Toll-Like Receptor 2–Mediated Intestinal Injury and Enteric Tumor Necrosis Factor Receptor I Contribute to Liver Fibrosis in Mice

PHILLIPP HARTMANN, MICHAEL HAIMERL, MAGDALENA MAZAGOVA, DAVID A. BRENNER, and BERND SCHNABL

Department of Medicine, University of California San Diego, La Jolla, California

BACKGROUND & AIMS: Progression of liver fibrosis in experimental models depends on gut-derived bacterial products, but little is known about mechanisms of disruption of the mucosal barrier or translocation. We used a mouse model of cholestatic liver disease to investigate mechanisms of intestinal barrier disruption following liver injury. **METHODS:** Liver fibrosis and bacterial translocation were assessed in Toll-like receptor 2 (TLR2)-deficient and tumor necrosis factor receptor I (TNFRI)-deficient mice subjected to bile duct ligation. Epithelial and lamina propria cells were isolated and analyzed by immunoblot analyses and flow cytometry. We analyzed bone marrow chimeras and mice with a conditional gain-of-function allele for the TNFRI receptor. By crossing TNFRI$^{flxneo/flxneo}$ mice with mice that expressed the VillinCre transgene specifically in intestinal epithelial cells, we created mice that express functional TNFRI specifically on intestinal epithelial cells (VillinCreTNFRI$^{flxneo/flxneo}$ mice). **RESULTS:** Following bile duct ligation, TLR2-deficient mice had less liver fibrosis and intestinal translocation of bacteria and bacterial products than wild-type mice. Mice with hematopoietic cells that did not express TLR2 also had reduced bacterial translocation, indicating that TLR2 expression by hematopoietic cells regulates intestinal barrier function. The number of TLR2$^+$ monocytes that produce tumor necrosis factor α increased in the intestinal lamina propria of wild-type mice following bile duct ligation; bacterial translocation was facilitated by TNFRI-mediated signals on intestinal epithelial cells. **CONCLUSIONS: Intestinal inflammation and bacterial translocation contribute to liver fibrosis via TLR2 signaling on monocytes in the lamina propria and TNFRI signaling on intestinal epithelial cells in mice. Therefore, enteric TNFRI is an important mediator of cholestatic liver fibrosis.**

Keywords: Microbiome; Endotoxin; Intestinal Inflammation; Microbiota Composition.

BASIC AND TRANSLATIONAL LIVER

Liver fibrosis is triggered upon chronic liver damage caused by various etiologies, including viral hepatitis, alcohol abuse, chronic cholestasis, and nonalcoholic steatohepatitis. Fibrosis can progress to cirrhosis as end-stage liver disease, which is the 12th most common cause of death in the United States.[1] There is an emerging concept that gut-derived bacterial products resulting from bacterial translocation play a central role in the initiation of acute liver injury and progression to chronic liver disease. In rodents, experimental bacterial overgrowth in the intestine induces bacterial translocation, as defined by crossing of viable bacteria or bacterial products across the intestinal epithelial lining, and subsequent liver damage.[2,3] In humans, patients with chronic liver disease show intestinal bacterial overgrowth with bacterial translocation, and severity of liver disease correlates with systemic levels of endotoxin (or lipopolysaccharide).[4] Reducing the intestinal bacterial burden by nonabsorbable antibiotics reduces endotoxemia and improves chronic liver disease in preclinical animal models[5,6] and patients.[7] Further evidence for the critical involvement of gut-derived bacterial products in liver fibrogenesis comes from genetically modified animals. Mice that are either unresponsive to bacterial products by a deficiency or mutation in Toll-like receptor (TLR)-4 and 9[6,8] or lack molecules propagating TLR signaling events triggered by bacterial products[9,10] are resistant to experimental liver fibrosis. Nevertheless, the exact molecular mechanism of increased intestinal permeability and liver fibrosis is not known. In this study, we used an animal model of cholestatic liver fibrosis to show that TLR2$^+$ monocytes in the intestinal lamina propria mediate intestinal barrier disruption by production of tumor necrosis factor (TNF)-α. Tumor necrosis factor receptor I (TNFRI) on enterocytes mediates tight junction disruption by activation of the RhoA/myosin II regulatory light chain (MLC) pathway.

Materials and Methods

Animal Models of Liver Disease

TNFRI$^{-/-}$[11] and transgenic mice expressing green fluorescent protein (GFP) under the control of a β-actin promoter[6] have been described. TNFRI$^{flxneo/flxneo}$[12] and VillinCreTNFRI$^{flxneo/flxneo}$[13] 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. TLR2-deficient mice that were originally purchased from Jackson Laboratory (Bar Harbor, ME) were rederived by cesarean section and housed in a specific pathogen-free vivarium. All transgenic and knockout mice were on a C57BL/6 genetic background. Age-matched male mice on a C57BL/6 genetic background were used for the study except when stated otherwise. All animals received

Abbreviations used in this paper: CFU, colony-forming unit; GFP, green fluorescent protein; MLC, myosin II regulatory light chain; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinases; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFRI, tumor necrosis factor receptor I; ZO, zonula occludens.

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humane care in compliance with institutional guidelines. Mice had access to water and irradiated chow diet (PicoLab Rodent Diet 20 5053; LabDiet, Brentwood, MO) ad libitum. Ligation of the common bile duct or sham operation as control has been described.[2] One day, 10 days, or 3 weeks after bile duct ligation or sham operation, mice were harvested for analysis.

Generation of Bone Marrow Chimeric Mice

Recipient mice were lethally irradiated with 1000 rad using a ^{137}Cs source and injected intravenously 2–3 hours later with approximately 5×10^6 bone marrow cells derived from the tibia and femur of donors as described.[14] Eight weeks after engrafting, animal experiments were performed. To assess reconstitution, GFP-expressing transgenic mice under a β-actin promoter were used as bone marrow donors with wild-type mice as recipients. Engraftment was assessed by fluorescence-activated cell sorter (FACS) analysis of peripheral blood and isolated intestinal lamina propria cells. After red blood cell lysis, peripheral blood and lamina propria cells were stained with PE-labeled CD45.2 (eBioscience, San Diego, CA). Cell suspensions were analyzed for the presence of intracellular GFP on a BD LSR II (BD Biosciences, San Jose, CA). Immunofluorescence was performed on frozen liver sections using anti-GFP (Abcam, Cambridge, MA) and anti-F4/80 (eBioscience) as primary antibodies and fluorescein isothiocyanate– or Texas Red–conjugated as secondary antibodies (Invitrogen, Grand Island, NY).

Epithelial Cell and Lamina Propria Cell Isolation

Intestines of mice were rinsed with cold phosphate-buffered saline, opened longitudinally, and cut into pieces of 1-cm lengths. Intestinal pieces were placed in ice-cold RPMI 1640 medium (Invitrogen) with 5% fetal calf serum containing dithiothreitol (1 mmol/L). After vigorous shaking, the supernatant was discarded and tissue was incubated for 20 minutes in RPMI 1640 with 5% fetal calf serum containing EDTA (1 mmol/L) at 37°C with shaking (250 rpm). This step was repeated for a total of 2 times. Intestinal epithelial cells were collected from supernatant after the first incubation. For lamina propria cell isolation, tissue was further incubated in RPMI 1640 with 5% fetal calf serum containing collagenase type VIII (Sigma, St. Louis, MO; 1 mg/mL) for 30 minutes at 37°C with shaking (250 rpm). Cell suspension was sieved through a cell strainer (100 μm; BD Biosciences). Lamina propria cells were collected by centrifugation; stained with CD45.2, CD11b, TLR2, Lys6C, Lys6G, CD11c, F4/80, interleukin-6, or TNF-α (all eBiosciences); and analyzed by fluorescence-activated cell sorting. Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions.

