Gut Microbiome Directs Hepatocytes to Recruit MDSCs and Promote Cholangiocarcinoma

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Gut dysbiosis is commonly observed in patients with cirrhosis and chronic gastrointestinal disorders; however, its effect on antitumor immunity in the liver is largely unknown. Here we studied how the gut microbiome affects antitumor immunity in cholangiocarcinoma. Primary sclerosing cholangitis (PSC) or colitis, two known risk factors for cholangiocarcinoma which promote tumor development in mice, caused an accumulation of CXCR2+ polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC). A decrease in gut barrier function observed in mice with PSC and colitis allowed gut-derived bacteria and lipopolysaccharide to appear in the liver and induced CXCL1 expression in hepatocytes through a TLR4-dependent mechanism and an accumulation of CXCR2+ PMN-MDSCs. In contrast, neomycin treatment blocked CXCL1 expression and PMN-MDSC accumulation and inhibited tumor growth even in the absence of liver disease or colitis. Our study demonstrates that the gut microbiome controls hepatocytes to form an immunosuppressive environment by increasing PMN-MDSCs to promote liver cancer.

**SIGNIFICANCE:** MDSCs have been shown to be induced by tumors and suppress antitumor immunity. Here we show that the gut microbiome can control accumulation of MDSCs in the liver in the context of a benign liver disease or colitis.

See related commentary by Chagani and Kwong, p. 1014.

**INTRODUCTION**

The gut and the liver are anatomically and physiologically connected, and this “gut–liver axis” controls not only the liver pathology but also intrahepatic and systemic immune responses. As such, the gut microbiome is an important modulator of antitumor immunity (1–3). The intestinal barrier is the first-line defense to separate intestinal lumen microbes from host (4). Defects in the gut barrier function have been described in different liver diseases (5, 6). An impairment of barrier function increases intestinal permeability and promotes transportation of microbial products, even intact bacteria, into portal circulation. Altered intestinal microbiome composition, known as “dysbiosis,” is associated with intestinal barrier dysfunction, both of which can be commonly observed in chronic gastrointestinal diseases such as inflammatory bowel diseases (IBD), primary sclerosing cholangitis (PSC), and cirrhosis (7–9), and are all risk factors for the development of cholangiocarcinoma (10). However, the knowledge of the influence of dysbiosis and intestinal barrier dysfunction on liver immunity and tumor development is limited.

Cholangiocarcinoma is the second most common primary hepatic malignancy (11). Most patients with cholangiocarcinoma present with unresectable disease at the time of diagnosis, and the prognosis is poor (12). PSC, a chronic liver disease characterized by progressive inflammation and scarring of the medium and large bile ducts of the liver or the extrahepatic bile tree, is recognized as an important risk factor for cholangiocarcinoma (13). Clinical data suggest that colitis, a chronic inflammatory colon disease, contributes to cholangiocarcinoma development (10, 14, 15). Intestinal dysbiosis has been described in patients with PSC (16) or colitis (17). Yet, the role of gut microbiome in cholangiocarcinoma progression is unknown.

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells with the ability to suppress both adaptive and innate immune responses through multiple mechanisms (18). The presence of MDSCs in patients with cancer and their tumor-promoting functions are well documented. MDSCs can be grouped into polymorphonuclear MDSCs (PMN-MDSC) and monocytic MDSCs (M-MDSC; ref. 19). Chemotaxis is important for MDSC accumulation and differs among these two MDSC populations. Specifically, M-MDSC recruitment is often mediated by CCL2–CCR2, whereas the binding of chemokine CXCL1 to its receptor CXCR2 is important for PMN-MDSC accumulation (19). Extrahepatic diseases such as tumors of other organs can induce MDSC accumulation in the liver (20),
and hepatic MDSCs are known to promote liver tumors (21). Recently, the involvement of MDSCs in patients with cholangiocarcinoma has been described, and it has been suggested that targeting PMN-MDSCs improves the efficacy of checkpoint inhibitor therapy in murine cholangiocarcinoma models (22).

Here, we studied how the gut microbiome can affect MDSCs in the liver. Combining different mouse models for PSC, colitis, and cholangiocarcinoma, we demonstrate that Gram-negative commensal gut bacteria control accumulation of hepatic MDSCs through a TLR4/CXCL1/CXCR2-dependent mechanism and thereby suppress antitumor immunity in the liver.

RESULTS

Impaired Gastrointestinal Barrier in PSC and Colitis Exposes the Liver to the Gut Microbiome

Gut dysbiosis has been observed in patients with PSC (23). We examined two different well-established murine PSC models (24). A PSC-like state was either modeled in mice using bile duct ligation (BDL; Supplementary Fig. S1A) or occurred spontaneously in Mdr2−/− mice (Supplementary Fig. S1B; refs. 25, 26). As reported (27), sequencing of the gut microbiome revealed a different spectrum of commensal gut bacteria from mice after BDL (Fig. 1A). Profound changes of the gut commensals were observed at the class level as shown in Fig. 1A. Especially notable are the differences in abundances of Lactobacillales, Actinobacteria, and Clostridiales. These taxa appear to be inversely related, with Lactobacillales and Actinobacteria seen in higher abundance in the control samples and Clostridiales relatively more abundant after BDL (Fig. 1A; Supplementary Fig. S1C). Emerging evidence suggests that dysbiosis contributes to intestinal barrier dysfunction in PSC (28). Next, changes in the intestine were studied. Microscopic analysis revealed signs of chronic colitis with mucosal fibrosis and a reduction of goblet cells in mice after BDL (Supplementary Fig. S1D). Tight junction proteins (Occludin and ZO-1) were decreased in duodenum, jejunum, ileum, and cecum of BDL mice, whereas no difference of Occludin and ZO-1 was observed in the colon (Fig. 1B; Supplementary Fig. S1E). Inflammatory cytokine mRNA levels (IL1β, IL17, IFNγ, TGFβ, TNFε, and IL10) were significantly elevated particularly in ileum tissue samples derived from mice after BDL (Fig. 1C; Supplementary Fig. S2A). These results indicate that PSC development led to inflammatory responses in the small bowel epithelium and an impairment of the gut barrier function. Next, we studied the portal vein, which drains the blood from the intestine to the liver and thereby forms a physical connection between the liver and the gut microbiome. Indeed, higher bacterial 16S rRNA levels were detected in portal vein blood in both PSC-like mouse models (Fig. 1D and E). We also observed a significant increase in serum levels of FITC-labeled dextran after oral administration, further supporting an impaired intestinal barrier function in murine PSC-like models (Fig. 1F and G). Finally, we examined liver tissue for the presence of translocated bacteria. Plating liver tissue lysate revealed more bacterial colonies from mice with PSC-like lesions than from control mice (Supplementary Fig. S2B and S2C).

Inflammatory bowel disease is well known to have impaired intestinal barrier function, so we decided to extend our gut microbiome–liver studies to a well-established murine colitis model induced by dextran sulfate sodium (DSS; ref. 29). Similar to mice with PSC-like lesions, FITC–dextran concentrations in serum (Supplementary Fig. S2D) and bacterial 16S rRNA in portal vein blood (Supplementary Fig. S2E) were higher in mice with colitis than in controls. Consistently, bacterial growth was detected in liver tissues and mesenteric lymph nodes of DSS–colitis mice but not in liver tissues from control mice (Supplementary Fig. S2F and S2G). Different strains including Lactobacillus species were detected in the livers of mice with DSS-induced colitis (Supplementary Fig. S2H). Bacterial translocation to liver was further confirmed with FISH using a probe specific for bacterial DNA (EUB338; Supplementary Fig. S2I). These results demonstrate that both PSC and colitis cause impaired intestinal barrier function, which permits microbes and their products to enter the portal circulation and enter the liver.

