejunum (n = 14-19). (B) TNF-α mRNA level in isolated lamina propria cells of the jejunum (n = 5-9). (C) Relative amount of TNF-α^+ inflammatory cells isolated from the jejunum and analyzed by FACS (n = 3-4). *$P < 0.05$, Abbreviations: EtOH, ethanol; N.S., no significance.

alcohol abuse were examined. Similar to findings in our preclinical model of ALD, TNF-α messenger RNA (mRNA) expression was significantly higher in duodenal biopsies from alcoholics (Fig. 2A). Using CD68 that is primarily expressed by human monocytes and macrophages, immunofluorescent (IF) staining demonstrated a significant increase of CD68/TNF-α double-positive cells in the lamina propria of alcoholic patients (Fig. 2B,C). As a result of limited availability of jejunal, ileal, and colonic samples from alcoholics, our experiments were restricted to examination of duodenal samples. These data suggest that chronic alcohol consumption results in increased intestinal TNF-α production in humans.

Enteric Dysbiosis Induces Intestinal TNFα During ALD Development. We and others have recently shown that chronic alcohol feeding results in dysbiosis with qualitative and quantitative disturbances in the microbiome using an intragastric feeding model for 3 weeks or Lieber-DeCarli feeding, respectively.[20-22] Interestingly, intestinal permeability is significantly increased in the jejunum after alcohol feeding (Supporting Fig. 3), and intestinal bacterial overgrowth is most pronounced in the jejunum,[20] the intestinal site with elevated levels of TNF-α. Given that dysbiosis is associated with intestinal inflammation,[23] we next investigated whether restoration of eubiosis abolishes the induction of intestinal TNF-α. Mice were subjected to alcohol or isocaloric diet feeding, and depletion of the commensal microflora was performed using nonabsorbable antibiotics Polymyxin B and Neomycin for the last 4 weeks. Intestinal bacterial overgrowth was induced after alcohol feeding, but completely abolished in mice receiving nonabsorbable antibiotics and subjected to alcohol feeding (Fig. 3A). Importantly, reducing the intestinal bacterial burden significantly reduced intestinal TNF-α expression after chronic alcohol feeding (Fig. 3B). Consistent with gene expression data, nonabsorbable antibiotics reduced the number of TNF-α-positive monocytes and macrophages in the lamina propria of the jejunum (Fig. 3C,D). Moreover, the intestinal barrier was stabilized by preventing a decrease of the tight-junction protein, occludin, after chronic alcohol administration (Fig. 3E). Nonabsorbable antibiotics prevented an increase of intestinal permeability in alcohol-fed mice,

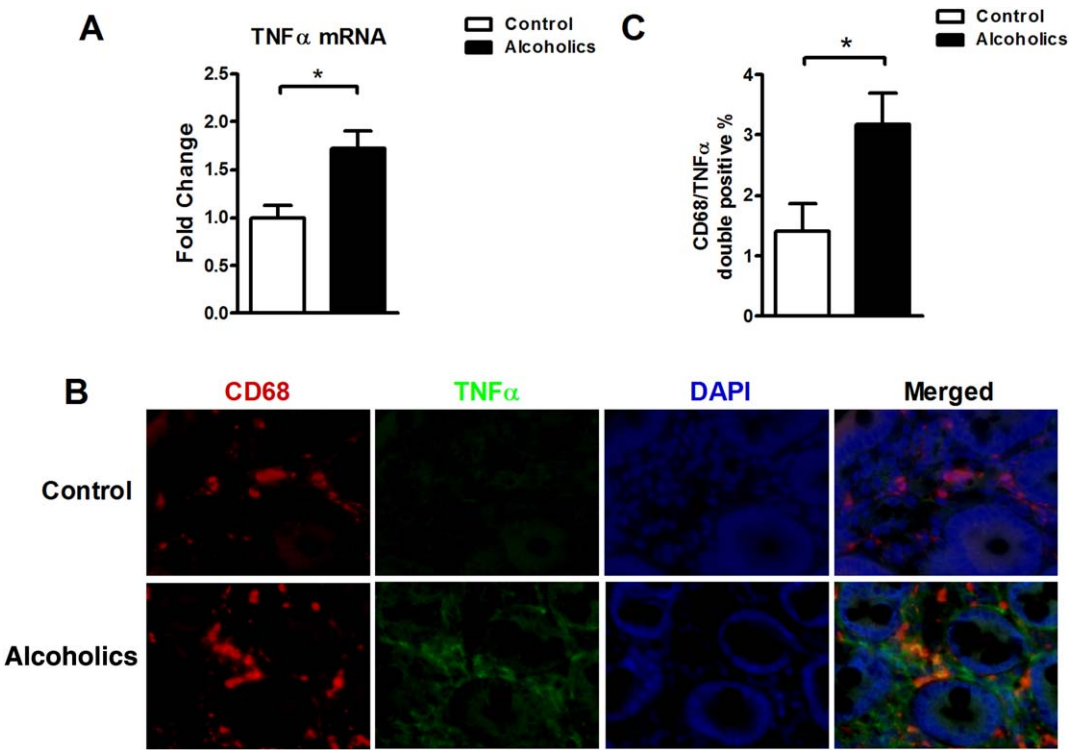

Fig. 2. Alcohol abuse increases intestinal inflammatory TNF-α^+ cells in humans. (A) TNF-α mRNA expression in duodenal biopsies obtained from healthy controls (n = 15) and patients with chronic alcohol abuse (n = 22). (B and C) Duodenal biopsies obtained from healthy controls (n = 11) and patients with chronic alcohol abuse (n = 8) were stained with CD68 (red) and TNF-α (green) by IF. Nuclei are stained in blue. (B) Representative intestinal sections are shown (magnification, 200×). (C) Quantification of CD68/TNF-α double-positive cells. *$P < 0.05$. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole.

