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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Western Diet—Induced Dysbiosis in Farnesoid X Receptor Knockout Mice Causes Persistent Hepatic Inflammation after Antibiotic Treatment



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Patients who have liver cirrhosis and liver cancer also have reduced farnesoid X receptor (FXR). The current study analyzes the effect of diet through microbiota that affect hepatic inflammation in FXR knockout (KO) mice. Wild-type and FXR KO mice were on a control (CD) or Western diet (WD) for 10 months. In addition, both CD- and WD-fed FXR KO male mice, which had hepatic lymphocyte and neutrophil infiltration, were treated by vancomycin, polymyxin B, and Abx (ampicillin, neomycin, metronidazole, and vancomycin). Mice were subjected to morphological analysis as well as gut microbiota and bile acid profiling. Male WD-fed FXR KO mice had the most severe steatohepatitis. FXR KO also had reduced Firmicutes and increased Proteobacteria, which could be reversed by Abx. In addition, Abx eliminated hepatic neutrophils and lymphocytes in CD-fed, but not WD-fed, FXR KO mice. Proteobacteria and Bacteroidetes persisted in WD-fed FXR KO mice even after Abx treatment. Only polymyxin B could reduce hepatic lymphocytes in WD-fed FXR KO mice. The reduced hepatic inflammation by antibiotics was accompanied by decreased free and conjugated secondary bile acids as well as changes in gut microbiota. Our data revealed that *Lactococcus*, *Lactobacillus*, and *Coprococcus* protect the liver from inflammation. (*Am J Pathol* 2017, 187: 1800–1813; <http://dx.doi.org/10.1016/j.ajpath.2017.04.019>)

The liver is constantly exposed to gut-derived components as 70% of hepatic blood supply comes from enterohepatic circulation. Gut-derived signaling dictates liver health. Bacteria-generated lipopolysaccharide (LPS), which stimulates IL-6—mediated Janus activating kinase 2—STAT3 activation, is essential for liver regeneration.¹ However, excessive LPS causes hepatic inflammation and dampens liver regeneration capability.^{2,3} Moreover, the transition of steatosis to steatohepatitis plays a crucial role in hepatocyte proliferation and liver carcinogenesis. Thus, it is important to understand the effect of the gut in contributing to liver inflammation. Increased hepatic inflammatory signaling can be due to excessive lipid and sugar intake that generates reactive oxygen species, stressing the metabolic system.⁴ It can also be due to inappropriate activation and homing of neutrophils.⁵ Moreover, hepatic inflammation is associated with intestinal

bacterial overgrowth, increased gut permeability, and reduced immunological defenses. Therefore, gut-derived signaling has a significant impact on the liver health.^{3,6}

Bile acids (BAs) are no longer considered solely for lipid absorption; they are signaling molecules with diverse effects on regulating host immunity and inflammation.^{6–14} Clinical data revealed that the receptor for BAs [ie, farnesoid X receptor (FXR)] is reduced in liver and colon cancers.^{15,16} Similarly, mice that lack FXR develop spontaneous liver cancer and have increased susceptibility to colitis, cholestasis, and colon cancer.¹⁷ However, tissue-specific FXR knockout (KO) mice, including liver and intestines, do not

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spontaneously develop liver cancer.^{18,19} These findings clearly indicate the interactive effect of hepatic and gut FXR in contributing to liver carcinogenesis. Moreover, elevated BAs found in FXR KO mice contribute to liver carcinogenesis because cholestyramine can prevent it.^{17,20,21} Because of the significance of BAs in inflammation and carcinogenesis, the current study uncovers whether the specific BAs that contribute to or protect from hepatic inflammation in response to a Western diet (WD) intake in FXR KO mice.

BAs that flow through the gut-liver axis have a pivotal role in maintaining liver and intestinal health. Free and conjugated primary and secondary BAs are generated by hepatic as well as microbial enzymes. Bile salt hydrolase in *Bifidobacterium* and *Lactobacillus* deconjugates BAs, and bacterial 7 α -dehydroxylase converts primary into secondary BAs.³ Thus, eubiosis is essential for BA homeostasis. In contrast, WD-induced dysbiosis leads to dysregulated BA synthesis and increases the risk of liver and colon cancer. Therefore, there is an intimate relationship between gut microbiota and BAs, and it would be important to identify the microbiota and their associated BAs, which contribute to hepatic inflammation.