Commensal Gram-Negative Gut Bacteria Cause Accumulation of Hepatic MDSCs through a TLR4-Dependent Mechanism

Next, we studied the immune cell subsets in livers of mice with PSC-like lesions. BDL caused a robust accumulation of hepatic CD11b+Gr1+ myeloid cells compared with other immune cells (Fig. 2A; Supplementary Fig. S3A and S3B). CD11b+Gr1+ myeloid cells consisted mainly of CD11b+Ly6G+Ly6C+ polymorphonuclear myeloid cells and, to a lesser extent, of CD11b+Ly6G+Ly6C− monocytic cells. In Mdr2−/− mice, the increase of hepatic CD11b+Ly6G+Ly6C+ cells was also found by flow cytometry (Fig. 2B; Supplementary Fig. S3C). Corroborating these findings, IHC analysis showed an increase of Ly6G+ cells in the livers of Mdr2−/− mice (Fig. 2C). To assess whether the PSC-like lesion–induced

Figure 1. PSC and colitis cause a leaky gut and bacterial translocation. A, BDL was performed in C57BL/6 mice. Two weeks later, stool samples from BDL and control (Ctr) mice were collected for 16S rRNA sequencing. n = 5 for Ctr and BDL. Bar plots of the order levels in BDL and Ctr mice are shown. Relative abundance is plotted for each mouse. B, BDL was performed in C57BL/6 mice. Two weeks later, duodenum, jejunum, ileum, cecum, and colon were collected for immunochemistry (IHC). Representative IHC samples for Occludin are shown. Arrows point to positive signaling of Occludin. C, BDL was performed in C57BL/6 mice. Two weeks later duodenum, jejunum, ileum, cecum, and colon were collected for RT-PCR. The relative mRNA expression analysis for IL1β, IL17, and IFNγ was performed. n = 5 for Ctr and BDL. Data, mean ± SEM. **, P < 0.01; ****, P < 0.0001, two-way ANOVA. D, Quantitative real-time PCR for relative 16S rRNA in portal vein blood of Ctr and BDL mice. n = 5 for Ctr and BDL. Data, mean ± SEM. ****, P < 0.0001, Student t test. E, Quantitative real-time PCR for relative 16S rRNA levels in portal vein blood of Ctr and MDR2−/− mice. n = 5 for Ctr and MDR2−/−. Data, mean ± SEM. ***, P < 0.001, Student t test. F, BDL was performed in C57BL/6 mice. Two weeks later, BDL and Ctr mice received 440 mg/kg body weight FITC–dextran by oral gavage. Four hours later, blood was collected. The concentration of FITC–dextran was measured in blood. n = 5 for Ctr and BDL. Data, mean ± SEM. ****, P < 0.0001, Student t test. G, Ten-week-old VBN/J Ctr and Mdr2−/− mice received 440 mg/kg body weight FITC–dextran by oral gavage. Four hours later, blood was collected. The concentration of FITC–dextran was measured. n = 5 for Ctr and Mdr2−/−. Data, mean ± SEM. ***, P < 0.001, Student t test.
The Gut Microbiome Controls Hepatic MDSCs

Order
- D_0__bacteria; D_1__bacteroidetes; D_2__bacteroidia; D_3__bacteroidales
- D_0__bacteria; D_1__firmicutes; D_2__bacilli; D_3__lactobacillales
- D_0__bacteria; D_1__firmicutes; D_2__clostridia; D_3__clostridiales
- D_0__bacteria; D_1__verrucomicrobia; D_2__verrucomicrobiae; D_3__verrucomicrobiales
- D_0__bacteria; D_1__firmicutes; D_2__eryspelotrichia; D_3__eryspelotrichales
- D_0__bacteria; D_1__patescbacteria; D_2__sacccharmonadina; D_3__sacccharmonadales
- D_0__bacteria; D_1__tenericutes; D_2__mollicutes; D_3__mollicutes RF39
- D_0__bacteria; D_1__proteobacteria; D_2__deltaaproteobacteria; D_3__desulfovibrionales
- D_0__bacteria; D_1__proteobacteria; D_2__gammaproteobacteria; D_3__enterobacteriales
- D_0__bacteria; D_1__proteobacteria; D_2__alphaproteobacteria; D_3__rhodospirillales
- D_0__bacteria; D_1__proteobacteria; D_2__gammaaproteobacteria; D_3__betaproteobacterales
- D_0__bacteria; D_1__proteobacteria; D_2__betaproteobacteria; D_3__xanthomonadales
- D_0__bacteria; D_1__cyanobacteria; D_2__melainabacteria; D_3__gastranaerophiles

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Jejunum

Ileum

Cecum

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Two weeks after BDL, the absolute numbers of hepatic M-myeloid, PMN-myeloid, and total myeloid cells were determined in BDL and control (Ctr) mice. Data, mean ± SEM. ****, *P < 0.0001, two-way ANOVA. B, The absolute numbers of hepatic M-myeloid, PMN-myeloid, and total myeloid cells were determined in FVB/N Ctr and Mdr2−/− mice at the age of 10 weeks. n = 4 for Ctr, 5 for Mdr2−/−. Data, mean ± SEM. **, P < 0.01, two-way ANOVA. C, Immunohistochemistry of Ly6G in liver of Ctr or Mdr2−/− mice at the age of 10 weeks. D, Inhibition of T-cell proliferation by myeloid cells was assayed by flow cytometry of T cells in coculture experiments. Hepatic CD11b+Gr1+ myeloid cells were purified from Ctr or BDL mice. Splenic T cells (T) were isolated from normal C57BL/6 mice. T cells were labeled with CFSE and activated using anti-CD3/anti-CD28. Myeloid cells and T cells were cocultured at different ratios. The percentage of diluted CFSE after 72 hours coculture was measured by FACS. Data, mean ± SEM. ****, P < 0.0001, two-way ANOVA. E, The absolute numbers of hepatic M-MDSCs, PMN-MDSCs, and total MDSCs were determined in C57BL/6 mice after one cycle of DSS treatment (2.5% DSS in drinking water for 1 week, followed by regular water for 2 weeks). n = 6 for H2O, 7 for DSS. Data, mean ± SEM. ****, P < 0.0001, two-way ANOVA. F, Immunochemistry of Ly6G in liver of WT or Mdr2−/− mice treated with vancomycin (Vanco), or neomycin (Neo) for 3 weeks before stool samples were collected for 16S rRNA sequencing. n = 5 for Ctr and BDL. Bar plots of the order levels in BDL and Ctr mice are shown. Relative abundance is plotted for each mouse. H, C57BL/6 mice received neomycin for 2 weeks prior to BDL (BDL + Neo). Two weeks after BDL, the absolute numbers of hepatic M-MDSCs, PMN-MDSCs, and MDSCs were determined. n = 5 for BDL and BDL + Neo. Data, mean ± SEM. ****, P < 0.0001, two-way ANOVA. J, C57BL/6 germ-free mice were colonized with stool samples from mice treated with vancomycin (Vanco Stool) or neomycin (Neo Stool) for 3 weeks by oral gavage. Two weeks later, mice were sacrificed, and the absolute numbers of hepatic M-MDSCs, PMN-MDSCs, and MDSCs were determined. n = 5 for control, Vanco Stool, and Neo Stool. Data, mean ± SEM. *, *P < 0.05; **, *P < 0.01; ****, *P < 0.0001, two-way ANOVA. K, BDL was performed in C57BL/6 mice. Two weeks later, the concentration of endotoxin in portal vein was detected. n = 5 for Ctr and BDL. Data, mean ± SEM. **, P< 0.01, Student t test. L, C57BL/6 mice received H2O, DSS, Neo, and DSS + Neo for 7 days. The concentration of endotoxin in portal vein was detected. n = 5 for H2O, DSS, Neo, and DSS + Neo. Data, mean ± SEM. **, P < 0.01; ****, *P < 0.0001, one-way ANOVA. M, PBS or 2.5 mg/kg LPS was i.p. injected in Tlr4−/− or C57BL/6 WT mice. Three days later, the absolute numbers of M-MDSCs, PMN-MDSCs, and total MDSCs were determined. Data, mean ± SEM. n = 6 for PBS and 7 for LPS in WT mice, n = 6 for PBS and LPS in Tlr4−/− mice, ns, not significant. ****, P < 0.0001, two-way ANOVA.
myeloid cells exhibited immunosuppressive function, we isolated hepatic CD11b^+Gr1^+ myeloid cells and tested their ability to suppress CD8^+ T-cell proliferation in vitro. Indeed, CD8^+ T-cell proliferation was inhibited by BDL–PSC-recruited hepatic myeloid cells (Fig. 2D), proving that these CD11b^+Gr1^+ cells were MDSCs. In addition, fewer CD69^+CD8^+ and Granzyme B^+CD8^+ hepatic T cells were seen in mice with PSC-like lesions (Supplementary Fig. S3D and S3E). PMN-MDSC depletion using 1A8 antibody significantly enhanced the frequency of TNF^+CD8^+ T cells, IFN^+CD8^+ T cells, and CD69^+CD8^+ T cells in liver tissues (Supplementary Fig. S3F). Finally, we studied livers from mice with DSS-induced colitis and observed similar results (Fig. 2E and F; Supplementary Fig. S3G). Accumulation of MDSC in mice after BDL ligation and in Mdr2^−/− mice was not limited to the liver and was also observed in spleen, gut, and lung (Supplementary Fig. S3H and S3I), and in mice after BDL ligation–increased bacterial 16S rRNA in lung tissues were found (Supplementary Fig. S3J).

Next, we studied the effects of commensal gut bacteria on the accumulation of hepatic MDSCs using neomycin and vancomycin treatment to selectively target Gram-negative and Gram-positive bacteria, respectively. As expected, vancomycin and neomycin treatment altered commensal gut bacteria (Fig. 2G), and neomycin treatment impaired the accumulation of hepatic PMN-MDSCs in BDL mice (Fig. 2H; Supplementary Fig. S3K). Because PMN-MDSCs were the major subset, total MDSCs were also reduced after neomycin treatment (Fig. 2H; Supplementary Fig. S3K). Similar results were obtained in neomycin-treated mice with DSS-induced colitis (Supplementary Fig. S3L and S3M), demonstrating that Gram-negative bacteria depletion by neomycin prevented MDSC accumulation in the liver. To study the immediate effects of neomycin–vancomycin-treated gut microbiota on hepatic MDSCs, fecal microbiota transplantation (FMT) was performed. Germ-free mice were fed with cecum stool derived from either neomycin–treated specific pathogen-free (SPF) mice (Neo-Stool, most Gram-positive bacteria left) or vancomycin-treated SPF mice (Vanco Stool, most Gram-negative bacteria left; Supplementary Fig. S3N). Oral gavage with Vanco Stool significantly increased hepatic PMN-MDSCs, whereas Neo-Stool had no effect (Fig. 2I; Supplementary Fig. S3O). Portal lipopolysaccharide (LPS) concentration in GF mice was higher in mice after FMT with stool samples derived from mice treated with vancomycin than with neomycin (Supplementary Fig. S3P). These results indicated that Gram-negative bacteria promote MDSC accumulation in the liver.