as determined by fecal albumin enzyme-linked immunosorbent assay (Fig. 3F). We confirmed previously published results from rats that intestinal decontamination reduces ALD,[24] as evidenced by decreased liver injury (Supporting Fig. 4A) and steatosis (Supporting Fig. 4B,C). Additionally, oral antibiotics restored liver/body-weight ratio (Supporting Fig. 5A), but did not alter intestinal ethanol absorption (Supporting Fig. 5B). Using a time-course experiment, we found that intestinal inflammation preceded the onset of increased intestinal permeability (Fig. 3G,H). We assessed whether changes in epithelial integrity contribute to gut barrier dysfunction, but found that the morphology of intestinal villi was normal in the jejunum after chronic alcohol feeding (Supporting Fig. 6A). In addition, there was no significant difference in the apoptosis rate of jejunal enterocytes between control and alcohol mice (Supporting Fig. 6B). Our findings suggest that alcohol-associated enteric dysbiosis induces intestinal inflammation and increases intestinal permeability.

TNFRI on Enterocytes Induces Intestinal Barrier Dysfunction After Chronic Alcohol Feeding. To further define the role of TNF-α in mediating intestinal barrier dysfunction after alcohol administration, we focused on the main receptor for TNF-α, TNFRI. Given that experimental ALD depends on increased intestinal permeability and translocation of microbial products from the intestinal lumen to the liver,[4] we investigated the contribution of TNFRI expressed on enterocytes to intestinal barrier regulation. We used mutant mice carrying a conditional gain-of-function allele for TNFRI with an introduced loxP-flanked neomycin-resistance cassette in intron 5 of the murine *p55TnfR* gene ($TNFRI^{flxneo/flxneo}$).[15] A nonfunctional TNFRI allele is engineered to be reactivated specifically in villin expressing intestinal epithelial cells by a Villin Cre-loxP-mediated recombination. By crossing $TNFRI^{flxneo/flxneo}$ mice with the intestinal epithelial cell-specific VillinCre transgenic mouse, a functional TNFRI was selectively expressed on intestinal epithelial cells ($VillinCreTNFRI^{flxneo/flxneo}$),[16,25] whereas all other tissues and organs (including the liver) were mutant for TNFRI. We confirmed, by western blotting, that TNFRI was absent in intestinal epithelial cells isolated from TNFRI mutant $TNFRI^{flxneo/flxneo}$ mice, whereas TNFRI was reactivated and expressed on enterocytes isolated from $VillinCreTNFRI^{flxneo/flxneo}$