Bacterial Cultures

To assess for bacterial translocation, mesenteric lymph nodes were harvested in a sterile fashion and cultured on blood agar plates (containing 5% sheep blood) for total bacteria as described.[2,15] Plates were incubated for 48–72 hours at 37°C in aerobic conditions, followed by the counting of colony-forming units (CFUs).

To assess bacterial translocation from gastrointestinal segments, we used an intestinal loop model as we have previously described.[2,14,16] *Escherichia coli* serotype O74:K:H39 isolated from a patient with peritonitis (ATCC #23535) was transfected with a GFP-expressing plasmid, which conferred chloramphenicol resistance.[17] After anesthesia, a midline laparotomy incision was made. A 4-cm-long segment of the gastrointestinal tract (proximal, mid, or distal small intestine, cecum, or colon) was created with 2 vascular hemoclips without disrupting the mesenteric vascular arcades. The length of intestine between the 2 clips was injected with approximately 10^6 CFUs. After 4 hours, mice were sacrificed and mesenteric lymph nodes were cultured on nutrient broth agar plates (containing chloramphenicol 20 μg/mL). GFP-expressing CFUs were counted after 48–72 hours using fluorescence microscopy. The number of translocated CFUs was adjusted for the total number of bacteria injected and expressed as CFUs per 1×10^6 injected bacteria.

Protein Expression Analysis

Formalin-fixed liver samples were embedded in paraffin and stained with Sirius red to assess fibrosis. The Sirius red–positive area was measured at a final magnification of 40×. The entire median lobe of the liver was imaged using a polarizing filter and analyzed using National Institutes of Health ImageJ as described.[18] Results are expressed relative to wild-type control mice. Frozen intestinal sections were stained using anti-occludin, anti–claudin-4, and anti–zonula occludens (ZO)-1 antibodies and a fluorescein isothiocyanate–conjugated secondary antibody (all Invitrogen) as described.[2] Nuclei were stained with Hoechst (blue). Control sections were stained with an isotype antibody instead of the primary antibody and showed no staining. Western blot analysis was performed as described[15] using anti–phospho-MLC (Cell Signaling, Danvers, MA), anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-tubulin (Santa Cruz Biotechnology) or anti–β-actin (Sigma) antibodies. Western blots were quantitated using National Institutes of Health ImageJ. Alanine aminotransferase levels were measured as described.[15] TNF-α was measured in the plasma using enzyme-linked immunosorbent assay (eBioscience) with a detection limit $\leq$3.9 pg/mL.

Statistical Analysis

Mann–Whitney rank sum test was used for statistical analysis. All data are presented as mean $\pm$ SEM. $P < .05$ was selected as the level of significance.

Other materials and methods are described in Supplementary Materials and Methods.

Results

TLR2-Deficient Mice Are Protected From Cholestatic Liver Fibrosis

TLR2 is the receptor for products from gram-positive bacteria such as peptidoglycan. Conflicting results about the importance of TLR2 in experimental liver injury and fibrosis have been reported. Although one study reported increased survival and protection from liver injury of TLR2-deficient mice following bile duct ligation for up to 30 days,[19] another report showed no difference in bile duct ligation–induced liver fibrosis.[6] Because the phenotype in experimental disease models changes with the intestinal microflora[20] and to eliminate potential opportunistic pathogens present in these mice, TLR2-deficient mice were rederived and housed in a specific pathogen-free environment. Mice were subjected to bile duct ligation as a common model for cholestatic liver fibrosis. Following bile duct ligation for 10 days or 3 weeks, TLR2$^{-/-}$ mice showed reduced fibrogenesis as evidenced by decreased hepatic gene expression of collagen α1(I) as com-

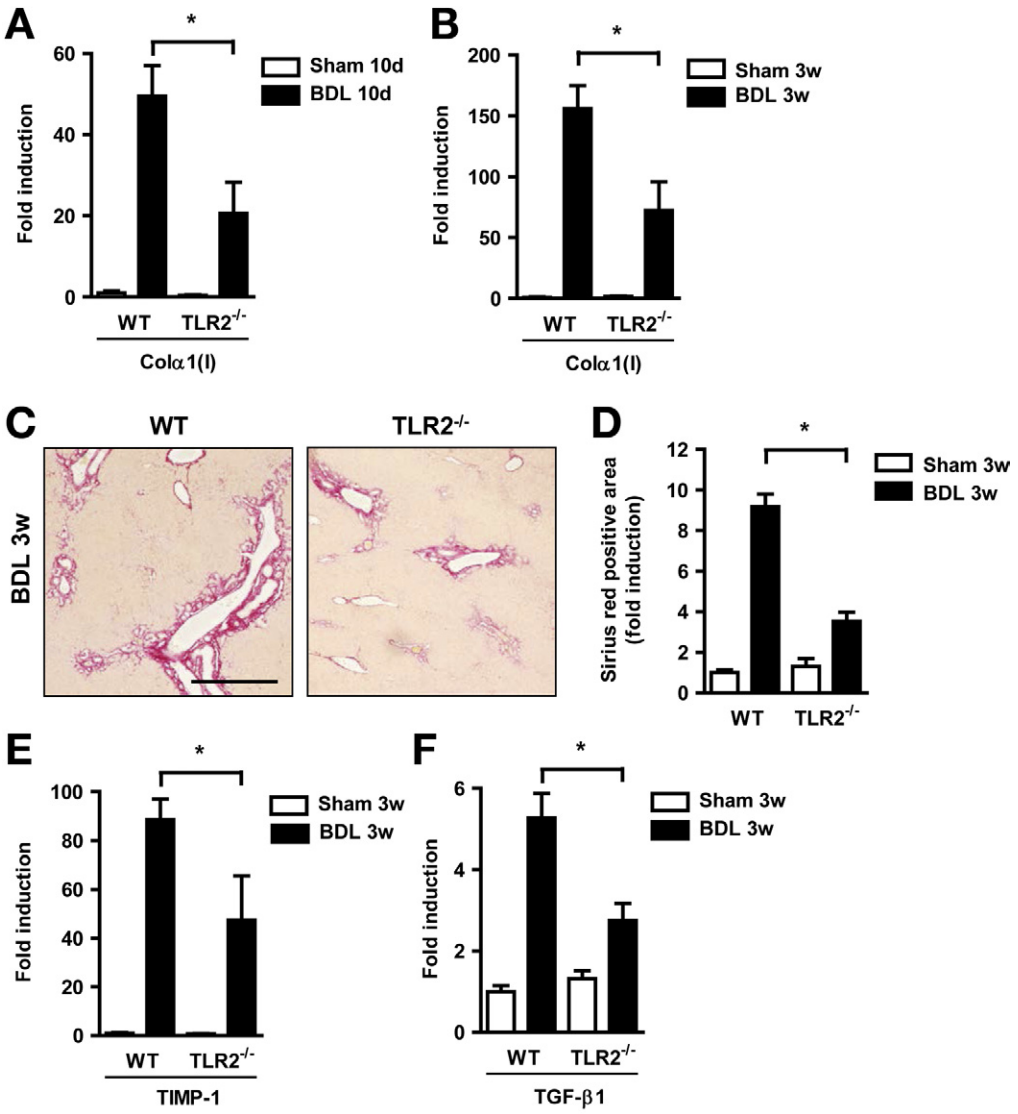

Figure 1. Diminished hepatic fibrosis in TLR2$^{-/-}$ mice. Wild type and TLR2$^{-/-}$ mice underwent sham operation (n = 4) or bile duct ligation (BDL; n = 8–13) for indicated time periods. (*A* and *B*) Collagen α1(I) messenger RNA expression was assessed in the liver using quantitative polymerase chain reaction. (*C* and *D*) Hepatic fibrosis was assessed by Sirius red staining and quantified using morphometric analysis. *Scale bar* = 50 μm. (*E* and *F*) Hepatic TIMP-1 and TGF-β1 messenger RNA levels were determined by quantitative polymerase chain reaction. *$P < .05$.