We next determined the portal blood concentration of LPS, which is a major component of Gram-negative bacteria. BDL increased LPS concentration in portal vein blood (Fig. 2J). In accordance with results from prior studies (30), higher portal LPS concentrations were also seen in mice after DSS treatment (Fig. 2K), which was reversed by neomycin treatment (Fig. 2K). Furthermore, LPS intraperitoneal challenge was sufficient to induce an accumulation of hepatic MDSCs in wild-type (WT) mice (Fig. 2L; Supplementary Fig. S3Q). Toll-like receptor 4 (TLR4) is the main receptor for LPS (31). Therefore, we studied its role in MDSC accumulation in more detail. Figure 2L and Supplementary Fig. S3Q show that TLR4 deficiency (TLR4^−/−) completely reversed the accumulation of hepatic CD11b^+Gr1^+ MDSCs upon LPS challenge. Similarly, colitis-induced MDSC accumulation was abrogated in Tlr4^−/− mice (Fig. 2M; Supplementary Fig. S3R). These data indicate that neomycin-sensitive Gram-negative bacteria control MDSC frequencies in the liver via LPS and TLR4 even in the absence of specific liver disease or colitis.

**Hepatocytes Mediate MDSC Accumulation via LPS/TLR4/CXCL1**

CXCR2 is a receptor crucial for neutrophil recruitment to inflammatory sites (32) and MDSC accumulation (33). In C57BL/6 mice, approximately 95% of CD11b^+Ly6G^-Ly6C^- polymorphonuclear myeloid cells expressed CXCR2, whereas approximately 5% of CD11b^+Ly6G^-Ly6C^- monocyte myeloid cells were CXCR2-positive (Fig. 3A). BDL and DSS treatment caused a significant increase of hepatic CXCR2^+ cells (Fig. 3B and C; Supplementary Fig. S4A and S4B). PMN-MDSCs accounted for around 85% of hepatic CXCR2^+ leukocytes (Fig. 3D), and the percentage of PMN-MDSCs in CXCR2^+ cells increased after BDL and DSS treatment (Fig. 3E; Supplementary Fig. S4C). CXCL1 is the main ligand of CXCR2 in mice (34). Therefore, we studied CXCL1 expression in the liver of mice with PSC-like lesions and colitis. Cxcl1 mRNA level in livers of BDL, Mdr2^−/−, and DSS–colitis mice increased significantly (Fig. 3F; Supplementary Fig. S4D and S4E), and CXCL1 overexpression in the liver led to an accumulation of hepatic PMN-MDSCs (Fig. 3G; Supplementary Fig. S4F). As expected, CXCL1 antibody neutralization decreased hepatic PMN-MDSCs as well as total MDSC (Fig. 3H; Supplementary Fig. S4G), showing that CXCL1 upregulation mediates accumulation of hepatic PMN-MDSCs in mice with PSC-like lesions and colitis. Similar results were obtained when SB225002, a potent and selective CXCR2 inhibitor (35), was used in BDL-induced PSC-like lesions and DSS-induced colitis model (Fig. 3I; Supplementary Fig. S4H and S4I). Together, these data indicate that the CXCL1/CXCR2 axis regulates PMN-MDSC accumulation in the liver.

Next, we studied the effect of LPS/TLR4 on CXCL1 expression in the liver. TLR4 deficiency abrogated the upregulation of Cxcl1 mRNA expression in both whole liver tissue (Fig. 3J) and isolated hepatocytes (Fig. 3K) from mice subjected to LPS intraperitoneal challenge, demonstrating that TLR4 on hepatocytes is essential for CXCL1 expression upon LPS challenge. Moreover, we found Cxcl1 mRNA level significantly increased only in hepatocytes after DSS treatment but not in macrophages, liver sinusoidal endothelial cells (LSEC), or hepatic stellate cells (HSC; Fig. 3L), indicating that hepatocytes are the main source of CXCL1 in this setting.

We next asked whether resident or bone marrow (BM)–derived/immune TLR4 is responsible for CXCL1 expression and PMN-MDSC accumulation in livers of TLR4–BM chimeric mice. We first confirmed successful BM transplantation (BMT) by flow cytometry of CD45.1 and CD45.2 in peripheral blood (Supplementary Fig. S4J). No MDSC accumulation upon DSS treatment was seen in mice lacking Tlr4 in radioreistant cells [WT to knockout (KO)], whereas MDSCs significantly increased after DSS treatment in chimeric mice expressing Tlr4 in radioresistant cells (KO to WT; Fig. 3M; Supplementary Fig. S4K). Next, we tested mice lacking Tlr4 expression on hepatocytes (Tlr4^−/−) by crossing Tlr4^−/− mice...
A gated on CD11b

PMN-MDSC

M-MDSC

B

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D

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with mice bearing an Albumin-Cre transgene (Alb-Cre) and found that DSS treatment could not induce hepatic MDSC accumulation in ThraKO mice (Fig. 3N; Supplementary Fig. S4L). Similarly, hepatic Cxcl1 mRNA expression was impaired in DSS-treated ThraKO mice (Fig. 3O). All these results demonstrated that TLR4 expression on hepatocytes was essential for LPS to induce CXCL1 expression in the liver.

**PSC and Colitis Promote Cholangiocarcinoma**

PSC is an important risk factor for cholangiocarcinoma, and clinical data suggest that colitis was associated with cholangiocarcinoma (14). Using two well-established murine cholangiocarcinoma models (36, 37), we next tested whether our mouse models of PSC or colitis would promote cholangiocarcinoma. Plasmids encoding activated AKT and YAP or AKT and Notch1 were delivered by hydrodynamic injection, which resulted in formation of cholangiocarcinoma after 4 weeks (36, 37). Microscopic analysis demonstrated tumor lesion with histologic features of cholangiocarcinoma as previously described by others (Supplementary Figs. S5A and S5B; ref. 38). Larger tumors were found in BDL mice upon AKT and YAP injection (Fig. 4A). We also tested AKT and YAP delivery into Mdr2−/− mice and again noticed that PSC-like lesions promoted cholangiocarcinoma growth (Fig. 4C). Similar results were found in mice with colitis. More tumor burden was found in DSS-treated mice upon hydrodynamic injection of either AKT and YAP (Fig. 4D) or AKT and Notch1 (Fig. 4E). Finally, accelerated cholangiocarcinoma growth was also observed in a second colitis mouse model [induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS); Supplementary Fig. S5B; ref. 38]. Larger tumors were found in BDL–PSC mice (Fig. 6A–C; Supplementary Fig. S7A and S7B) compared to Control Cholangiocarcinoma (36, 37), we next tested whether our mouse models of PSC or colitis would promote cholangiocarcinoma models (36, 37), we next tested whether our mouse models of PSC or colitis would promote cholangiocarci- noma. We found that DSS treatment could not induce hepatic MDSC accumulation in ThraKO mice (Fig. 3N; Supplementary Fig. S4L). Similarly, hepatic Cxcl1 mRNA expression was impaired in DSS-treated ThraKO mice (Fig. 3O). All these results demonstrated that TLR4 expression on hepatocytes was essential for LPS to induce CXCL1 expression in the liver.

**Targeting PMN-MDSCs Reduces Cholangiocarcinoma**

Next, we investigated the contribution of the MDSC/CXCL1/CXCR2 axis to cholangiocarcinoma progression. PMN-MDSC depletion by 1A8 antibody (Supplementary Fig. S6A) significantly reduced cholangiocarcinoma growth in mice with colitis (Fig. 5A). Hepatic CXCL1 overexpression by hydrodynamic delivery promoted cholangiocarcinoma growth (Fig. 5B) and was accompanied by an increase of hepatic PMN-MDSCs (Supplementary Fig. S6B). Consistently, CXCL1 neutralization reduced cholangiocarcinoma burden progression in DSS–colitis mice (Fig. 5C) and decreased hepatic PMN-MDSCs (Supplementary Fig. S6C). Similar results were obtained when SB225002 was used to pharmacologically block CXCR2 (Fig. 5D; Supplementary Fig. S6D).