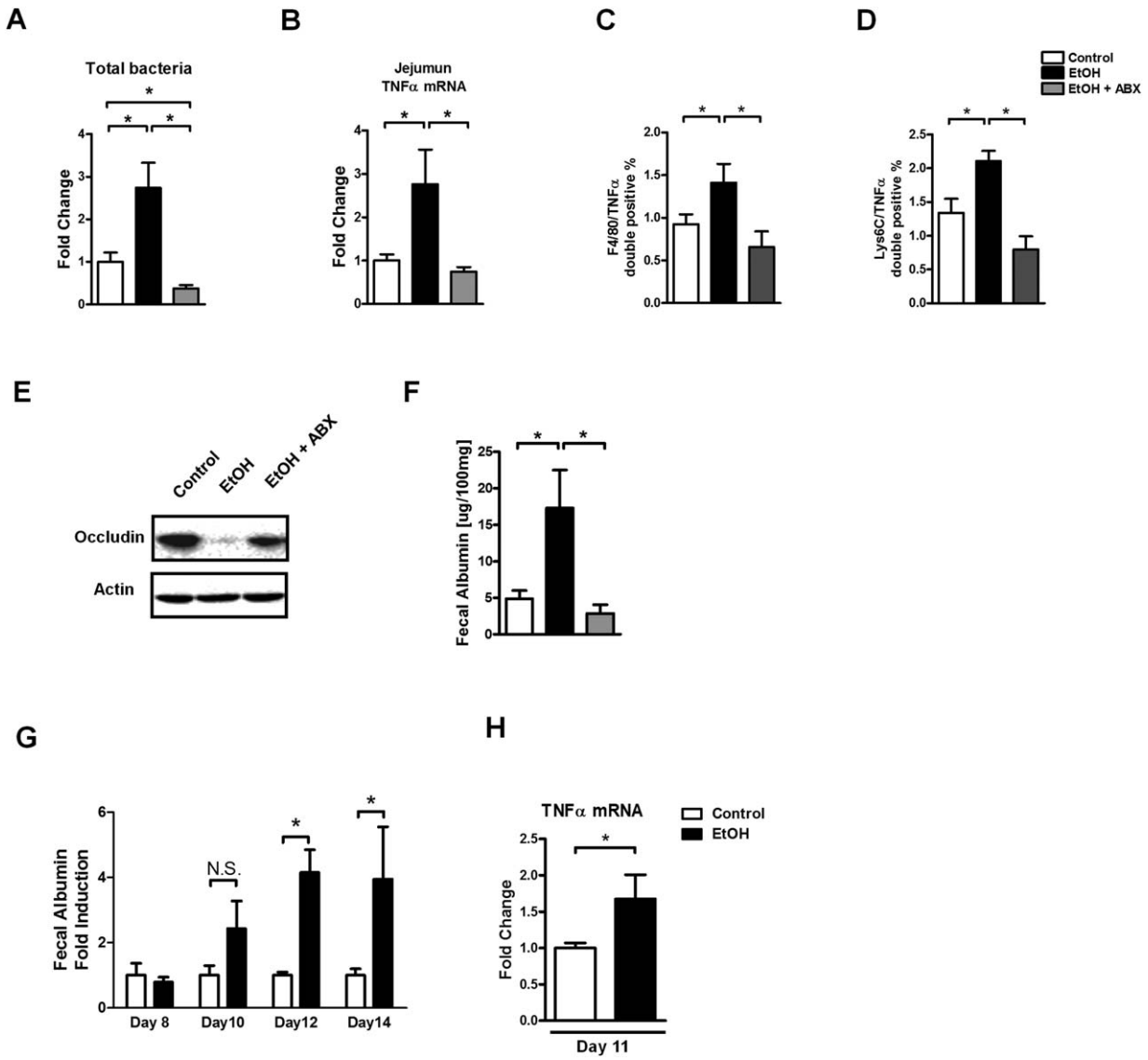

Fig. 3. Intestinal decontamination inhibits alcohol-induced dysbiosis, intestinal inflammation, and barrier dysfunction. (A-F) C57BL/6 mice were orally fed a control diet (n = 9), alcohol diet (n = 9), and alcohol diet plus antibiotics (ABX; n = 9). (A) Total cecal bacteria. (B) Jejunal TNF-α mRNA level. (C) Quantification of F4/80 TNF-α double-positive cells in the jejunum, as assessed by IF staining. (D) Quantification of Lys6C TNF-α double-positive cells in the jejunum, as assessed by IF staining. (E) Representative western blotting for occludin in the jejunum. (F) Fecal albumin content. (G-H) C57BL/6 mice were orally fed a control or alcohol diet for indicated time points (n = 4-7). (G) Fecal albumin content. Values are presented relative to control-fed animals. (H) Jejunum TNF-α mRNA level after 11 days of control and alcohol diet feeding. *$P < 0.05$. Abbreviations: EtOH, ethanol; N.S., no significance.

mice (Supporting Fig. 7A). In addition, TNFRI was not expressed in liver of both strains. WT mice showed TNFRI expression on intestinal epithelial cells and in liver (Supporting Fig. 7A,B).

TNFRI mutant ($TNFRI^{flxneo/flxneo}$) mice were protected from an increase in intestinal permeability after alcohol feeding for 8 weeks, compared to WT mice, as measured by fecal albumin. Levels of fecal albumin were significantly higher in mice with a selective reactivation of TNFRI on intestinal epithelial cells ($VillinCreTNFRI^{flxneo/flxneo}$), as compared to TNFRI mutant ($TNFRI^{flxneo/flxneo}$), but similar to C57BL/6 WT mice (Fig. 4A). Hepatic contents of *Escherichia coli* proteins were determined as the measure of bacterial translocation originating from the intestine. After alcohol feeding, livers from $TNFRI^{flxneo/flxneo}$ mice showed significantly less translocated *E. coli* proteins, as compared to WT mice. However, $VillinCreTNFRI^{flxneo/flxneo}$ mice demonstrated more *E. coli* translocation than their $TNFRI^{flxneo/flxneo}$ littermates (Fig. 4B). Changes in tight-junction protein expression are mediating the paracellular leakage pathway in the intestine. Therefore, we assessed protein level and integrity of the tight-junction protein, occludin, in the jejunum using western blotting and IF. WT mice