In the current study, we challenged wild-type (WT) and FXR KO mice of both sexes with a control diet (CD) and WD, followed by antibiotic treatments, to uncover gut bacteria as well as BAs that have a protective or detrimental effect on hepatic inflammation. Our data showed that WD and FXR inactivation increased hepatic inflammatory signaling with lymphocyte and neutrophil infiltration. Diet, sex, and different antibiotic treatments altered the gut microbiota and had different effects on hepatic inflammation. The specific bacteria and BAs that may contribute to or protect the liver from inflammation are identified.

Materials and Methods

Mice

Specific pathogen-free C57BL/6 mice and FXR KO mice²² of both sexes were housed in steel microisolator cages at 22°C with a 12-hour light/dark cycle and were given a CD (5.2% fat, 12% sucrose, and 0.01% cholesterol, w/w) or WD (21.2% fat, 34% sucrose, and 0.2% cholesterol, w/w) from Harlan Teklad (Madison, WI), after weaning (3 weeks, 6 to 10 mice per group) and were euthanized at the age of 10 months. Experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*²³ under protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Sacramento, CA).

Antibiotic Treatment

The treatment regimens included vancomycin (Vcm; 500 mg/L, Gram-positive coverage), polymyxin B (PolyB;

100 mg/L, Gram-negative coverage), and Abx (broad-spectrum coverage) that included ampicillin (1 g/L), neomycin (1 g/L), metronidazole (1 g/L), and vancomycin (500 mg/L) in drinking water for 12 weeks. All three treatments have been shown to disrupt the gastrointestinal microbiota.^{24,25}

Biochemical Analysis

Serum alanine aminotransferase (ALT; Pointe Scientific, Canton, MI) and lipopolysaccharide (Thermo Fisher Scientific, Rockford, IL) levels were quantified according to the manufacturers' instructions.

Quantification of Bile Acids

Sample preparation was performed based on published methods.¹¹ Hepatic BAs were detected on a Prominence Ultra Fast Liquid Chromatograph system (Shimadzu, Kyoto, Japan) coupled to an API 4000 QTRAP mass spectrometer (Sciex, Redwood City, CA) operated in the negative ionization mode. Chromatography was performed on a Kinetex C₁₈ column (50 × 2.1 mm, 2.6 μ m particle size) maintained at 40°C preceded by a high-pressure column prefilter. The mobile phase consisted of a gradient of methanol delivered at a flow rate of 0.4 mL/minute. Mass spectrometer parameters were described in our previous publication.¹¹

Gene Expression Profiling

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA. Real-time quantitative RT-PCR was performed on an ABI 7900HT Fast real-time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The mRNA levels were normalized to the level of *Gapdh* mRNA.

Flow Cytometry

Freshly isolated spleen cells (10⁶) were first incubated with Fc block and then labeled with anti-mouse antibodies, including phycoerythrin-Cy5-conjugated anti-CD62L, allophycocyanin (APC)-Cy7-conjugated anti-CD25, phycoerythrin-Cy5-conjugated anti-CD44, fluorescein isothiocyanate-conjugated anti-CD4, and APC-Cy7-conjugated CD25, APC anti-F4/80, and APC Cy7 anti-CD11b (BD Pharmingen, San Diego, CA), and Alexa Fluor 700-conjugated anti-CD8 and Pacific Blue-conjugated anti-CD44 (BioLegend, San Diego, CA) and a staining buffer consisting of 1% fetal bovine serum, 1 mmol/L EDTA, and 0.02% azide in Dulbecco's phosphate-buffered saline (Mediatech, Herndon, VA). List mode data files were collected using a Fortessa cell analyzer with FACSDiva software version 8.0 (BD Biosciences, San Jose, CA). All data sets were analyzed using FlowJo software version 10 (Tree Star Inc., Ashland, OR).