MDSCs have been reported to promote liver cancer through inhibiting natural killer (NK)– or T-cell function (21, 39). Next, we asked if NK or T cells mediated the cholangiocarcinoma-promoting function of MDSC. NK, CD4+ T, or CD8+ T cells were depleted, respectively, in cholangiocarcinoma-bearing mice with or without colitis (Fig. 5E; Supplementary Fig. S6E). Tumor volume in mice after depletion of CD4+ or CD8+ T cells with and without DSS treatment was similar to what we had seen before depletion (Fig. 4E). However, DSS treatment had no further effect on already increased tumor load in mice depleted of NK cells (Fig. 5F and G; Supplementary Fig. S6F), suggesting that MDSCs, which were accumulated in mice after DSS treatment, could no longer promote intrahepatic cholangiocarcinomas (iCCA) without suppressing NK-cell function.

**Gut Microbiome Directs Hepatocytes to Control Cholangiocarcinoma**

The specific role of the gut microbiome on MDSCs in the liver and tumor growth was further investigated. Neomycin treatment was used to eliminate Gram-negative bacteria in the gut, which resulted in fewer cholangiocarcinoma in BDL mice (Fig. 6A–C; Supplementary Fig. S7A and S7B) and DSS–colitis mice (Supplementary Fig. S7C–S7E). To...
Figure 4. PSC and colitis promote cholangiocarcinoma. A, C57BL/6 mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT and YAP. One week later, BDL was performed on the mice. Mice were sacrificed 3 weeks after BDL. Representative liver images and hematoxylin and eosin (H&E) staining are shown. Microscopic tumors were counted. n = 5 for Ctr and BDL. Data, mean ± SEM. **, P < 0.01; ****, P < 0.0001, Student t test. B, Intrahepatic injection using 3 x 10⁶ LD1 cells was performed on C57BL/6 mice, followed by BDL. Two weeks later, the mice were sacrificed. Representative liver images and tumor images were shown. Tumor weights were detected. The ratio of tumor weight to whole liver weight was measured. n = 4 for LD1 and LD1 + BDL. Data, mean ± SEM. *, P < 0.05; **, P < 0.01; ****, P < 0.0001, Student t test. C, FVBN/J (Ctr) or Mdr2−/− mice at 10 weeks were used to induce cholangiocarcinoma via hydrodynamic injection of AKT and YAP. Mice were sacrificed 7 weeks after injection. Representative liver images and H&E staining are shown. Microscopic tumors were counted. n = 6 for Ctr and Mdr2−/−. Data, mean ± SEM. ****, P < 0.0001, Student t test. D, C57BL/6 mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT and YAP. One week later, the mice were treated with 2.5% DSS in drinking water for 1 week, followed by regular water for 2 weeks (one cycle). Mice were sacrificed after two cycles of DSS treatment. Representative liver images and H&E staining are shown. Microscopic tumors were counted. n = 9 for H2O, 8 for DSS. Data, mean ± SEM. ****, P < 0.0001, Student t test. E, C57BL/6 mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT and NICD1. One week later, the mice were treated with 2.5% DSS in drinking water for 1 week, followed by regular water for 2 weeks (one cycle). Mice were sacrificed after two cycles of DSS treatment. Representative liver images and H&E staining are shown. Microscopic tumors were counted. n = 18 for H2O, 18 for DSS. Data, mean ± SEM. *, P < 0.05; ****, P < 0.0001, Student t test.

directly address the effect of gut microbiome on liver tumor growth, we repeated the fecal transplant studies in gut-sterilized mice using antibiotic treatment and monitored tumor growth of intrahepatic injected RIL175 tumor cells (Fig. 6D) in the absence of colitis or PSC-like lesions. Higher tumor burden was found in mice colonized with stool from mice treated with vancomycin (Fig. 6E–G), which was accompanied by a higher number of liver-infiltrating PMN-MDSCs (Supplementary Fig. S7F). Interestingly, in the absence of PSC-like lesions or colitis, gut sterilization using an antibiotic cocktail (ABX, 0.5 g/L vancomycin, 0.5 g/L neomycin, and 0.5 g/L primaxin) did not reduce cholangiocarcinoma growth (Supplementary Fig. S7G and S7H). The lack of effect was likely due to the low PMN-MDSC numbers in the absence of a leaky gut.

Our study demonstrated that TLR4 expression on hepatocytes was critical for PMN-MDSC hepatic accumulation...
Figure 5. The CXCL1/CXCR2/MDSC/NK axis regulates liver cancer development. A, AKT+ NICD1 tumor-bearing C57BL/6 mice with DSS colitis were treated with isotype control (Iso) or anti-Ly6G antibody (1A8; 200 μg, i.p. every other day). Microscopic tumors were counted after two cycles of DSS treatment. Data, mean ± SEM, n = 10 for Iso and 1A8. ****, P < 0.0001, Student t test. B, CXCL1 overexpression in cholangiocarcinoma was induced by hydrodynamic injection using AKT + NICD1 + CXCL1 (CXCL1). Hydrodynamic injection using AKT + NICD1 was used as control (Ctr). Microscopic tumors were counted after 7 weeks. Data, mean ± SEM, n = 8 for Ctr and CXCL1. ****, P < 0.0001, Student t test. C, C57BL/6 mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT and YAP. Mice were treated with CXCL1 neutralization antibody (α-CXCL1) or isotype (Iso) control (4 mg/kg, i.v.) and sacrificed after 2 cycles of DSS treatment. The microscopic tumors were counted. Data, mean ± SEM, n = 5 for Iso and 4 for α-CXCL1. ***, P < 0.001, Student t test. D, Cholangiocarcinoma was induced via hydrodynamic injection of AKT + NICD1, followed by two cycles of DSS treatment. Mice were treated with vehicle or SB225002 (10 mg/kg, i.p. every other day). Microscopic tumors were counted. Data, mean ± SEM, n = 6 for vehicle and SB225002. ****, P < 0.0001, Student t test. E–G, Cholangiocarcinoma was induced via hydrodynamic injection of AKT + NICD1. Mice were treated with anti-CD4, anti-CD8, or NK depletion antibodies and killed after two cycles of DSS treatment. Microscopic tumors were counted. n = 5 for CD8 depletion in DSS-treated mice, 4 for some other groups. Data, mean ± SEM, *, P < 0.05; **, P < 0.01; ***, P < 0.001, two-way ANOVA.
Figure 6. Gut microbiome directs hepatocytes to control cholangiocarcinoma. A–C, Cholangiocarcinoma was induced by hydrodynamic injection of AKT + YAP, and mice were treated with neomycin (Neo). One week later, BDL was performed. Control (Ctr), BDL, Neo, and BDL + Neo mice were sacrificed 3 weeks after BDL (A). Representative H&E staining of livers is shown (B). Microscopic tumors (C) were counted. n = 5 for each group. Data represent mean ± SEM. **, P < 0.01; ***, P < 0.001, one-way ANOVA. D–G, C57BL/6 mice were treated with an antibiotics cocktail (0.5 g/L vancomycin, 0.5 g/L neomycin, and 0.5 g/L primaxin) for 3 weeks, followed by oral gavage of cecum stool samples derived from mice treated for 3 weeks with vancomycin (Vanco Stool) or neomycin (Neo Stool). Two weeks later (week 5), intrahepatic injection of 3 × 10^5 RIL175 cells was performed and mice were sacrificed at week 8 (D). Representative tumors are shown (E). Tumor weight (F) and the ratio of tumor in whole liver (G) are shown. n = 6 for Vanco Stool, 7 for Neo Stool. Data, mean ± SEM. **, P < 0.01; ***, P < 0.001, Student t test. H and I, Tlr4−/− mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT + YAP, then treated with H2O or DSS for two cycles (H). The microscopic tumors and liver weight (I) were determined. Data, mean ± SEM. n = 9 for H2O, 8 for DSS, ns, not significant, Student t test. J–L, Alb-Cre; Tlr4 WT (Tlr4 WT) or Alb-Cre; Tlr4 KO (Tlr4 KO) mice were used to induce cholangiocarcinoma via hydrodynamic injection of AKT + YAP or AKT + YAP plus or without CXCL1. Mice were sacrificed after two cycles of DSS treatment (J). Representative H&E staining is shown (K). Microscopic tumors were counted (L), n = 5 for Tlr4 WT, 4 for Tlr4 KO and Tlr4 KO + CXCL1. Data, mean ± SEM. *, P < 0.05; ****, P < 0.0001, two-way ANOVA.
The Gut Microbiome Controls Hepatic MDSCs

The Gut Microbiome from Patients with Cirrhosis Affects Myeloid Cells in the Liver