pared with wild-type C57BL/6 control mice (Figure 1*A* and *B*). TLR2-deficient mice had a reduction in fibrosis as measured by Sirius red staining (Figure 1*C* and *D*). This was associated with decreased expression of fibrosis-related genes, such as tissue inhibitor of matrix metalloproteinases (TIMP)-1 and transforming growth factor (TGF)-β1 as potent profibrogenic cytokines (Figure 1*E* and *F*). Liver injury as measured by systemic alanine aminotransferase levels was not significantly different in wild-type and TLR2$^{-/-}$ mice 1 day or 3 weeks following bile duct ligation (Supplementary Figure 1), indicating that TLR2 does not mediate cholestatic hepatotoxicity but fibrosis progression. Reduced hepatic fibrosis in TLR2$^{-/-}$ mice was confirmed in a second model of toxic liver injury and fibrosis using chronic injections of carbon tetrachloride (Supplementary Figure 2).

Translocation of Bacteria and Their Products Is Abolished in TLR2$^{-/-}$ Mice Following Bile Duct Ligation

Liver fibrosis is believed to be promoted by translocation of bacteria or their products from the gut to the portal and systemic circulation.[6,8] We therefore investigated whether TLR2 is involved in regulating intestinal barrier function and bacterial translocation following the onset of liver injury and fibrosis. As a measure of bacterial translocation across the mucosal barrier, viable bacteria were quantified in mesenteric lymph nodes as the first organ encountered in the translocation route from the gastrointestinal tract to the portal vein. TLR2$^{-/-}$ mice showed significantly less positive mesenteric lymph node cultures as compared with wild-type mice following bile duct ligation for 1 day (Figure 2*A*). Significantly lower systemic endotoxin levels were found in TLR2$^{-/-}$ mice following bile duct ligation for 1 day or 3 weeks as compared with wild-type mice (Figure 2*B* and *C*). To determine the gastrointestinal site of bacterial translocation in vivo, a ligated intestinal loop model was used as previously described by us.[2,14] A 4-cm loop of the proximal, mid, or distal small intestine, cecum, or colon was ligated (without disrupting the mesenteric vascular arcades and blood supply) in anesthetized mice that had

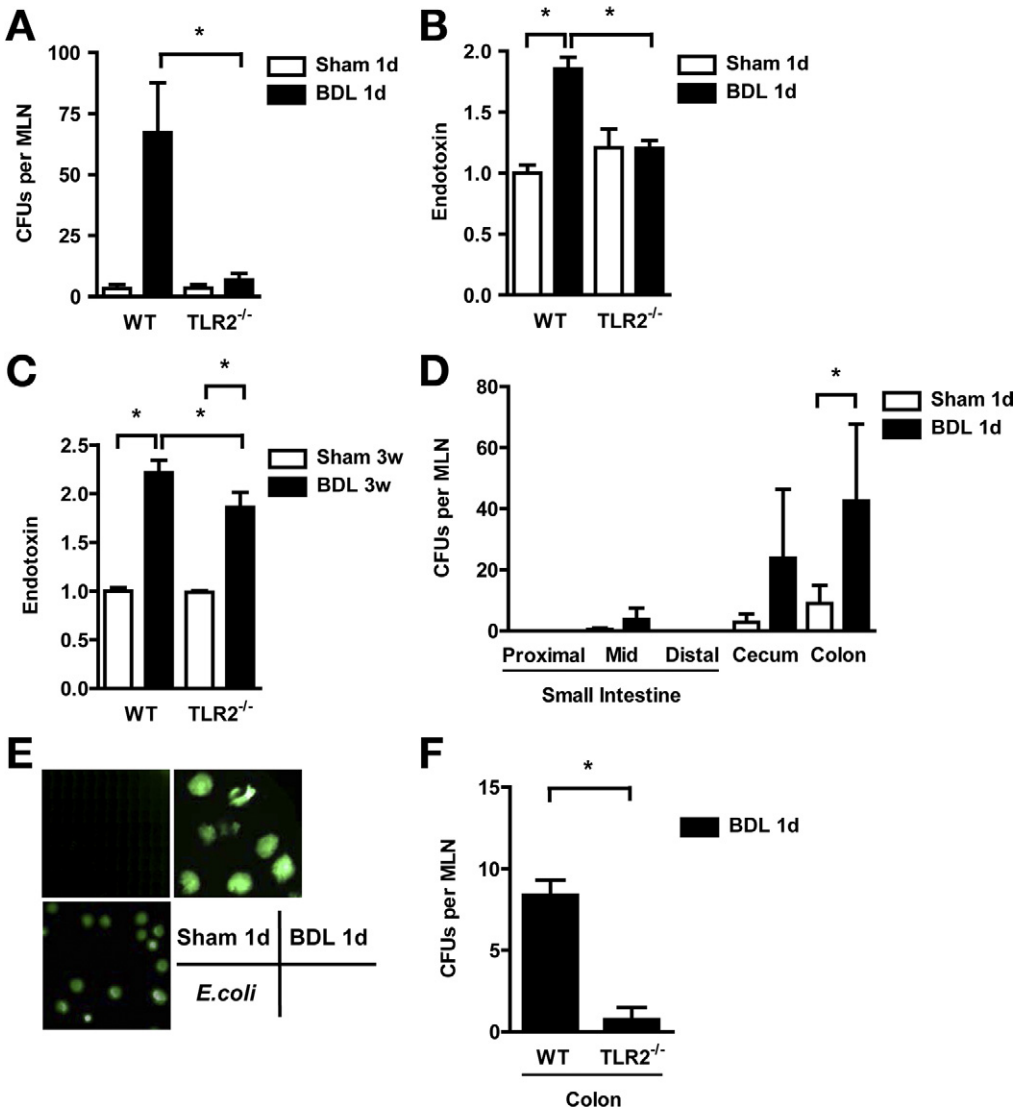

Figure 2. TLR2$^{-/-}$ mice show less bacterial translocation following ligation of the common bile duct. (*A*) Aerobic bacteria were cultured and quantified in mesenteric lymph nodes (MLN; n = 10–12). (*B* and *C*) Plasma endotoxin was measured (n = 4 for sham, n = 8–21 for bile duct ligation [BDL]). (*D* and *E*) GFP-expressing *E coli* were injected into intestinal loops of BALB/c mice that underwent sham or BDL operations 1 day before. MLN were cultured 4 hours after injection (*D*; n = 4 for small intestine, n = 17 for cecum, n = 28 for colon). Positive cultures showed GFP expression using fluorescence microscopy. An image with GFP-expressing *E coli* as positive control is shown (*E*). (*F*) Translocation of GFP-*E coli* from colonic loops to MLN was assessed in wild type (n = 4) and TLR2$^{-/-}$ mice (n = 5) 1 day after BDL. *$P < .05$.

undergone sham operations or bile duct ligation 1 day before, and viable GFP-expressing *E coli* were injected. At 4 hours, mice were harvested, mesenteric lymph nodes cultured, and translocated bacteria counted using fluorescent microscopy. The large intestine and in particular the colon showed a significant increase in translocated *E coli* (Figure 2*D* and *E*). This is consistent with our study demonstrating that the colon shows the largest increase in intestinal permeability following 1 day of bile duct ligation.[2] Translocation of GFP-expressing *E coli* from colonic loops to mesenteric lymph nodes was markedly attenuated in TLR2-deficient mice as compared with wild-type mice, suggesting that TLR2 is involved in regulation of the intestinal barrier function (Figure 2*F*).