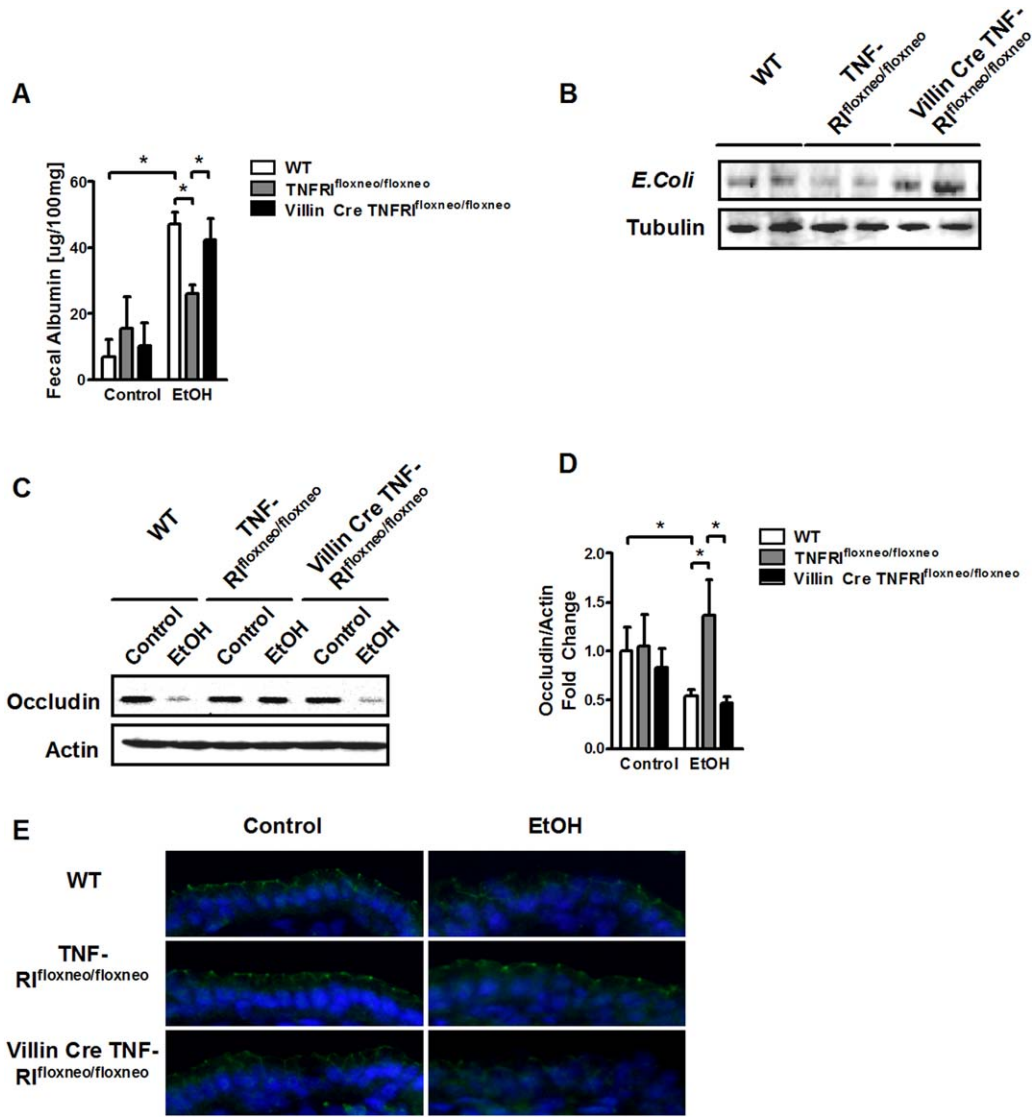

Fig. 4. Reactivation of TNFRI on intestinal epithelial cells mediates alcohol-induced intestinal permeability in TNFRI mutant mice. WT, TNFRI$^{flxneo/flxneo}$, and VillinCre TNFRI$^{flxneo/flxneo}$ mice were orally fed a control (n = 3-4) and alcohol diet (n = 7-9). (A) Fecal albumin content. (B) Western blotting for *E. coli* proteins in liver. (C) Representative western blotting for occludin in the jejunum. (D) Occludin quantification of western blottings. (E) Representative IF staining for occludin; nuclei are stained blue. $^*P < 0.05$. Abbreviation: EtOH, ethanol.

showed a lower protein expression of occludin after ethanol feeding for 8 weeks, as compared to isocaloric-diet–fed mice. Whereas TNFRI$^{flxneo/flxneo}$ mice carrying a mutation in TNFRI are protected from tight-junction disruption in the jejunum, VillinCreTNFRI$^{flxneo/flxneo}$ mice, which have a functional TNFRI selectively expressed on intestinal epithelial cells, show a disruption of tight-junction proteins (Fig. 4C-E). TNFRI expression in intestinal epithelial cells was not affected by alcohol feeding (Supporting Fig. 8). TNF-α mRNA level and TNF-α protein expression by monocytes and macrophages were comparable in the jejunum of all groups after ethanol feeding (Supporting Fig. 9). In contrast to a local secretion of TNF-α by activated monocytes and macrophages in the intestinal lamina propria, it is also conceivable that systemic TNF-α is elevated during ALD and activates TNFRI on intestinal epithelial cells to induce tight-junction disruption. However, plasma TNF-α levels were below the detection limit, both in control diet- and ethanol-fed mice (Supporting Fig. 10). Taken together, mice with reactivation of TNFRI on enterocytes lose their protection against tight-junction disruption, as compared to TNFRI mutant mice, suggesting that TNFRI on intestinal epithelial cells mediates intestinal barrier loss after chronic alcohol administration.

TNFRI on Intestinal Epithelial Cells Mediates ALD. We next determined whether changes in intestinal permeability directly translate into differences in ALD. Consistent with previous findings[26], TNFRI