The Gut Microbiome Controls Hepatic MDSCs

**Figure 7.** TLR4 gene signatures are associated with poor survival of patients with cholangiocarcinoma. A and B, Human HepG2 or Hep3B cell line was stimulated with 100 ng/mL LPS overnight. CXCL1 (A) and IL8 (B) concentrations in supernatant were determined by ELISA. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ****, P < 0.0001, one-way ANOVA. C, Total of 81 iCCA cases were divided into two groups (low risk and high risk) according to the expression of TLR4 activation–associated genes. Log-rank (Mantel–Cox) test was performed to detect overall survival of the two groups. Log-rank P value and permutation P value are provided. D, HRs with 95% confidence interval (CI) for the three cohorts ICGC, Thailand, and Japan are shown. E–G, CIBERSORT was applied to estimate the abundance of different immune cells based on TLR4 gene-expression level. The abundance of different immune cells in each ICCA patient is shown (E). The abundance of different immune cells in low-risk and high-risk ICCA patients is shown (F). The overall abundance of immune cells in low-risk and high-risk groups is shown (G). (continued on next page)

in leaky gut. The role of TLR4 in cholangiocarcinoma progression was tested. DSS–colitis failed to accelerate tumor growth upon AKT and YAP injection in Tlr4−/− mice (Fig. 6H and I), and no changes in hepatic MDSC levels were seen (Supplementary Fig. S7I). Next, we studied the specific role of TLR4 expression on hepatocytes and how changes in the gut microbiome affect hepatic MDSCs. As expected, tumor growth was dramatically suppressed in mice lacking Tlr4 expression on hepatocytes (Tlr4−/−) after DSS treatment and injection with AKT + YAP (Fig. 6J; Supplementary Fig. S7J). In contrast, CXCL1 overexpression (Supplementary Fig. S7K) rescued impaired tumor growth in Tlr4−/− mice (Fig. 6J; Supplementary Fig. S7J). These results indicated that TLR4 expression on hepatocytes was important for cholangiocarcinoma tumor growth in the context of leaky gut. Together, these data demonstrate that targeting Gram-negative bacteria inhibits cholangiocarcinoma through a hepatocyte selective TLR4 signaling pathway, which leads to hepatic PMN-MDSC accumulation via CXCL1/CXCR2.

The Gut Microbiome from Patients with Cirrhosis Affects Myeloid Cells in the Liver

We conducted complementary studies to assess the significance of our findings observed in animal models to the situation in patients. First, LPS was detected in liver samples from cirrhotic patients (Supplementary Fig. S8A) by IHC. Next, the ability of LPS to induce the expression of CXCL1 and IL8, an important CXCR2 ligand in humans, in human hepatic cell lines was tested. Similar to the observation in mice, LPS incubation induced a strong release of CXCL1 and IL8 from both HepG2 and Hep3B cells (Fig. 7A and B). We also studied TLR4 signature genes
and patient outcome using bulk transcriptomic data from three cohorts of patients with iCCA [International Cancer Genome Consortium (ICGC; ref. 40), Japan (https://www.ebi.ac.uk/ega/home), and Thailand (ref. 41; Supplementary Table S2)]. The TLR4 pathway activity can be detected using a panel of 153 TLR4 signature genes (PathCards, pathway unification database; Supplementary Table S3). BRB-Array analysis separated iCCA cases into two groups (low risk and high risk) according to TLR4 gene signature expression levels (Supplementary Table S4). Patients with high TLR4 gene signature expression displayed worse overall survival in all three cohorts (Fig. 7C; Supplementary Fig. S8B). The mean HRs (95% CI) for the three cohorts were 3.54 (1.9–6.59, ICGC), 2.27 (1.21–4.26, Thailand), and 2.52 (1.66–3.82, Japan; Fig. 7D). We also applied CIBERSORT (42) to estimate the abundance of tumor-infiltrating immune cell subsets in each tumor sample of the high-risk and low-risk groups of ICGC, Japan, and Thailand cohorts based on the gene-expression data (Fig. 7E; Supplementary Fig. S8C and S8D). Here, we did not find significant differences of each immune cell subset (Fig. 7F; Supplementary Fig. S8E and S8F) as well as the overall immune cell abundance (Fig. 7G; Supplementary Fig. S8G and S8H) in all three cohorts, suggesting that the high-risk and low-risk groups were not simply separated by the abundance of immune cells.

Next, we conducted two different studies to investigate a link between the gut microbiome and MDSCs in patients. The level of hepatic myeloid cells in patients with PSC was measured. We used a cohort of patients with PSC with active ulcerative colitis (PSC + aUC), inactive ulcerative colitis (PSC + iUC), or without ulcerative colitis (PSC–noUC; Supplementary Table S3). Myeloid cells in liver sections were detected by IHC using CD15 staining. The presence of CD15+ cells in PSC with active colitis was significantly higher than in those patients with PSC with inactive colitis or patients with PSC without colitis (Fig. 7H), suggesting that active colitis in patients may increase the number of intrahepatic myeloid cells. In addition, we interrogated a gene-expression data set derived from patients with PSC (GSE118373) and found that CXCL1 expression was positively correlated with most of the MDSC signature genes (ref. 43; Fig. 7I; Supplementary Table S6; ref. 44).

Finally, we decided to extend our studies beyond cholangiocarcinoma and PSC and asked whether stool samples derived from other patients with gut dysbiosis may affect intrahepatic MDSC. We performed FMT into germ-free mice using stool samples from patients with liver cirrhosis due to alcoholic hepatitis or healthy control (Supplementary Fig. S9A and S9B; Supplementary Tables S7 and S8). FMT from patients with cirrhosis into germ-free mice increased myeloperoxidase (MPO) myeloid cells and Ly6G mRNA expression level in the liver of mice (Supplementary Fig. S9C–S9E). An increase of CXCL1 expression was also observed, but this change did not reach statistical significance (Supplementary Fig. S9F). In summary, a set of different human studies support our murine studies and demonstrate that the gut microbiome can induce myeloid cell accumulation in the liver, which is associated with worse clinical outcome in cholangiocarcinoma.

**DISCUSSION**

The gut microbiome has profound effects on systemic immune responses, and it has been recognized as an important regulator of antitumor immunity (1, 3). The portal vein, which drains blood from the small and large intestine, delivers large amounts of commensal gut bacteria–derived products to the liver (45). There are several studies suggesting that gut commensal bacteria promote development and growth of hepatocellular carcinoma (HCC; refs. 46, 47).
Most of these studies focused on how the gut microbiome promotes carcinogenesis and malignant transformation of HCC. In contrast, there is only very limited knowledge on how the gut microbiome controls antitumor immunity in the liver and primary liver cancer. Here we studied how the gut microbiome may affect antitumor immunity in cholangiocarcinoma, the second most common type of liver cancer. We studied how two different well-known risk factors for cholangiocarcinoma, PSC and colitis, promote the development of cholangiocarcinoma and demonstrate that gut-derived commensal bacteria are being exposed to the liver and cause an accumulation of immunosuppressive MDSCs. Unexpectedly, TLR4 expression in the liver. Thus, our study suggests that gut commensal bacteria can direct hepatocytes to form a tumor-promoting environment by recruiting PMN-MDSCs.

Studying cholangiocarcinoma and risk factors for biliary cancer using animal models is complicated by the fact that CCAs are classically subdivided into three groups depending on the anatomic site of origin: iCCA, perihilar CCA, and distal CCA (48). Currently, there is no ideal mouse model available for extrahepatic cholangiocarcinoma (49), so we decided to use two different iCCA models, which have previously been established by others. A recent meta-analysis identified inflammatory bowel disease and cirrhosis among others as risk factors for iCCA and extrahepatic CCA with an odds ratio of up to 15 (50). PSC is another well-known risk factor for iCCA (51, 52). In the absence of a well-characterized and highly reproducible PSC animal model, in which mice will develop fibrous-obliterative cholangitis of the intra- and extrahepatic bile ducts in association with inflammation of the gut with predominant right-sided colitis and the development of cholangiocellular carcinoma (24), we decided to combine well-established animal models for colitis and PSC with models for cholangiocarcinoma. As expected, both colitis and PSC promoted growth of cholangiocarcinoma significantly.

The contribution of MDSCs to colitis-associated tumorigenesis is well established (53, 54), and gut-derived commensal bacteria have emerged as important factors during initiation and promotion of colon cancer (55, 56). Hepatic macrophages play a central role in the pathogenesis of chronic liver injury (57). Resident macrophages (Kupffer cells) recognize pathogen-associated molecular patterns and can promote the formation of the inflammasome (58). NLRP3 inflammasome activation was also described in Mdr2-associated cholestasis (59). We noticed a specific accumulation of PMN-MDSCs in mice with colitis, PSC, and upon focal transplant of stool samples derived from vancomycin-treated mice. PMN-MDSCs were CXCR2+ in contrast to CD11b+F4/80+ macrophages or CD11b+Ly6G-Ly6C+ M-MDSCs. This promoted us to study CXCL1 and TLR4 expression in the liver. Unexpectedly, TLR4 expression, which has previously been described to also be expressed by hepatocytes (60, 61), induced CXCL1 expression and an accumulation of PMN-MDSCs much more than TLR4 on M-MDSCs and macrophages.

Our studies show a TLR4-dependent accumulation of hepatic PMN-MDSCs by Gram-negative commensal bacteria, but we did not investigate the role of other TLRs and therefore can only conclude that TLR4 is necessary but not necessarily sufficient for the responses observed, and we cannot exclude that other components in the microenvironment, such as stroma or paracrine signaling, may also contribute to enhanced tumor growth.