TLR2-Mediated Signaling in Hematopoietic Cells Is Required for Bacterial Translocation Following Cholestatic Liver Injury

To investigate whether innate immune signaling in hematopoietic or nonhematopoietic cells mediates bacterial translocation following liver injury, we generated bone marrow chimeric mice in which donor and/or recipients were either wild type or TLR2 deficient. To confirm successful bone marrow transplantation, control animals were transplanted with bone marrow cells from mice expressing GFP under a β-actin promoter and studied 8 weeks after transplantation. Mice were found to be fully chimerized in peripheral blood, colonic lamina propria, and liver, as assessed 1 day after bile duct ligation (Supplementary Figure 3*A* and *B*).

To determine the impact of TLR2-mediated signaling in hematopoietic or nonhematopoietic cells on bacterial translocation, mesenteric lymph nodes from chimeric mice were cultured 1 day after bile duct ligation. Chimeric mice lacking TLR2 on hematopoietic cells were protected from translocation of bacteria to the mesenteric lymph nodes. In contrast, transplant recipients expressing TLR2 on hematopoietic cells showed bacteria translocated to the mesenteric lymph nodes, regardless of whether the bone marrow recipient was wild type or TLR2 deficient (Figure 3*A*). Similarly, plasma endotoxin levels were lowest in transplant recipients lacking TLR2 on hematopoietic cells (Figure 3*B*). These findings indicate that TLR2

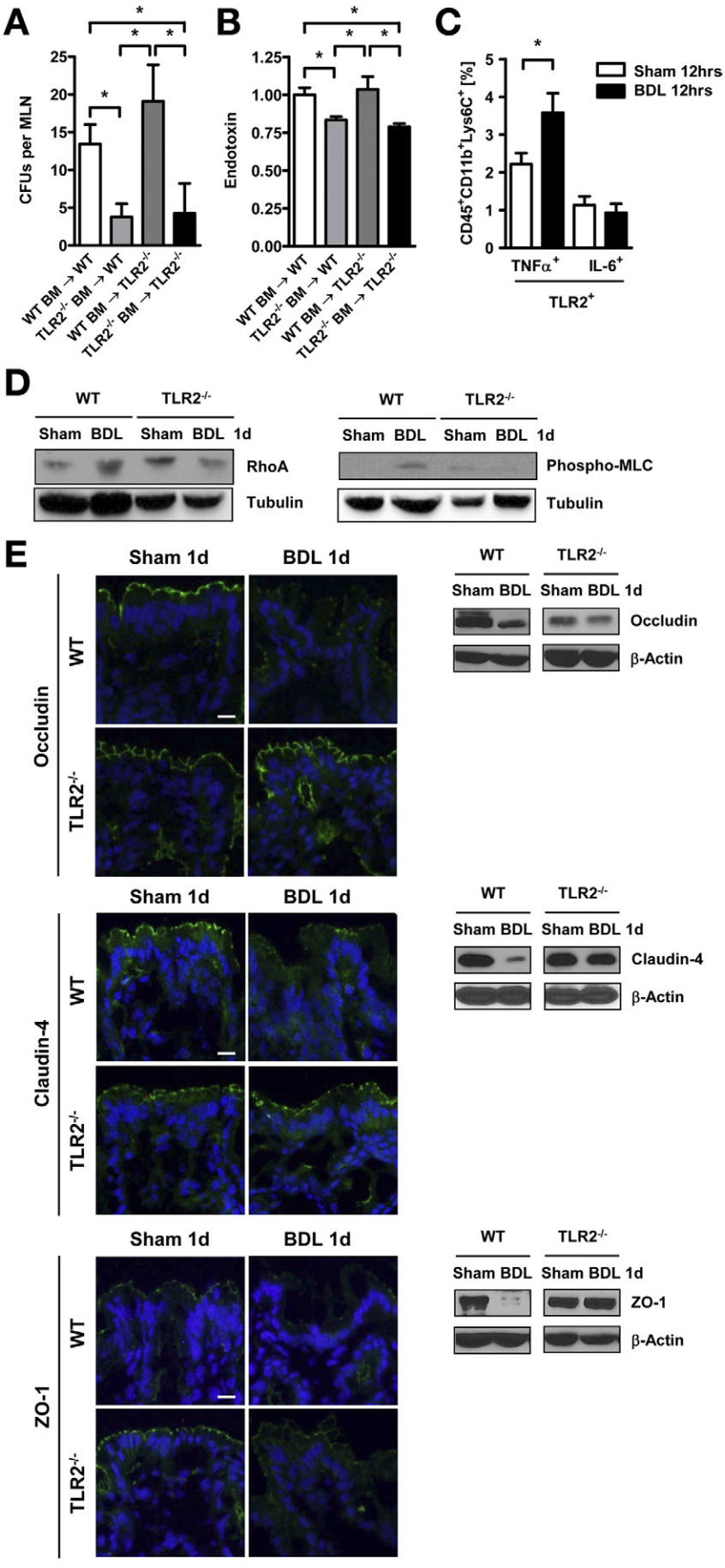

Figure 3. Bacterial translocation is dependent on TLR2 signals from hematopoietic cells. (*A* and *B*) CFUs were counted of mesenteric lymph node cultures (n = 8–23), and plasma endotoxin (n = 4–15) was measured in wild-type and TLR2 bone marrow chimeric mice 1 day after bile duct ligation (BDL). (*C*) $TLR2^+$, intracellular TNF-α^+ (n = 4–5 independent experiments), and $TLR2^+$, IL-6^+ expression (n = 3 independent experiments) of $CD45.2^+$, $CD11b^+$, $Lys6C^+$ cells were assessed in colonic lamina propria of wild-type mice. Lamina propria cells from 3 mice were pooled in each independent experiment. (*D*) RhoA, phospho-MLC, and tubulin expression was assessed in isolated colon epithelial cells in wild-type and $TLR2^{-/-}$ mice. A representative Western blot is shown. (*E*) Occludin, claudin-4, and ZO-1 protein expression in intestinal sections of wild-type and $TLR2^{-/-}$ mice were analyzed by immunofluorescence and Western blot analysis. Representative fluorescent images of 3–4 independent experiments are shown. *Scale bar* = 1 μm. *$P < .05$.

expression on hematopoietic cells mediates bacterial translocation following cholestatic liver injury.