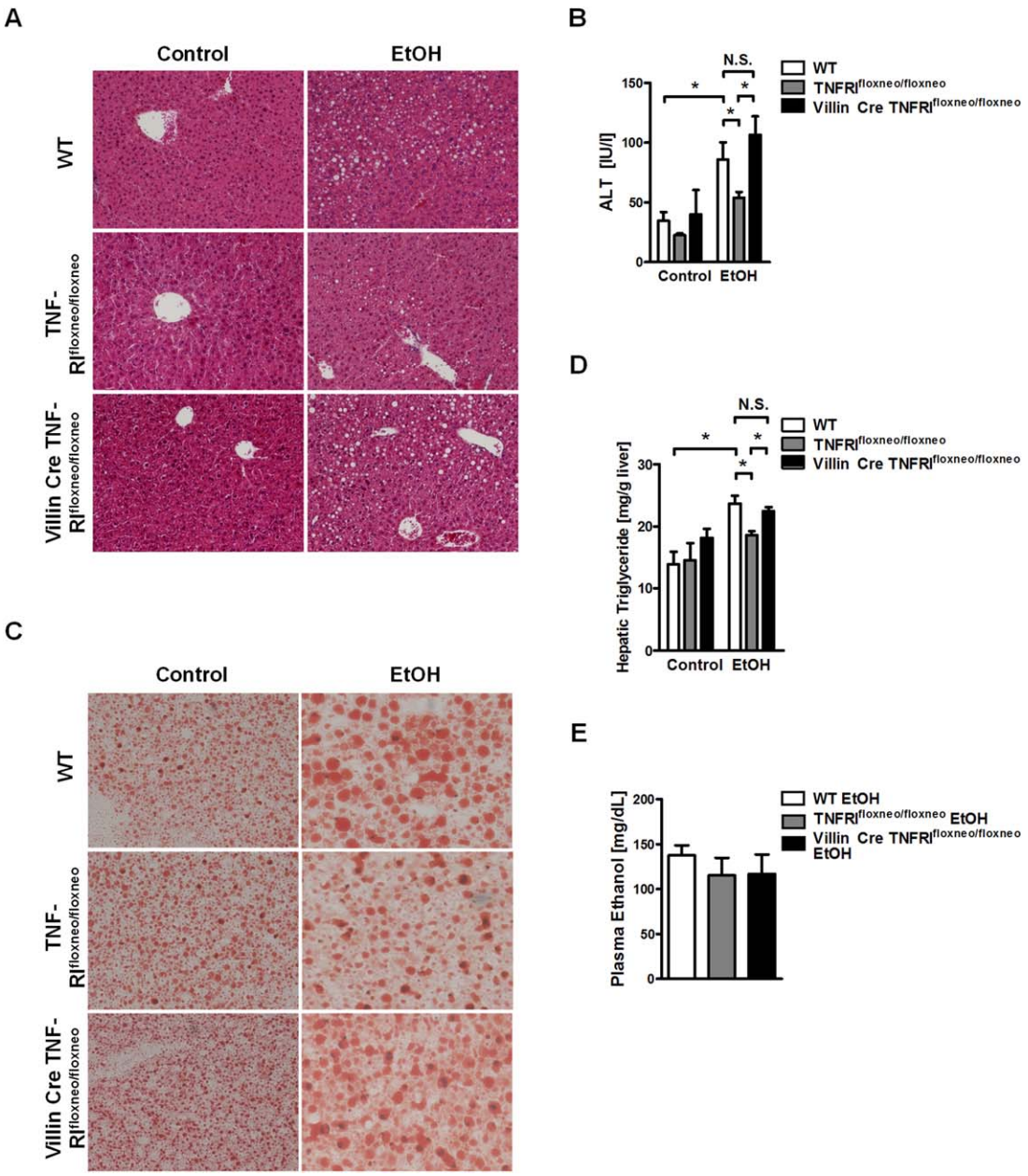

Fig. 5. Reactivation of TNFRI on intestinal epithelial cells promotes ALD in TNFRI mutant mice. WT, TNFRI$^{flxneo/flxneo}$, and VillinCre TNFRI$^{flxneo/flxneo}$ mice were orally fed a control (n = 3-4) and alcohol diet (n = 7-9). (A) Representative liver sections after hematoxylin and eosin staining. (B) Plasma ALT level. (C) Representative liver sections after Oil Red O staining. (D) Hepatic TG content. (E) Plasma ethanol concentration. Magnification, 200×. *$P < 0.05$. Abbreviations: EtOH, ethanol; N.S., no significance.

mutant (TNFRI$^{flxneo/flxneo}$) mice showed less liver injury, as assessed by plasma alanine aminotransferase (ALT) level (Fig. 5A,B), and decreased hepatic triglyceride (TG) contents after alcohol feeding, compared to WT mice (Fig. 5C,D), confirming that a nonfunctional TNFRI protects mice from ALD. VillinCreTNFRI$^{flxneo/flxneo}$ mice that have a functional TNFRI selectively on intestinal epithelial cells showed a significantly higher liver/body-weight ratio (Supporting Fig. 11A), more liver injury (Fig. 5A,B), and increased hepatic steatosis (Fig. 5C,D) than TNFRI$^{flxneo/flxneo}$ mice, but comparable with WT mice after chronic alcohol feeding. There is no significant difference in alcohol-induced liver injury and hepatic steatosis between VillinCreTNFRI$^{flxneo/flxneo}$ and WT mice. Plasma ethanol levels (Fig. 5E) and hepatic ethanol metabolism (Supporting Fig. 11B,C) were similar among the three groups of mice. In summary, TNFRI on intestinal epithelial cells contributes to the paracellular leakage pathway, resulting in bacterial translocation, and promotes ALD.