16S rRNA Gene Sequencing of Gut Microbial Communities

Cecal DNA was isolated using the ZR Fecal DNA miniprep kit (Zymo Research, Irvine, CA). Illumina sequencing of barcoded 16S rRNA gene amplicons of genomic DNA was performed based on published methods.^{11,26–28} Variable region 4 of the 16S rRNA gene was amplified and sequenced. Sequence reads were analyzed using QIIME software version 1.9.1.²⁹

Bioinformatics and Statistical Analysis

Functional profiles of microbial communities were predicted using phylogenetic investigation of communities by reconstruction of unobserved states, and linear discriminant analysis effect size was performed to identify pathways that were statistically different.³⁰ Spearman correlations were performed with the R program (The R Foundation; <http://www.r-project.org>). Unpaired *t*-test, Mann-Whitney *U* test, and one-way analysis of variance were performed by using GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA). Data are expressed as means \pm SD. Differences between groups in microbiota family and genus level were calculated by Kruskal-Wallis tests. *P* values were adjusted for multiple comparisons using false discovery rate. *P* < 0.05 was considered statistically significant.

Results

The Effect of Diet and FXR Inactivation on Hepatic Phenotypes

When mice were 10 months old, WD intake and FXR inactivation both induced steatosis, which was more severe in males than females (Figure 1A). However, the most striking finding was that only FXR KO mice had massive lymphocyte and neutrophil infiltration, which was more severe in males than females (Figure 1, A–C). In addition, only WD-fed male FXR KO mice had fatty adenoma, which was grossly visible (Figure 1A). Serum LPS levels were much higher in WD-fed male FXR KO mice than the female counterparts (Figure 1D). In addition, serum ALT level was increased by both WD intake and FXR inactivation (Figure 1E). Together, our data support that the induced serum endotoxin, ALT, and hepatic inflammatory cell infiltration contributed to the development of steatohepatitis in FXR KO mice in a male-dominant manner.

The Effect of Antibiotics on Hepatic Inflammation

To determine the role of gut microbes in hepatic inflammation, antibiotics were used to treat male FXR KO mice. Broad-spectrum coverage Abx (a cocktail of ampicillin, neomycin, metronidazole, and vancomycin) completely