TNF-α–Expressing TLR2$^+$ Monocytes in the Colonic Lamina Propria Disrupt Epithelial Tight Junctions, Resulting in Bacterial Translocation

Several studies show that intestinal inflammation caused by inflammatory cells in the lamina propria is involved in increasing intestinal permeability leading to translocation of bacteria or their products.[21,22] It is therefore possible that TLR2-mediated signals in cells from the mucosal innate immune system are required for the paracellular leakage pathway in the intestine. We therefore focused on the lamina propria cell infiltrate in the colon because the colon had the largest rate of bacterial translocation and increase in intestinal permeability (Figure 2*D*). TNF-α is a known downstream target of TLR2 in monocytes and macrophages,[23] and it increases the permeability of the tight junction barrier in the intestine.[24] We found that the number of infiltrating TLR2$^+$ and TNFα$^+$ monocytes (Figure 3*C*), but not TLR2$^+$ and TNFα$^+$ macrophages, dendritic cells, or neutrophils (Supplementary Figure 4*A*–*C*), significantly increased 12 hours after bile duct ligation. The percentage of TLR2$^+$ monocytes expressing interleukin-6 as another proinflammatory cytokine was unchanged 12 hours after bile duct ligation as compared with sham operation (Figure 3*C*). In contrast to a local secretion of TNF-α, it is also conceivable that systemic TNF-α is elevated in response to cholestatic liver injury. However, plasma TNF-α levels were below the detection limit, both in sham and bile duct–ligated animals 12 hours after surgery (Supplementary Figure 4*D*). TNF-α has been reported to activate RhoA or MLC kinase with a subsequent phosphorylation of MLC.[25–27] The degree of MLC activation correlates with local disease activity in patients with inflammatory bowel disease, suggesting that MLC may be regulated by local cytokine signaling rather than systemically.[28] Colonic epithelial cells isolated from C57BL/6 mice that have undergone bile duct ligation showed higher protein levels of RhoA and more MLC phosphorylation, a downstream target of RhoA, compared with sham-operated mice. This increase in RhoA activation and MLC phosphorylation was blunted in epithelial cells isolated from the colon of TLR2$^{-/-}$ mice following bile duct ligation (Figure 3*D* and Supplementary Figure 5*A*). However, no increase in the gene expression or phosphorylation of MLC kinase was observed in isolated colonic enterocytes following various time intervals of bile duct ligation (not shown). Although subsequent molecular events that cause increased intestinal permeability following phosphorylation of MLC are less clear, changes in tight junction protein expression such as occludin are essential effectors of the paracellular leakage pathway.[21,29] Therefore, the integrity of tight junction proteins of colonic epithelial cells was investigated using immunofluorescence and confirmed by Western blotting. Following 1 day of cholestatic liver injury, staining for occludin protein was diminished as compared with sham-operated wild-type animals, as we have reported previously.[2] Occludin expression was unchanged in the colon of TLR2-deficient mice after bile duct ligation (Figure 3*E* and Supplementary Figure 5*B*). Similarly, expression of claudin-4 and ZO-1 was decreased 1 day after bile duct ligation in wild-type mice but not in TLR2$^{-/-}$ mice (Figure 3*E* and Supplementary Figure 5*B*). We also investigated whether increased cell death of colonic enterocytes might contribute to bacterial translocation, but we did not find a significant increase in apoptosis of the lining epithelial cells between sham and bile duct–ligated wild-type mice after 1 day (not shown). Taken together, bacterial translocation occurs following cholestatic liver injury and is facilitated by a disruption of tight junction proteins that involves a TLR2-induced TNF-α secretion in lamina propria monocytes activating the RhoA/MLC pathway in enterocytes.

TNFRI-Deficient Mice Are Protected From Liver Fibrosis and Show Decreased Bacterial Translocation

To further define the role of TNF-α in mediating tight junction disruption following liver injury, we used TNFRI-deficient mice and subjected them to bile duct ligation. TNFRI$^{-/-}$, but not TNFR2$^{-/-}$, mice show decreased hepatic fibrogenesis after 4 days of bile duct ligation.[30] Consistent with this report, TNFRI$^{-/-}$ mice were protected from liver fibrosis as shown by decreased collagen α(I) gene expression and deposition of extracellular matrix proteins following bile duct ligation for 3 weeks (Figure 4*A*–*C*). Hepatic gene expression of TIMP-1 and TGF-β1 was also lower in TNFRI$^{-/-}$ mice following bile duct ligation as compared with C57BL/6 mice (Figure 4*D* and *E*). Liver injury as measured by alanine aminotransferase levels was not significantly different in wild-type and TNFRI$^{-/-}$ mice 1 day or 3 weeks after bile duct ligation, consistent with previous studies[31,32] (Supplementary Figure 1). Moreover, bacterial translocation of viable bacteria to the mesenteric lymph nodes was abrogated in TNFRI$^{-/-}$ mice as compared with C57BL/6 mice after 1 day of cholestatic liver injury. Inhibition of bacterial translocation did not require TNFRI-mediated signals on hematopoietic cells but instead was required in nonhematopoietic cells, presumably in intestinal epithelial cells, as shown by bone marrow transplantation (Figure 5*A*). Translocation of endotoxin to the systemic circulation was similarly abolished in TNFRI$^{-/-}$ mice following bile duct ligation for 1 day or 3 weeks (Figure 5*B* and *C*). Consistent with decreased bacterial translocation, RhoA protein levels and MLC phosphorylation were not increased (Figure 5*D*). Expression of the tight junction components occludin, claudin-4, and ZO-1 remained intact following bile duct ligation in TNFRI$^{-/-}$ mice (Figure 5*E*). Thus, TNFRI on nonhematologic cells mediates bacterial translocation following cholestatic liver injury.