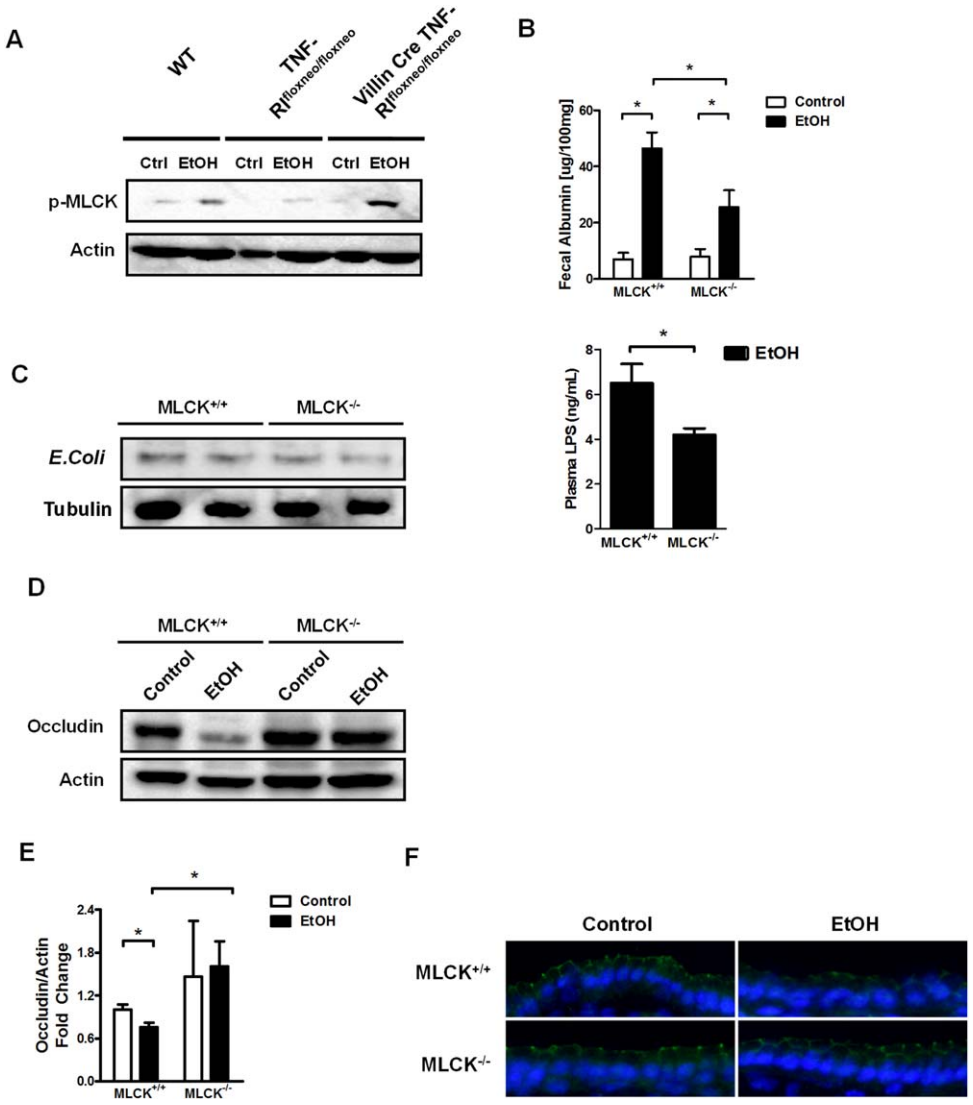

Fig. 6. MLCK is involved in TNF-α signaling and contributes to alcohol-induced barrier loss. (A) WT, TNFRI$^{flx-neo/flxneo}$, and VillinCre TNFRI$^{flxneo/flxneo}$ male mice were treated with ethanol or dextrose as control (ctrl) by gavage once (n = 3-4). Representative western blotting for phosphorylated MLCK (p-MLCK) in epithelial cells isolated from the jejunum. (B-F) MLCK$^{+/+}$ and MLCK$^{-/-}$ littermate mice were orally fed a control (n = 4) and alcohol diet (n = 10-14). (B) Fecal albumin content. (C) Western blotting for *E. coli* proteins in liver (left panel); plasma LPS level (right panel). (D) Representative western blotting for occludin in the jejunum. (E) Occludin quantification of western blottings. (F) Representative IF staining for occludin; nuclei are stained blue. $^*P < 0.05$. Abbreviation: EtOH, ethanol.

Intestinal MLCK Contributes to Increased Intestinal Permeability and Liver Disease After Chronic Alcohol Feeding. TNF-α signaling phosphorylates and activates MLCK, which subsequently redistributes tight-junction proteins and increases intestinal permeability in many intestinal diseases.[14,27] To further identify TNFRI downstream signaling that mediates tight-junction disruption after alcohol administration, we assessed phosphorylation of MLCK. Jejunal epithelial cells isolated from WT mice that were administered alcohol by oral gavage once showed higher levels of phosphorylated MLCK (long isoform) than WT mice gavaged with dextrose as a control. This increase in MLCK phosphorylation was blunted in epithelial cells isolated from TNFRI mutant (TNFRI$^{flxneo/flxneo}$) mice after alcohol administration. Reactivation of TNFRI selectively on intestinal epithelial cells restored MLCK phosphorylation to a level similar to that of WT mice after alcohol administration (Fig. 6A). However, MLCK phosphorylation is comparable between WT and TNFR2-deficient enterocytes after ethanol administration (Supporting Fig. 12). These data indicate that TNFRI, but not TNFR2, mediates MLCK activation in the intestinal epithelium after alcohol administration.

To dissect the functional role of MLCK in increasing intestinal permeability after chronic alcohol feeding, MLCK-deficient and WT littermate mice were subjected to the Lieber-DeCarli alcohol feeding model for 8 weeks. Alcohol feeding increased intestinal permeability in WT mice, as compared to isocaloric-diet–fed WT mice. Intestinal permeability is significantly lower in MLCK$^{-/-}$ than WT mice after alcohol administration. However, MLCK$^{-/-}$ mice still show higher levels of fecal albumin, compared to isocaloric-diet–fed mice (Fig. 6B). MLCK$^{-/-}$ mice showed a nonsignificant trend toward a lower amount of *E. coli*