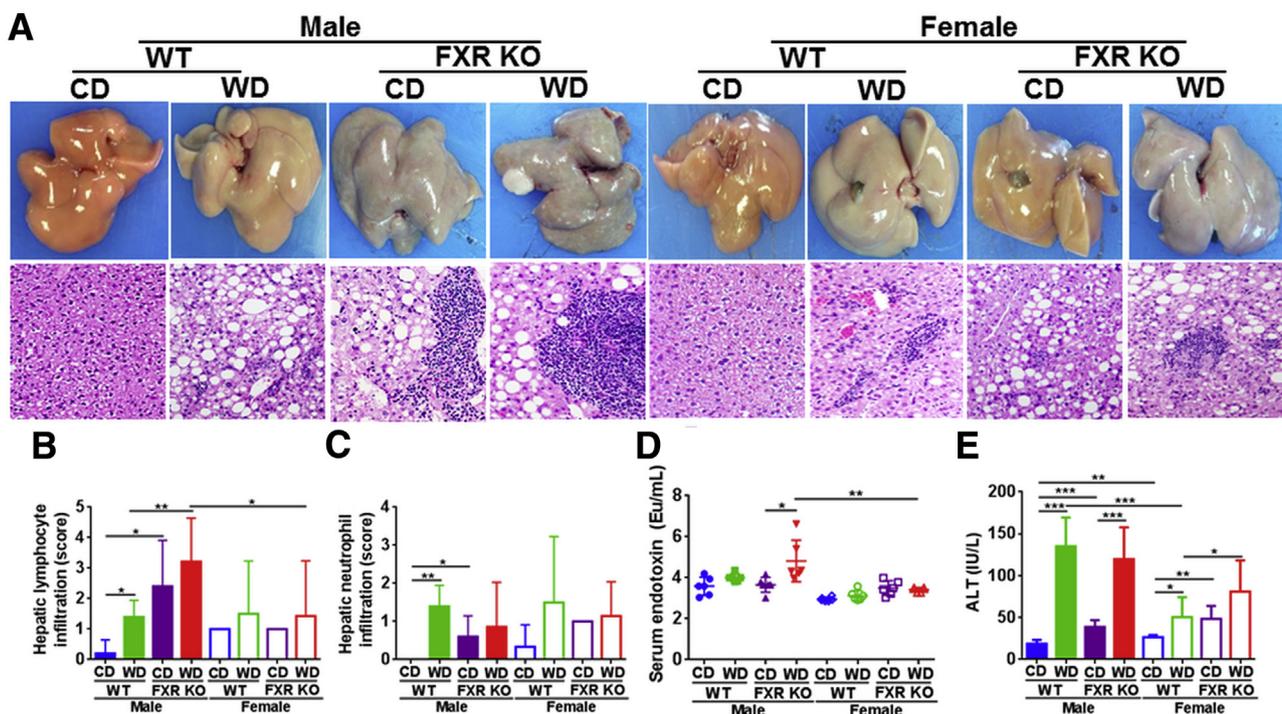


Figure 1 Histology and inflammation score in control diet (CD)– and Western diet (WD)–fed wild-type (WT) and FXR knockout (KO) mice of both sexes. **A:** Representative liver morphology and hematoxylin and eosin–stained liver sections. Hepatic lymphocyte (**B**) and neutrophil (**C**) infiltration score was graded on a scale of 0 (absent), 1 (rare), 2 (mild), 3 (moderate), and 4 (severe). Serum endotoxin (**D**) and alanine (**E**) level. Data are expressed as means \pm SD (**B–E**) $n \geq 6$ per group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 [based on a single variable of comparison (ie, diet, genotype, or sex)]. Original magnification, $\times 40$ (**A**). ALT, serum alanine aminotransferase.