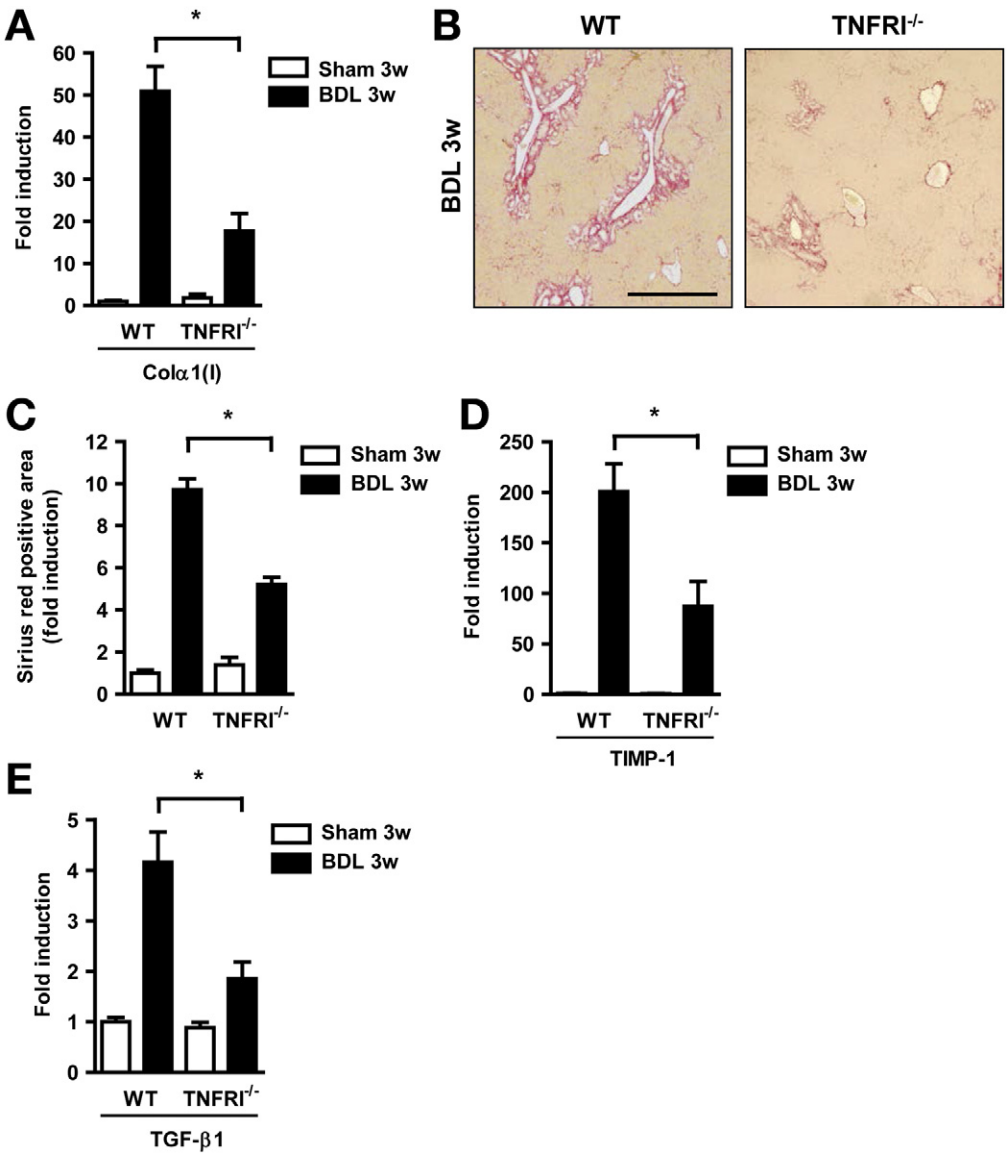

Figure 4. TNFRI$^{-/-}$ mice are protected from liver fibrosis after bile duct ligation. Wild-type and TNFRI$^{-/-}$ mice underwent sham operation (n = 4) or bile duct ligation (BDL; n = 9–13) (*A*) Hepatic collagen α1(I) messenger RNA was measured by quantitative polymerase chain reaction. (*B* and *C*) Collagen deposition was evaluated by Sirius red staining and quantitated by image analysis. *Scale bar* = 50 μm. (*D* and *E*) Hepatic TIMP-1 and TGF-β1 gene expression were assessed by quantitative polymerase chain reaction. *$P <$ .05.

BASIC AND TRANSLATIONAL LIVER

TNFRI-Deficient Mice With Reactivation of TNFRI on Intestinal Epithelial Cells Lose Their Protection Against Bile Duct Ligation–Induced Liver Fibrosis

To confirm the intestinal epithelial cell–specific function of TNFRI in mediating bacterial translocation in liver fibrosis, we used mutant mice carrying a conditional gain-of-function allele for this receptor with an introduced loxP-flanked neomycin-resistance cassette in intron 5 of the murine *p55TnfR* gene (TNFRI$^{flxneo/flxneo}$).[12] A nonfunctional TNFRI allele is engineered to be reactivated specifically in villin-expressing intestinal epithelial cells by a Villin Cre-loxP–mediated recombination (VillinCreTNFRI$^{flxneo/flxneo}$).[12] By crossing TNFRI$^{flxneo/flxneo}$ mice with the intestinal epithelial cell–specific VillinCre transgenic mouse, a functional TNFRI is selectively expressed on intestinal epithelial cells, as analyzed by polymerase chain reaction.[13] We confirmed the selective reactivation on isolated colonic epithelial cells (Figure 6*A*). TNFRI$^{flxneo/flxneo}$ mice carrying a mutation in TNFRI are protected from tight junction disruption in the colon, while wild-type mice show diminished expression of tight junction proteins 1 day after bile duct ligation. VillinCreTNFRI$^{flxneo/flxneo}$ mice that have a functional TNFRI selectively on intestinal epithelial cells show a disruption of tight junction proteins (Supplementary Figure 6). VillinCreTNFRI$^{flxneo/flxneo}$ mice lose their protection against bile duct ligation–induced liver fibrosis as compared with TNFRI$^{flxneo/flxneo}$. However, they still develop significantly less liver fibrosis as compared with C57BL/6 wild-type mice (Figure 6*B* and *C*). In addition, endotoxin levels were higher in VillinCreTNFRI$^{flxneo/flxneo}$ as compared with TNFRI$^{flxneo/flxneo}$ mice but lower as compared with C57BL/6 wild-type mice (Figure 6*D*). In summary, TNFRI on intestinal epithelial cells contributes to the paracellular leakage pathway, resulting in bacterial translocation and liver fibrogenesis.

Discussion

Bacterial translocation is a well-characterized phenomenon in patients with liver disease.[4] Although experimental studies suggest that progression of liver disease,