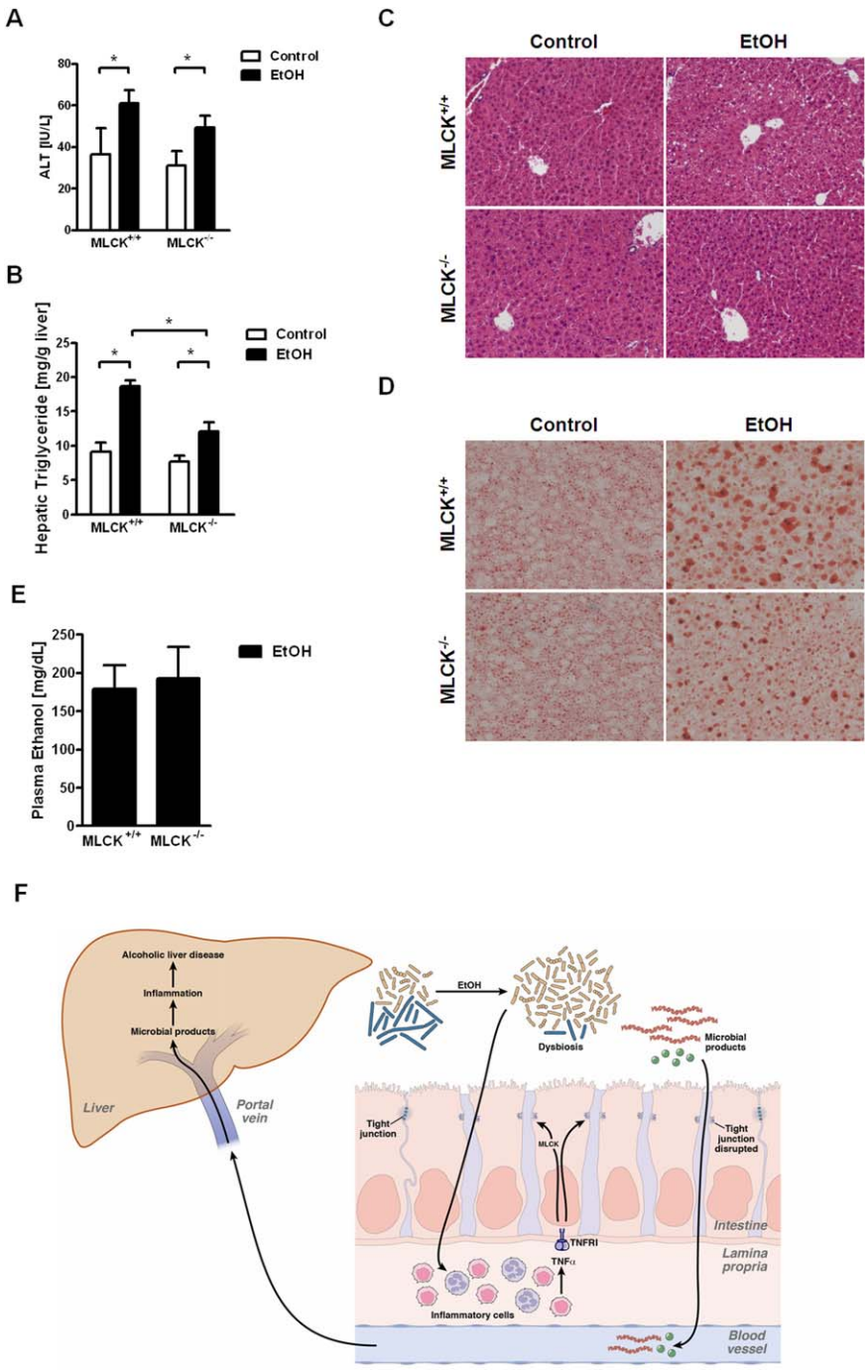

Fig. 7. MLCK contributes to ALD. $MLCK^{+/+}$ and $MLCK^{-/-}$ mice were orally fed a control (n = 4) and alcohol diet (n = 10-14). (A) Plasma ALT level. (B) Hepatic TG content. (C) Representative liver sections after hematoxylin and eosin staining. (D) Representative liver sections after Oil Red O staining. (E) Plasma ethanol concentration. $*P < 0.05$. (F) Schematic representation of the proposed model of microbial translocation during ALD: After the onset of alcoholic dysbiosis, monocytes and macrophages of the intestinal lamina propria are activated and produce TNF-α. TNF-α binds to TNFRI on enterocytes and increases intestinal permeability. This is, in part, mediated by the activation of MLCK, resulting in disruption of tight junctions. Microbial products cross the mucosal barrier to reach the liver through the portal circulation. Microbial products cause hepatic inflammation and liver disease. The model has been reproduced from a previous work[39] with permission from Elsevier and modified with additional permission from Elsevier. Abbreviation: EtOH, ethanol.

proteins in the liver, but significantly lower LPS plasma level, as compared to WT littermates after alcohol feeding (Fig. 6C). Expression of occludin protein was diminished in the jejunum of alcohol-fed WT mice, but not of alcohol-fed $MLCK^{-/-}$ mice (Fig. 6D-F). These results indicate that MLCK is only partially mediating the intestinal barrier loss pathway after chronic alcohol feeding.

Administration of ethanol leads to an increase of liver/body-weight ratio in WT, but not $MLCK^{-/-}$ mice (Supporting Fig. 13A). Plasma ALT levels, as measures for liver injury, were reduced in alcohol-fed $MLCK^{-/-}$ mice, compared to WT littermates, but there was no significant difference (Fig. 7A). Hepatic chemokine expression, such as chemokine (C-C motif) ligand (Ccl)2 (also known as monocyte chemoattractant protein 1) and Ccl3 (macrophage inflammatory protein 1 alpha) was increased after ethanol treatment in WT mice, but showed comparable level between alcohol-fed WT and $MLCK^{-/-}$ mice (Supporting Fig. 14). Hepatic

fat accumulation was significantly lower in MLCK-deficient mice, as compared to WT mice, after 8 weeks of ethanol feeding (Fig. 7B-D). However, hepatic TGs were still higher in $MLCK^{-/-}$ mice after alcohol feeding, as compared to mice fed an isocaloric diet. MLCK deficiency did not affect plasma ethanol levels or hepatic metabolism of ethanol (Fig. 7E and Supporting Fig. 13B,C). Taken together, MLCK deficiency reduces, but does not completely prevent, ALD. These data are consistent with a partial contribution of MLCK to the intestinal barrier loss pathway after chronic alcohol feeding.

Discussion

Although ALD depends on gut-derived bacterial products, the mechanism of mucosal barrier disruption and bacterial translocation *in vivo* is unknown. In this study, we propose that, after chronic alcohol use, monocytes and macrophages in the intestinal lamina propria activate and produce TNF-α, both in humans and mouse models. Activation of innate immune cells is dependent on alcohol-induced dysbiosis. Intestinal barrier disruption and bacterial translocation are facilitated by TNFRI expressed on intestinal epithelial cells. TNFRI-mediated activation of MLCK in enterocytes and disruption of tight junctions partially contributes to reduced barrier function. Increased intestinal permeability results in translocation of microbial products to reach the liver and promote steatohepatitis (Fig. 7F). This is the first study functionally linking an intestinal gene to gut leakiness and ALD *in vivo*.