Although we were able to demonstrate that commensal bacteria can be found in the liver of mice with colitis, and that transfer of stool samples can induce PMN-MDSCs, it is important to note that it is not clear whether intact bacteria or bacteria-derived components are necessary to induce CXCL1 expression in the liver.

It has previously been shown that PSC results in gut dysbiosis in patients (16, 23), which prompted us to study in more detail the underlying mechanisms of how bile duct changes may cause an increase of LPS in portal vein blood, leading to an accumulation of MDSCs. It has been suggested that changes in bile acid composition found in PSC shape the gut microbiota (16). Here we show that PSC-dependent dysbiosis caused local gut barrier dysfunction associated with local inflammatory responses, ultimately leading to a leaky gut and the ability of Gram-negative bacteria (Gram-negative bacteria–derived products) to transmigrate in the portal vein blood. These observations are supported by recent findings from Nakamoto and colleagues (28), who demonstrated that Klebsiella pneumoniae in the microbiota of patients with PSC disrupts the epithelial barrier to initiate bacterial translocation and liver inflammatory responses. Studies in Mdr2−/− mice indicated that intestinal dysbiosis amplifies the hepatic NLRP3-mediated innate immune response (59) and activation of γδ T cells (62).

Although largely generated from PSC or colitis models, the finding that Gram-negative bacteria induce hepatic MDSC accumulation has more general application. Our results demonstrate that colonization of Gram-negative bacteria–enriched stool in germ-free mice is sufficient to increase hepatic MDSCs, showing that the MDSC-regulating mechanism is independent of PSC or colitis condition. Only hepatic PMN-MDSCs, but not M-MDSCs, increased in Gram-negative bacteria–colonized germ-free mice, which could be explained by the different receptors on the two subsets (19, 33). MDSCs have broad immunosuppressive functions and inhibit both adaptive and innate immune responses (18). Their tumor-promoting function is general and has been reported in many kinds of tumors (18). Indeed, our results show that the increased hepatic MDSCs not only promote cholangiocarcinoma, but also promote the growth of orthotopic RIL175 HCC tumor, suggesting that our finding applies to all liver tumor types. Here we show that MDSCs exert their immunosuppressive function, leading to accelerated tumor growth, but it is possible that other components in the microenvironment, such as stroma or paracrine signaling, may also promote tumor growth (63).

Dysbiosis and increased gut permeability are commonly presented in chronic gastrointestinal diseases (64). Increased
blood LPS has been reported in patients with different chronic liver diseases, such as nonalcoholic fatty liver disease and cirrhosis (65). In addition, patients with liver cirrhosis often present bacterial translocation (66). Our study suggests that gut microbiome–induced hepatic MDSCs may be a common contributor to liver tumor development in the context of chronic gastrointestinal diseases.

It has been demonstrated that gut microbiota promoted diethylaminoamine (DEN)–hepatoxin carbon tetrachloride (CCL4)-induced HCC in a TLR4-dependent manner (67), indicating the essential role of Gram-negative bacteria/LPS/TLR4 in HCC promotion. In addition, Gram-negative bacteria have also been shown to be crucial in infection and inflammation (68, 69). Indeed, in cholangiocarcinoma, we used two PSC-like models (BDL and Mdr2−/−), two colitis models (DSS and TNBS), three cholangiocarcinoma models (AKT/YAP, AKT/Notch, and intrahepatic injection of LD1) in three mouse strains and both genders, and demonstrated that PSC and colitis accelerate cholangiocarcinoma progression in mice. More importantly, Gram-negative bacteria depletion by neomycin treatment inhibited cholangiocarcinoma progression and hepatic PMN-MDSC accumulation. Colonization of Gram-negative bacteria into antibiotics cocktail (ABX)-treated mice promoted RIL175 HCC tumor growth. Therefore, Gram-negative bacteria are a potential therapeutic target for cholangiocarcinoma.

Our studies suggest that TLR4 expression on hepatocytes promotes growth of iCCA via recruiting PMN-MDSCs. Interestingly, in Tlr4−/− mice, colitis had no effect on tumor growth and was similar to what was seen in Tlr4 WT mice without colitis, suggesting that TLR4 signaling may also control tumor growth through MDSC-independent pathways, and TLR4’s role in cholangiocarcinoma may be even more complex. As a matter of fact, both protumor and antitumor functions have been reported, and activation of TLR4 on dendritic cells has been found to improve antigen presentation and lead to better activation of cytotoxic T cells (70), whereas the opposite has been seen in DEN–CCL4-induced HCC (67).

The principle of our finding can also be applied to humans. We detected LPS in livers of patients with liver cirrhosis, and LPS induces human hepatocyte cells to produce CXCL1. More CD11b+ myeloid cells were found in patients with PSC with active colitis compared with patients with PSC with inactive colitis or without colitis. Of note, these samples are extremely rare and we had to restrict our analysis to patients with PSC. Complementary to that, we studied three different cohorts of patients with cholangiocarcinoma from Japan, Thailand, and from the ICGC. Although these data lack complete clinical data, we were able to show that a TLR4 gene signature gene expression was associated with worse survival of patients with cholangiocarcinoma.

Our understanding of how the gut microbiome controls immunologic mechanisms in cancer is largely derived from findings observed in mice. We are aware that animal models can never completely mimic the situation in a patient. Both PSC and CCA models used in this study have limitations: CCA induced by hydrodynamic injections arises in hepatocytes and not in cholangiocytes (71). BDL as well as Mdr2−/− mice cause PSC-like lesions, which can only resemble certain aspects of PSC seen in patients (24). However, experiments done using human cells and gene-expression data from patient cohorts described here clearly support our main conclusions derived from experiments in mice, and only future clinical trials will answer how we can translate findings described here into therapeutic applications (72).

In summary, our study shows that Gram-negative bacteria/LPS controls hepatocytes to form an immunosuppressive microenvironment by inducing CXCR2+ PMN-MDSC accumulation through TLR4-dependent CXCL1 production, thus promoting liver tumor growth (Supplementary Fig. S10). The finding is not limited to PSC or colitis-promoting cholangiocarcinoma, but also applies to other liver tumors with increased liver exposure to gut microbiome. Our study also suggests gut bacteria as a target for prevention and treatment of liver cancer with underlying chronic gastrointestinal conditions.

METHODS

Animal Studies

CS7BL/6 and BALB/c mice were purchased from Charles River. Mdr2−/− (Stock No: 002539), FVB/N (Stock No: 001800), B6 C57D1 (JAX No: 002014), C57BL/10 (Stock No: 000665), Albumin-Cre (Stock No: 003574), and Tbr4−/− (Stock No: 024872) were purchased from The Jackson Laboratory. Tbr4−/− mice were kindly provided by Dr. Giorgio Trinchieri (Cancer and Inflammation Program, NIH). Mice were randomly divided into five mice per cage. After one week, the mice were used for experiments. All experiments were conducted according to local institution guidelines and approved by the Animal Care and Use Committee of the NIH, Bethesda, MD. Hydrodynamic injection was used to induce intrahepatic cholangiocarcinoma (73). For NICD + AKT cholangiocarcinoma, 20 μg NICD, 4 μg AKT, and 1 μg hyperactive sleeping beauty 2 (HSB2) transposase plasmids were diluted into 1.6 mL PBS, and injected into tail vein within 5 to 7 seconds (36). For YAP + AKT cholangiocarcinoma, 30 μg YAP, 20 μg AKT, and 2 μg HSBB were injected hydrodynamically (37). Mice were treated with indicated dose of DSS for 7 days and regular water for 14 days (1 cycle). Fresh DSS water was replaced every other day. For the intrahepatic LD1 cholangiocarcinoma model, 3 × 105 LD1 cholangiocarcinoma cells were injected into the liver. Two weeks later, the mice were sacrificed for further detection. Mice were treated with 2.5 mg/kg LPS (i.p. L2880, Sigma), 200 μg anti-CD4 (i.p. once a week, clone OKT4A, Sigma), 200 μg anti-CD8 (i.p. every other day, clone2.43, Bio X Cell), or 600 μg anti-NK (i.v. every other day, clone PK136, Bio X Cell) for depletion, 200 μg anti-Ly6G (i.p. every other day, clone 1A8, Bio X Cell), 4 mg/kg CXCL1 neutralization antibody (i.v. every other day, MAB453, R&D Systems) for neutralization, 10 mg/kg CXCR2 inhibitor (i.p. every other day, SB225002, Tocris Bioscience) for inhibition. At the end of experiments, mice were sacrificed to collect organs for RNA isolation, flow cytometry, and histologic analysis. All tumor measurements were performed by a blinded investigator.