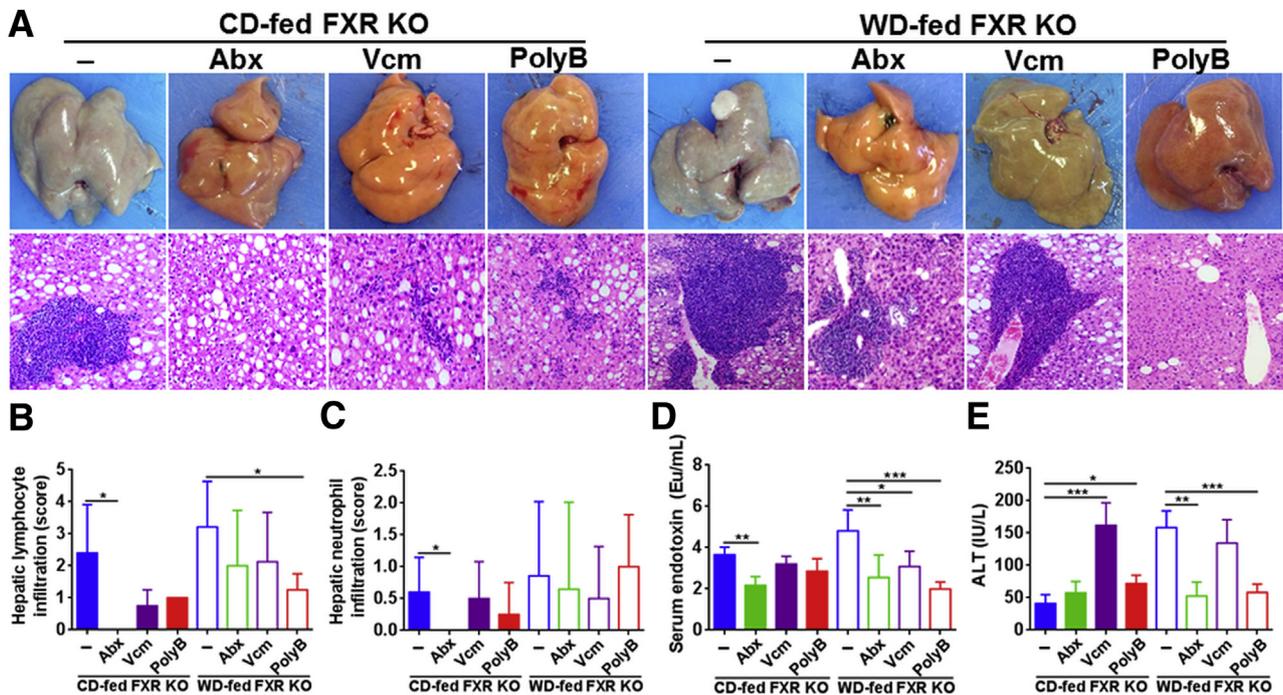


Figure 2 Histology and inflammation score in control diet (CD)- and Western diet (WD)-fed FXR KO mice treated with antibiotics Abx, Vcm, and PolyB. **A:** Representative liver morphology and hematoxylin and eosin-stained liver sections. Hepatic lymphocyte (**B**) and neutrophil (**C**) infiltration score was graded on a scale of 0 (absent), 1 (rare), 2 (mild), 3 (moderate), and 4 (severe). Serum endotoxin (**D**) and ALT (**E**) level. Data are expressed as means \pm SD (**B–E**). $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Original magnification, $\times 40$ (**A**). ALT, serum alanine aminotransferase; PolyB, polymyxin B; Vcm, vancomycin.

prohibited neutrophils and lymphocytes in CD-fed FXR KO mice (Figure 2, A–C), which was accompanied by a reduced serum LPS level (Figure 2D). Vcm and PolyB seem to reduce hepatic lymphocytes, but the effect was not statistically significant ($P = 0.07$ and $P = 0.11$, respectively) (Figure 2B). In addition, neutrophil infiltration remained after the Vcm and PolyB treatment (Figure 2C). In contrast to CD-fed mice, Abx was not able to eliminate neutrophils and lymphocytes in WD-fed FXR KO mice. Only PolyB significantly reduced lymphocyte infiltration in WD-fed FXR KO mice, which was consistent with the reduction of serum LPS and ALT (Figure 2, D and E). However, elimination of Gram-positive bacteria by Vcm increased serum ALT in CD-fed FXR KO mice (Figure 2E).

The Effect of Diet and FXR Status on Hepatic Inflammatory Signaling

WD intake and FXR inactivation increased the mRNA levels of hepatic profibrogenic markers collagen type 1 α 1, plasminogen activator inhibitor-1, and tissue inhibitor of metalloproteinases (Figure 3). Similarly, monocyte chemoattractant protein 1 (Mcp-1), macrophage inflammatory protein 2 α , intercellular adhesion molecule 1, and leukocyte integrin α (Cd11c) mRNA levels were induced by WD or FXR KO (Figure 3). The enhanced effect due to WD plus FXR KO in inducing inflammatory signaling was revealed by the mRNA level of serum amyloid a1, forkhead box P3,

chemokine (C-C motif) ligands 17 and 20, and lysophosphatidylcholine receptor [G-protein-coupled receptor (Gpr) 132], which had the highest expression levels in WD-fed FXR KO male mice, consistent with the observed liver pathology (Figure 3).

Because Abx reduced hepatic neutrophils and lymphocytes, gut microbiota likely contributed to hepatic inflammation. Thus, we studied the inflammatory signaling in the ileum, where the enterohepatic circulation takes place. Consistently, ileal mRNA levels of Il-6 and Mcp-1 were increased by WD intake and FXR deficiency (Figure 4). Gram-positive bacteria that generated short-chain fatty acids (SCFAs) increase the expression of intestinal tight junction genes to prevent leaky gut and have an anti-inflammatory effect.³¹ WD intake and/or FXR inactivation reduced the mRNA levels of SCFA receptors that include Gpr 41, 43, and 109a (Figure 4). These findings suggested WD intake as well as FXR inactivation might have a negative impact on SCFA receptor-regulated signaling. In addition, regenerating islet-derived protein 3 γ , an antimicrobial peptide, was also reduced by WD intake and FXR inactivation in the ileum (Figure 4).