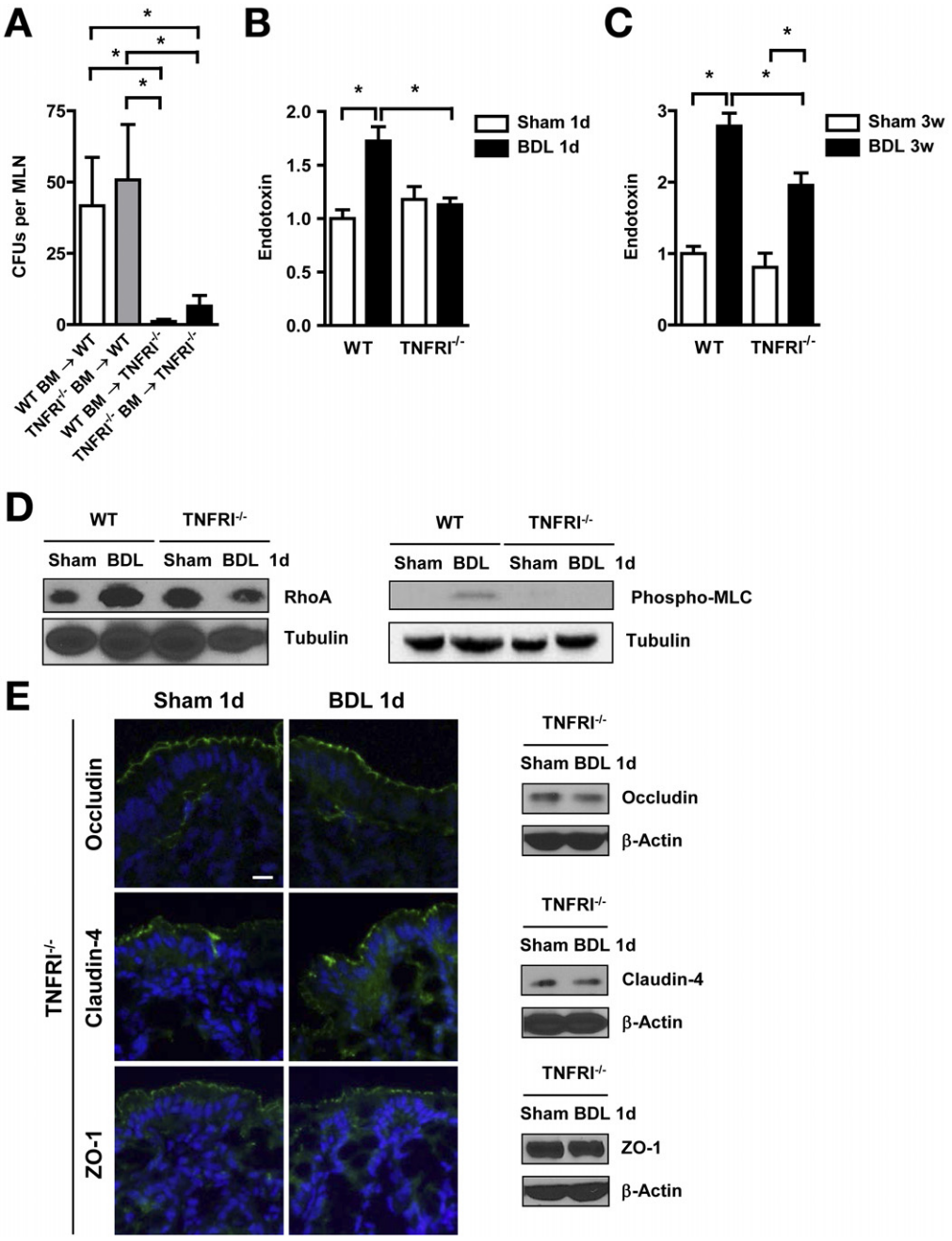

Figure 5. TNFRI on nonhematopoietic cells mediates bacterial translocation. (*A*) Aerobic bacteria were cultured and quantified in mesenteric lymph nodes (n = 7–10). (*B* and *C*) Endotoxin levels in the plasma were measured (n = 4 for sham, n = 6–10 for bile duct ligation [BDL]). (*D*) RhoA, phospho-MLC, and tubulin expression were analyzed in isolated epithelial cells from the intestine by Western blotting. Images are representative of at least 4 independent Western blots. (*E*) Immunofluorescent staining or Western blotting for occludin, claudin-4, and ZO-1 was performed on colonic sections. Representative images of 3–4 independent experiments are shown. *Scale bar* = 1 μm. *$P < .05$.

particularly progression of fibrosis, is dependent on gut-derived bacterial products,[6,8–10] the molecular mechanisms leading to a leaky gut barrier are unknown. Our study provides important insights into changes of the intestinal barrier function associated with cholestatic liver injury that result in bacterial translocation and liver fibrosis. Following cholestatic liver injury, TLR2-expressing inflammatory monocytes in the lamina propria of the colon produce and secrete TNF-α. TNFRI on enterocytes activates the RhoA/MLC pathway, ultimately resulting in a disruption of tight junctions. We hypothesize that bacteria and their products[33] translocate across the mucosal barrier, reach the liver via the portal circulation, and enhance liver fibrosis (Figure 7). Taken together, this is the first study functionally linking an intestinal gene to progression of liver fibrosis *in vivo*.

TNF-α levels produced by monocytes are increased in mesenteric lymph nodes, indicative of an immune response to enteric bacteria in rats with cirrhosis. Nonabsorbable antibiotics, which reduce the intestinal microflora and bacterial translocation to mesenteric lymph nodes, attenuate the expansion of monocytes in the mesenteric lymph nodes and normalize TNF-α in the mesenteric lymph nodes.[34] Most interestingly, bacterial translocation is reduced in cirrhotic rats treated with anti–TNF-α antibodies.[35] These findings support a role for TNF-α in the disruption of the intestinal barrier and in intestinal leakiness.

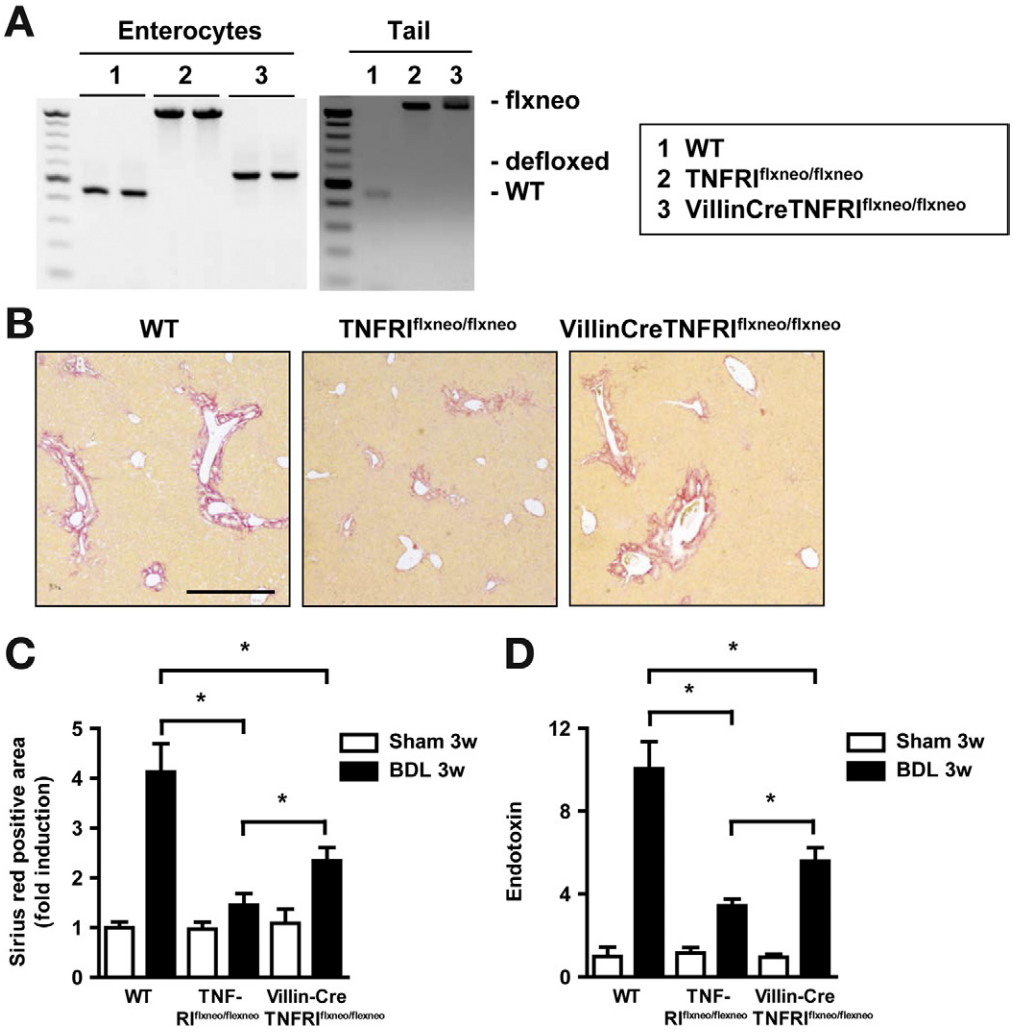

Figure 6. Reactivation of TNFRI on intestinal epithelial cells contributes to liver fibrosis. (*A*) Reactivation of TNFRI on isolated colonic epithelial in VillinCreTNFRI$^{flxneo/flxneo}$ was analyzed by polymerase chain reaction (*left panel*). Tails from respective mice served as controls (*right panel*). (*B–D*) Wild-type, TNFRI$^{flxneo/flxneo}$, and VillinCreTNFRI$^{flxneo/flxneo}$ mice underwent sham operation (n = 3–4) or bile duct ligation (BDL; n = 6 for C57BL/6, n = 9–10 for TNFRI$^{flxneo/flxneo}$ and for VillinCreTNFRI$^{flxneo/flxneo}$ mice). (*B* and *C*) Collagen deposition was evaluated by Sirius red staining and quantitated by image analysis. *Scale bar* = 50 μm. (*D*) Plasma endotoxin levels were measured. *$P < .05$.