Cell Lines

Three human HCC cell lines, HepG2, Hep3B, and Huh7, one murine HCC cell line, RIL175 (74), one murine B-cell lymphoma A20, one murine cholangiocarcinoma cell line, LD1, were used in this study. HepG2, Hep3B, Huh7, and A20 were purchased from ATCC. RIL175 has been described previously (20). The LD1 cholangiocarcinoma cell line in CS7BL/6 background was established in our lab and is derived from a mouse after hydrodynamic injection with AKT and YAP plasmids. The sequencing data of
The Gut Microbiome Controls Hepatic MDSCs

Flow Cytometry

For surface marker staining, cells were stained with antibodies for 15 minutes at 4°C, followed by washing with flow cytometry buffer. For intracellular staining, cells were stained with FOXP3/transcription factor staining buffer (eBioscience) according to the manufacturer’s instructions. Antibodies used in this study were as follows: anti-CD19-PerCP/Cy5.5 (clone eB101D3), eBioscience, anti-CD3-FITC (clone 17A2, BD Pharmingen), anti-CD4-Alexa Fluor 700 (clone GK1.5, BioLegend), anti-CD8-Pacific Blue (clone 53-6.7, BioLegend), anti-TCRb-BV510 (clone H57-587, BioLegend), PBSS7/CD1d-tetramer-APC (NH core facility), anti-CD11b-Pacific Blue (clone M1/70, BioLegend), anti-Gr1-PerCP/Cy5.5 (clone RB6-8C5, BioLegend), anti-Ly6G-Alexa Fluor 700 (clone 1A8, BioLegend), anti-Ly6C-APC (cloneHK1.4, BioLegend), anti-F4/80-FITC (clone BM18, BioLegend), anti-CXCR2-PE (clone SA04H64, BioLegend), anti-CD69-Pacific blue (clone H1.2F3, BioLegend), anti-Granulocyte-B220-FITC (clone G011, BioLegend), anti-TNFα-PE (clone MP6-XT22, BioLegend), anti-IFNγ-APC (clone XM1G12, BioLegend). The immune cell subsets were identified by markers: B cells: CD3−CD19−; hepatic CD4+ T cells: CD3−CD4+; CD8+ T cells: CD3−CD8+; NK cells: TCRβ1−CD1d-1Tetramer−; PMN-MDSC: CD11b+Ly6G−Ly6C−; M-MDSC: CD11b+Ly6G+Ly6C−; macrophage: CD11b+F4/80+CD163+. The absolute number of immune cells was calculated by multiplying frequency by the total live cells and then divided by liver weight.

Microscopic Tumor Analysis

Liver tissues were fixed in formalin overnight, followed by fixation in 70% alcohol. Three liver tissues with similar size from the same part of the liver were fixed on one slide. After hematoxylin and eosin staining, microscopic tumors in each slide were calculated.

Endotoxin Assay

Portal vein blood was collected in endotoxin-free tubes. After centrifugation at 10,000 × g for 5 minutes, the plasma was used to detect endotoxin concentration. ToxinSensor Chromogenic LAL Endotoxin Assay Kit (L00250, GenScript) was used according to the manufacturer’s instructions. All determinations were performed in duplicates.

Gut Permeability Assay

Mice were administered with FITC-dextran (4 KDa, Sigma) at a dose of 440 μg/kg body weight 4 hours after fasting. After 4 hours of the administration, blood was collected by cardiac puncture. The concentration of FITC in serum was detected by fluorescence plate reader at excitation 485 nm/emission 528 nm. Serum from mice not administered with FITC-dextran was used as a control.

Bacteria Translocation

Liver tissues were aseptically harvested from BDL-induced PSC mice, Mdr2−/− mice, and normal C57BL/6 mice treated with or without DSS. The cell suspension of liver was plated on Luria-Bertani (LB) medium without any antibiotics, then incubated at 37°C for 24 hours. Colony formation units were counted and calculated as colony forming units (CFU) per mL stool sample (1 g stool dissolved in 30 mL LB medium containing 15% glycerol under anaerobic conditions) at weeks 5 to 6 and repeated 2 weeks later. Two weeks after the second gavage, mice were placed on the control liquid diet for 15 days as described previously (75).

Bacteria Colonization

C57BL/6 mice in an SPF animal room were treated with vancomycin (0.5 g/L) or neomycin (0.5 g/L) for 3 weeks. Then, the cecum stool from five mice was collected into 10 mL anaerobic sterile glyceral. Germ-free mice in C57BL/6 background received 200 μL cecum solution in anaerobic sterile glyceral by oral gavage. Two weeks later, the germ-free mice were sacrificed for further detection. Stool samples from two cirrhotic patients and one healthy donor were used for fecal transplantation in germ-free mice. Mice were gavaged with 100 μL stool sample (1 g stool dissolved in 30 mL LB medium containing 15% glycerol under anaerobic conditions) at weeks 5 to 6 and repeated 2 weeks later. Two weeks after the second gavage, mice were placed on the control liquid diet for 15 days as described previously (75).

16S rRNA Sequencing

DNA extraction and amplification were performed using Eppendorf liquid handling robots. The V4 region of the 16S rDNA gene (515F-806R) was sequenced for 10 samples for Fig. 1A, and 15 samples for Fig. 2G, generating paired-end, overlapping reads on the Illumina MiSeq platform (76). The demultiplexed paired-end fastq files were preprocessed and analyzed using QIIME 2 version 2-2020.2 (https://qiime2.org; ref. 77). The DADA2 algorithm (78), implemented in QIIME2, was used for error modeling and filtering the raw fastq files. Post denoising and chimera removal, a total of 573,739 sequences were retained for 10 samples, with an average of 57,373 sequences per sample for Fig. 1A; a total of 835,408 sequences were retained for 15 samples, with an average of 55,693 sequences per sample for Fig. 2G. Taxonomic classification was performed using the QIME2 feature-classifier (https://github.com/qiime2/q2-feature-classifier) plugin trained on the Silva 132 database (79). The Alpha and Beta diversity analyses were performed using the diversity plugin (https://github.com/qiime2/q2-diversity) at rarefied sampling depths of 50,000 for Fig. 1A and 49,700 for Fig. 2G. The Bioproject accession number of the 16S rRNA sequencing for mouse stools is PRJNA680370.

DNA extraction and 16S rDNA sequencing for human samples (Supplementary Fig. S9A) were performed as described previously (75). Raw 16S rRNA sequencing reads of human stool samples can be found in the NCBI SRA associated with Bioproject PRJNA517994 (Cirrhosis) and PRJNA525701 (Healthy), under the following BioSample IDs: SAMN11083186 (Healthy), SAMN10856936 (Cirrhosis #1), and SAMN10856982 (Cirrhosis #2).

Human Cohort

Patient cohorts have been described previously (75). Patients with cirrhosis due to alcoholic hepatitis were enrolled from the InTeam Consortium (ClinicalTrials.gov identifier number: NCT02075918). Inclusion and exclusion criteria have been published (75). Liver biopsies were done if clinically indicated as part of routine clinical care for diagnostic purposes. The baseline characteristics are shown in Supplementary Table S7. The protocol was approved by the Ethics Committee of each participating center, and patients were enrolled after written informed consent was obtained from each patient.

FISH

Bacteria in liver tissues were visualized using FISH. Liver tissues were fixed in 5% paraformaldehyde overnight at 4°C, followed by washing with PBS, and dehydrated in 15% sucrose overnight at 4°C. Then, the samples were embedded in optimal cutting temperature compound. The slides with frozen samples were applied to probes at the concentration of 2 pmol/μL in prewarmed hybridization buffer (900 mmol/L NaCl, 20 mmol/L Tris pH 7.5, 0.01% SDS, 20% formamide). Next, the slides were incubated at 46°C in a humid chamber for 2 hours and washed at 48°C for 15 minutes in wash buffer.
(215 mmol/L NaCl, 20 mmol/L Tris pH 7.5, 5 mmol/L EDTA). Then the slides were dipped in water, then in 100% ethanol, air-dried, and coverslips were mounted using ProLongGold antifade reagent (Life Technologies). Finally, slides were analyzed using confocal microscopy. The probe used to detect bacteria in liver is EUB338-Alexa 488: all bacteria 16S rRNA GCTGCCTCCCGTAGGAGT (80).

**CXCL1 Overexpression in Mice**

To perform CXCL1 overexpression, 20 μg CXCL1 overexpression plasmid (catalog no. MG50150-UT; Sino Biological) was hydrodynamically injected into mice. In tumor-free mice, the mice were sacrificed for further determination 7 days later. In tumor-bearing mice, the mice were sacrificed for further determination 7 weeks later.

**Quantitative Reverse Transcription PCR**

Total RNA from whole liver tissues, hepatocytes, LSECs, macrophages, and HSCs was isolated using the RNeasy Mini Kit (catalog no. #74104, Qiagen) according to the manufacturer’s instructions. cDNA synthesis was performed using an iScript cDNA synthesis kit (catalog no. 170-8891, Bio-Rad). For germ-free mice colonized with human stool samples, RNA was extracted from liver tissues using TRIzol reagent (catalog no. 15596018, Thermo Fisher), and cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (catalog no. A21280, Thermo Fisher). RT-PCR was performed using qSYBR Green Supermix (catalog no. 1708882, Bio-Rad). The following primers were used for RT-PCR: CXCL1: forward, 5′-CXCL1-CCT GCA CCA CCA ACT GCT TA-3′; reverse, 5′-CTT GAG ATG GGA AGG CAG AGA TT-3′. GAPDH: forward, 5′-GAA GCT TCA GAA GGT GTT GCC CTC AG-3′; reverse, 5′-TCA GCC CTT CCA CAA ACT GT TA-3′. GAPDH expression level was used as a control.