Our data indicate that intestinal inflammation initiates the process of intestinal barrier dysfunction and translocation of microbial products during ALD. Intestinal inflammation is also present in the duodenum of patients with liver cirrhosis.[28] But what causes the onset of intestinal inflammation? Inflammasome-mediated enteric dysbiosis results in intestinal inflammation and microbial translocation during the development of nonalcoholic steatohepatitis (NASH). Bacterial products subsequently reach the liver to induce an inflammatory response that promotes progression of nonalcoholic fatty liver disease to NASH.[23] We have previously demonstrated that chronic alcohol feeding is associated with dysbiotic microbiome changes and pronounced intestinal bacterial overgrowth in the jejunum of mice.[20] As shown in our current study, intestinal decontamination not only suppresses intestinal bacterial overgrowth, but it also inhibits intestinal inflammation after alcohol feeding. Currently, we can only speculate about how alcohol-induced dysbiosis triggers intestinal inflammation. Whether bacterial pathogen-associated molecular patterns stimulate innate immune cells in the lamina propria or whether microbial metabolites (e.g., acetaldehyde) affect the innate immune system requires future studies.

We further demonstrate that TNFRI on enterocytes mediates disruption of the intestinal barrier after chronic alcohol feeding. TNF-α is an important immune-mediated tight-junction regulator in the intestine.[29] TNFRI-deficient, but not TNFR2-deficient, mice are protected from CLDs, such as cholestatic liver fibrosis[30] or ALD.[26] Using a bile duct ligation model in mice, we have previously demonstrated that intestinal TNFRI contributes to protection from cholestatic liver fibrosis by stabilizing the intestinal barrier.[25] However, reactivation of TNFRI specifically on enterocytes increased liver fibrosis, as compared to TNFRI mutant mice, but the degree of fibrosis is still lower, compared to WT mice,[25] suggesting that liver TNFRI (e.g., on hepatic stellate cells and/or Kupffer cells) might account for differences in the fibrogenic response.[30] In contrast, reactivation of TNFRI on intestinal epithelial cells increased intestinal permeability and liver disease similar to WT mice after alcohol feeding. These findings support a major role for enteric TNFRI in the disruption of the intestinal barrier during ALD, whereas hepatic TNFRI expression is dispensable for alcohol-induced liver injury and steatosis. Other hepatic inflammatory mediators, such as interleukin (IL)-1β might be more important in inducing downstream effects of TLRs, which are activated by translocated bacterial products. And, indeed, IL-1β signaling is required for the development of alcohol-induced liver steatosis, inflammation, and injury, as recently reported.[31] TNFR2 is important in the regulation of disease entities, such as experimental inflammatory bowel disease. TNFR2 activates tight-junction dysregulation to cause apoptosis-mediated barrier loss in experimental models of colitis.[32] In contrast to colitis models, intestinal inflammation is mild and does not involve an increased intestinal epithelial cell death after chronic alcohol feeding.

MLCK is phosphorylated as a downstream target of TNF-α after alcohol administration. MLCK is known to be activated and to play a central role as a common final pathway of barrier disruption in response to TNF-α in enterocytes. TNF-α-induced MLCK activation triggers caveolin-1-dependent endocytosis and removal of occludin.[33] The role of MLCK in TNF-α-induced and disease-associated barrier loss was linked specifically to occludin function.[17,33,34] Ethanol

stimulated MLCK activation, and the specific MLCK inhibitor, 1-(5-iodonaph-thalene-1-sulfonyl)−1H-hexhydro-1,4-diazapine, inhibited both ethanol-induced MLCK activity and tight-junction disruption in Caco-2 monolayers.[35] Furthermore, inhibition of MLCK activation with the chemical inhibitor, phosphoinositide kinase, reduced intestinal tight-junction disruption and intestinal damage after binge ethanol exposure and burn injury.[36] These studies suggest that MLCK mediates barrier regulation after an acute ethanol challenge. In contrast, our study, using a chronic model of alcohol feeding, intestinal barrier dysfunction, bacterial translocation, and ALD, are only partially suppressed in MLCK-deficient mice. Paracellular permeability is controlled by many tight-junction proteins. For example, claudins regulate permeability to specific ions, and the overall ionic permeability is the result of different combinations and ratios of claudin proteins.[37] After chronic alcohol administration, tight-junction disruption of occludin is prevented in TNFRI mutant and MLCK-deficient mice. However, alcohol-induced upregulation of claudin-8 is abrogated in TNFRI mutant mice, whereas claudin-8 induction is not inhibited in MLCK-deficient mice (Supporting Fig. 15). This indicates that pathways other than MLCK are activated downstream of TNFRI that regulate tight junctions and induce paracellular permeability during chronic stimulation. iNOS could be such a mediator, given that it is a known nuclear factor kappa B–dependent gene downstream of the TNF receptor.[38] Indeed, intestinal iNOS expression was lower in TNFRI mutant mice after chronic alcohol feeding, whereas iNOS protein was similarly in MLCK-deficient, compared to WT, mice (Supporting Fig. 16). These data further support that iNOS may serve as another TNF downstream target to mediate ALD.

In conclusion, using tissue-specific genetically modified mice, our study is the first to causatively link intestinal permeability to ALD *in vivo*. Alcoholic dysbiosis is associated with intestinal inflammation and is the switch to turn on barrier dysfunction. We identified intestinal TNFRI as the master regulator of intestinal barrier function after chronic alcohol administration. Based on this evidence, ALD might be treated by modulating the intestinal microbiome.

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