BASIC AND TRANSLATIONAL LIVER

We demonstrate that enteric TNFRI mediates bacterial translocation in early stages after cholestasis. We show that MLC as a downstream target of RhoA activation is phosphorylated following cholestatic liver injury. MLC is activated and plays a central role as a common final pathway of barrier disruption in response to TNF-α in enterocytes.[21] Although bacterial translocation is reduced to baseline in early stages of cholestatic liver disease, endotoxin levels are higher in TNFRI-deficient mice following 3 weeks of bile duct ligation as compared with sham-operated mice. This indicates that later in disease, other pathways contribute to gut leakiness and subsequent bacterial translocation. This might include TNFR2 or other cytokines and their receptors such as interleukin-1β or interferon gamma, which are known to play a role in the regulation of tight junction permeability.[21] Similar to bacterial translocation, reactivation of TNFRI specifically on enterocytes increases liver fibrosis as compared with mice deficient in TNFRI, but the degree of fibrosis is still lower compared with wild-type mice. TNF-α regulates proliferation and extracellular matrix production in hepatic stellate cells by binding to TNFRI,[30] which might account for differences in the fibrogenic response.

The question remains: how is TNFRI on enterocytes activated? We have no evidence that systemic TNF-α possibly coming from the injured liver reaches the TNFRI receptor on enterocytes via the bloodstream to cause tight junction disruption, because TNF-α was undetectable in plasma early after bile duct ligation. Our experiments, however, established that TLR2$^+$ monocytes, but not other inflammatory cells, are activated locally in the lamina propria of the colon to secrete TNF-α and disrupt the tight junction barrier. We have previously reported a rapid onset of increased intestinal permeability and intestinal bacterial overgrowth along the entire intestinal tract following bile duct ligation.[2] It is therefore conceivable that an abundance of bacterial ligands in the intestinal lumen results also in a higher concentration of TLR2 ligands in the lamina propria to activate these cells and to cause intestinal inflammation. It is less likely that the monocytes are activated in the liver and migrate to the lamina propria to cause intestinal inflammation and initiate a paracellular leakage pathway. TLR2 is expressed on Kupffer cells (Supplementary Figure 7), and future studies need to address its contribution to liver fibrogenesis.

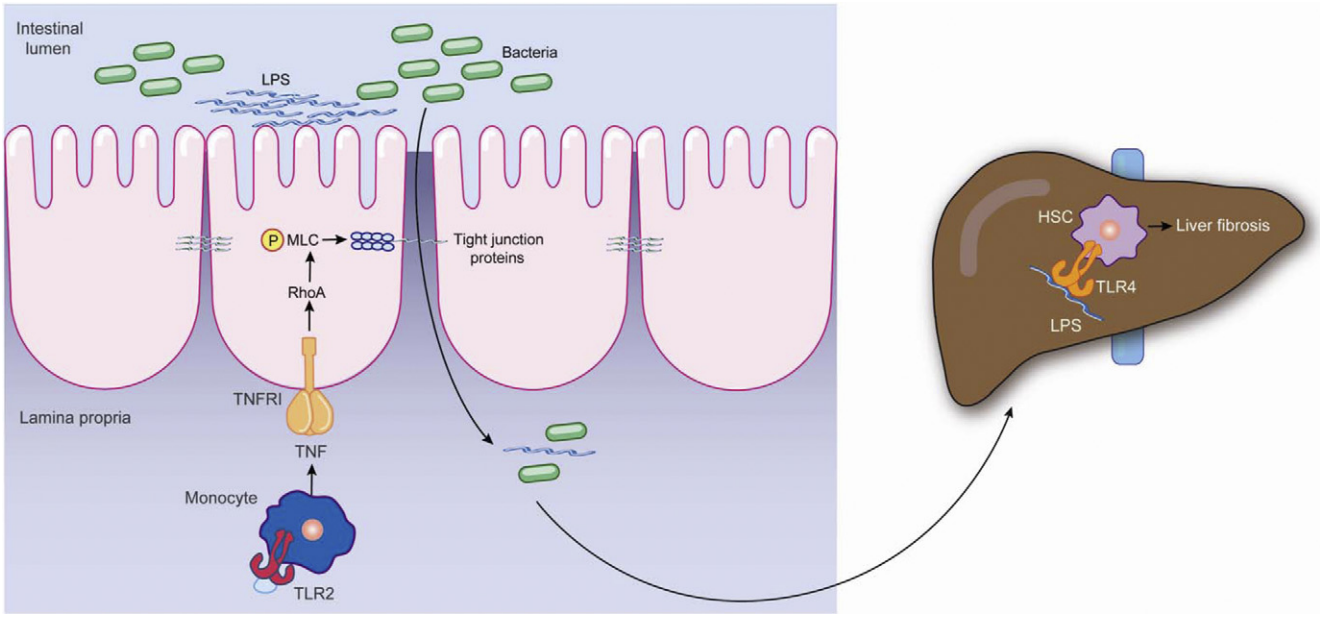

Figure 7. Proposed model of bacterial translocation in cholestatic liver disease. Following cholestatic liver injury, TLR2$^+$ monocytes of the intestinal lamina propria are activated and produce TNF-α. TNF-α binds to TNFRI on enterocytes to activate the RhoA/MLC pathway, resulting in disruption of tight junctions. Bacteria and bacterial products such as lipopolysaccharide (LPS) cross the mucosal barrier to reach the liver via the portal circulation. LPS binds to TLR4 on hepatic stellate cells (HSC) and causes liver fibrosis.

Our study shows that intestinal TNFRI functionally contributes to cholestatic liver fibrosis by increasing intestinal permeability and facilitating translocation of bacterial products. Our findings are consistent with the large body of evidence that chronic liver disease and in particular liver fibrogenesis is driven by the gut, emphasizing the importance of the gut-liver axis. Treatment targeting intestinal inflammation and bacterial translocation might contribute to the clinical management of patients with chronic liver disease.

Supplementary Material

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at http://dx.doi.org/10.1053/j.gastro.2012.07.099.

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Reprint requests

Address requests for reprints to: Bernd Schnabl, MD, Department of Medicine, University of California San Diego, MC0702, 9500 Gilman Drive, La Jolla, California 92093. e-mail: beschnabl@ucsd.edu; fax: (858) 822-5370.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Animal Models of Liver Disease and Isolation of Liver Cells

Mice were treated with carbon tetrachloride (2 μL/g body wt; 1:4 dilution with corn oil) or corn oil as control (2 μL/g body wt) by intraperitoneal injections 3 times per week. Injections were repeated for a total of 12 times.[1] Livers were harvested 2 days following the last injection. Isolation of liver cell fractions from normal mouse liver using magnetic cell sorting has been described.[1]

Endotoxin Assay

All material used for harvesting blood and measuring endotoxin was pyrogen free. An end point chromogenic Limulus amebocyte lysate (LAL) endotoxin kit (Lonza, Basel, Switzerland) was used according to the manufacturer's protocol. To adjust for the intrinsic color of plasma, plasma was diluted 1:10 and the absorbance of each sample was measured before the incubation with the test substrates and subtracted from the final absorbance. Results are expressed relative to wild-type control mice.

RNA Analysis

RNA was extracted from liver using TRIzol (Invitrogen). RNA was digested with deoxyribonuclease using the DNA-free kit (Ambion, Grand Island, NY) and reverse transcribed using the High Capacity cDNA Reverse Transcription kit (ABI, New York, NY). Real-time quantitative polymerase chain reaction was performed with SYBR Green using primer sequences obtained from National Institutes of Health qPrimerDepot.[2]

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