**Western Blot**

Whole-cell lysates were obtained by adding ice-cold lysis buffer (Mammalian Protein Extract Reagent, Thermo Fisher Scientific) containing protease inhibitors (Halt Protease and Phosphatase Inhibitor, Thermo Scientific). The lysed cells were then centrifuged at 12,000 rpm for 20 minutes to remove cellular debris. Protein concentration of supernatant was determined by the BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were then resolved by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were incubated with primary antibodies at 4°C overnight in 2% nonfat milk in TBS-Tween. The primary antibody dilution was 1:1,000 unless otherwise indicated. The following primary antibodies were used for immunoblotting analysis: CK-7 (ab181598) and CK-19 (ab52625) from Abcam and β-actin (4970S) from Cell Signaling Technology. After overnight incubation, membranes were washed in TBS-Tween and then corresponding horseradish peroxidase-conjugated secondary antibodies were added to membrane at a concentration of 1:2,000 and incubated for 1 hour at room temperature. Immunoblots were visualized with enhanced chemiluminescence reagents (Clarity/Clarity Max ECL, Bio-Rad).

**IHC**

IHC was carried out using 5-μm paraffin-embedded sections of mouse or human liver tissues using the Universal Animal IHC Kit (Lot: KT4418A, InnoVex) according to the manufacturer’s instructions. The primary antibodies anti-CX19 (Abcam, ab52625), anti-HNF4a (Santa Cruz Biotechnology, sc-374229), anti-Ly6G (clone IA8, Bio X Cell for Fig. 2D and F; BD Biosciences for Supplementary Fig. S2G), anti-MPO (Biocare Medical; catalog no. PP023AA), anti-CD15 (DAKO, M3631), anti-LPS Core, mAb WN1 222-S (Hycult-Biotech, catalog no. HM6011; RRID:AB_2750644) were used. ImageJ software was used to count the positive staining cells.

**ELISA**

A total of 5 × 10⁶ human hepatocyte cells from cell lines HepG2 or Hep3B were seeded into 6-well plate with 100 ng/mL LPS (L2880, Sigma) challenge overnight. CXCL1 and IL8 concentration in supernatant was determined using the Human GRO alpha ELISA Kit (Invitrogen, Ref: BMS2122) and the human IL8 ELISA Kit (Invitrogen, catalog no. KHC0081), respectively, according to the manufacturer’s instructions.

**T-cell Proliferation Assay**

Hepatic MDSCs were isolated using Myeloid-Derived Suppressor Cell Isolation Kit (order no. 130-093-538, Miltenyi Biotec) according to the manufacturer’s instructions. T cells from spleen were labeled with CFSE and stimulated using T-cell Activation/Expansion Kit (order no. 130-093-627, Miltenyi Biotec). A total of 1 × 10⁶ CFSE-labeled splenic T cells were seeded into 96-well plate and cocultured with MDSCs with different ratios (MDSC:T = 0.1, 0.2, 1, 11). Seventy-two hours later, diluted CFSE+ CD8+ T cells were measured as proliferated T cells using flow cytometry.

**Isolation of Lymphocytes from Gut**

Lympocyte isolation from gut was performed as previously described (81). Briefly, tissue segments were incubated in 30 mL of extraction media [30 mL RPMI + 93 μL 5% (w/v) dithiothreitol (DTT) + 60 μL 0.5 mol/L EDTA + 500 μL FBS] for 15 minutes at 37°C. Minced tissues were added to 25 mL of digestion media (25 mL RPMI + 12.5 mg dispase + 37.5 mg collagenase II + 300 μL FBS) and stirred at 500 rpm for 30 minutes at 37°C. Digested tissue was filtered through a 100-μm cell strainer into a 50 mL tube. The strainer was rinsed with 20 mL of RPMI containing 10% FBS. The filtered solution was centrifuged at 500 × g for 10 minutes at 4°C. Pellets were resuspended in 1 mL of RPMI containing 10% FBS. The cells were then ready for flow cytometry analysis.

**Isolation of Hepatocytes, LSECs, Macrophages, and HSCs**

A previously reported protocol was followed to isolate the four types of cells from the same liver (82, 83). Mice were euthanized with CO₂. The liver was perfused with 5 mL of extraction media containing 10% FBS and 0.5 mL/L EDTA in Hank’s Balanced Salt Solution (HBSS) at 37°C for 10 minutes. The liver was then perfused with 5 mL of digestion media containing 0.5 mL/L EDTA in HBSS supplemented with 5 mL of RPMI and 0.5 mL/L CaCl₂ at 37°C for 5 minutes. The livers were excised and homogenized and then passed through a 70-μm filter. The suspension was centrifuged at 500 × g for 5 minutes to separate hepatocytes (pellet) from nonparenchymal cells (NPC, supernatant). Hepatocytes were further enriched by magnetic bead depletion of anti-CD45 and anti-CD146 to deplete most immune cells and endothelial cells, respectively. Nonspecific binding was blocked with 1% FBS and 1% serum to ensure optimal cell attachment. The suspension was centrifuged at 1,500 × g for 5 minutes at 4°C. The pellets were resuspended in 20 mL of RPMI containing 10% FBS. The filtered solution was centrifuged at 500 × g for 10 minutes at 4°C. Pellets were resuspended in 1 mL of RPMI containing 10% FBS. The cells were then ready for flow cytometry analysis.

**Isolation of Hepatocytes, LSECs, Macrophages, and HSCs**

A previously reported protocol was followed to isolate the four types of cells from the same liver (82, 83). Mice were euthanized with CO₂. Then, the liver was perfused with 5 mL/L HEPES and 0.5 mL/L EDTA in Hank’s Balanced Salt Solution (HBSS) at 37°C for 5 minutes, followed by perfusion with 0.05% collagenase IV (Sigma, C1318) in HBSS supplemented with 5 mL/L HEPES and 0.5 mL/L CaCl₂ at 37°C for 5 minutes. The livers were excised and homogenized and then passed through a 70-μm filter. The suspension was centrifuged at 500 × g for 3 minutes to separate hepatocytes (pellet) from nonparenchymal cells (NPC, supernatant). Hepatocytes were further enriched by magnetic bead depletion of anti-CD45 and anti-CD146 to deplete most immune cells and endothelial cells, respectively. The NPC fraction was then submitted to a 15% OptiPrep density gradient and then centrifuged at 1,500 × g for 5 minutes at room temperature. The well-defined interface of cells was carefully collected, and then centrifuged at 500 × g for 5 minutes at 4°C. The NPCs were then ready for flow cytometry analysis; Antibodies used for staining were as follows: anti-CD146-APC (clone ME-9F1, BioLegend), anti-Tie2-PE (clone TEK4, BioLegend), anti-F4/80-FITC (clone BM8, BioLegend), and anti-CD3-PE/Cy7 (clone 17A2, BioLegend). LSECs were stained as CD146+Tie2+ F4/80+ CD11b+ CD3⁺. Macrophages were stained as F4/80+CD11b+CD146+ Tie2+ CD3⁻ cells, and HSCs were negatively stained as CD3⁻ CD146⁻ Tie2⁺ F4/80⁻ CD11b⁻ cells. Cell sorting was then carried out with FACSAria II (BD Biosciences). All experiments were performed with 95% yield of purity for each subset.
The Gut Microbiome Controls Hepatic MDSCs

**BMT**

CD45.1 WT C57BL/6 mice and CD45.2 Tbr4−/− mice were lethally irradiated with 900 rad. Four to six hours later, 2 × 107 BM cells were intravenously injected into the irradiated mice. All experiments were performed 6 weeks after BMT. Successful BMT was confirmed by flow cytometry of CD45.1 and CD45.2 in peripheral blood.

**Survival Analysis of Patients with Cholangiocarcinoma**

To indicate the potential association of TLR4-related genes and patient outcomes, we used bulk transcriptomic data from three cohorts, that is, ICGC, Japan, and Thailand. Survival information was available for 115 patients of the ICGC cohort, 162 patients of the Japan cohort, and 85 patients of the Thai cohort. To perform survival analysis based on 153 TLR4-related gene signatures (Supplementary Table S2), we applied BBR-Array Tools (version 4.6.0; ref. 84) developed by the Biometric Research Branch of NCI. Kaplan–Meier curves were provided for two risk groups obtained by 10-fold cross-validation. We performed permutation for the log-rank test 100 times. Both log-rank $P$ value and permutation $P$ value were provided to indicate statistical significance.

**Statistical Analysis**

The sample sizes for animal studies were guided by previous murine studies in our laboratory. Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software). The significant differences between groups were calculated by Student unpaired $t$ test, one-way, or two-way ANOVA (Tukey and Bonferroni multiple comparisons test). Log-rank (Mantel–Cox) test was used to determine significance between survival curves. $P < 0.05$ was considered statistically significant.

**Authors’ Disclosures**

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**Authors’ Contributions**

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