All three antibiotics reduced the expression of hepatic inflammatory genes, such as *Il6*, *Il1b*, *Tnfa*, and *Ccl20*, in both CD- and WD-fed FXR KO mice (Figure 5). However, Abx had the best effect in reducing the expressions of hepatic collagen type 1 α 1, macrophage inflammatory protein 2 α , and Mcp-1, but Vcm increased them in CD-fed FXR KO mice (Figure 5). In addition, Abx and PolyB

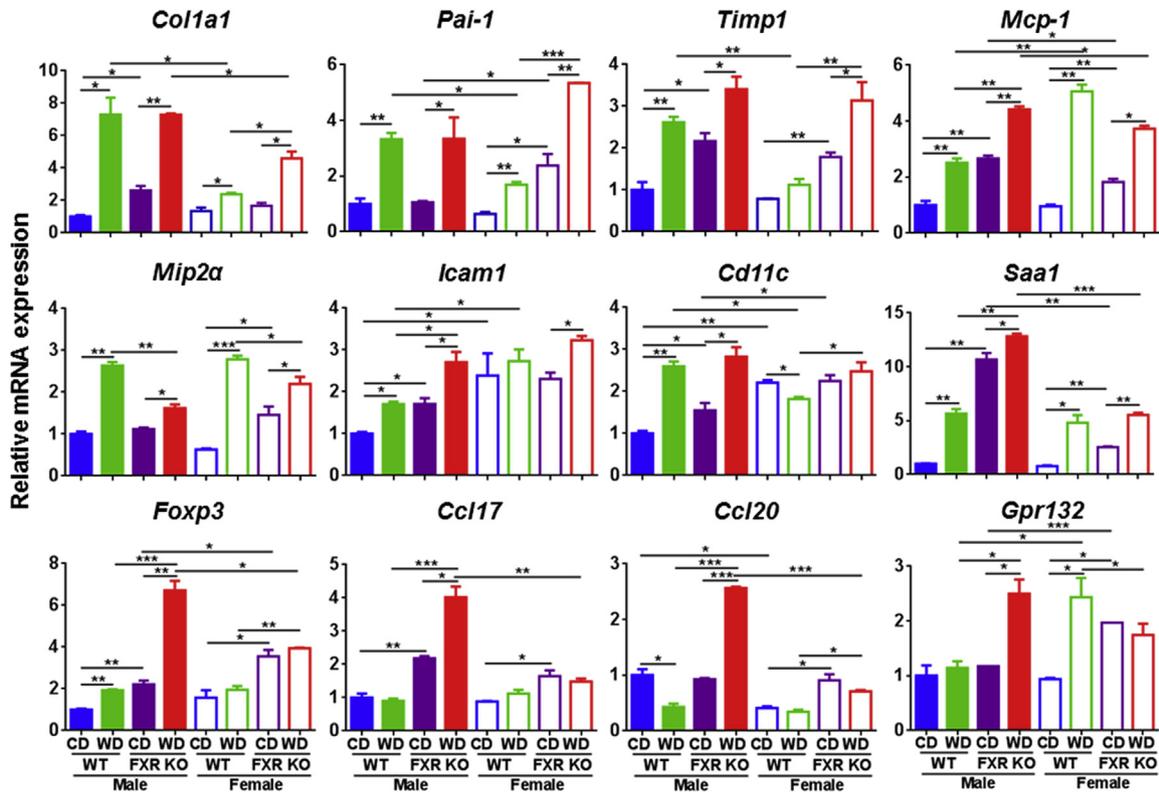


Figure 3 Hepatic gene expression in control diet (CD)– and Western diet (WD)–fed wild-type (WT) and FXR knockout (KO) mice of both sexes. Data are expressed as means ± SD. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ [based on a single variable of comparison (ie, diet, genotype, or sex)].

reduced tissue inhibitor of metalloproteinases mRNA in both CD- and WD-fed FXR KO mice. Moreover, many of the inflammatory genes, such as *Cxcl2*, *Ccl2*, *Foxp3*, *Ccl17*, and *Ccl20*, had higher expression levels in WD- than CD-fed FXR KO after Abx treatment (Figure 5). Furthermore, PolyB-reduced lymphocytes in WD-fed FXR KO mice were accompanied by reduced levels of many hepatic inflammatory genes as well as ileal *Mcp-1* and increased ileal regenerating islet-derived protein γ mRNA level (Figure 5).

The spleen plays an important role in modulating immune response by removing antibody-coated bacteria and blood cells. Flow cytometry data revealed that WD or FXR inactivation substantially increased total macrophages, tumor necrosis factor- α^+ macrophages, and $CD4^+Tim3^+$ (T-cell immunoglobulin domain and mucin domain-3) in both sexes (Figure 6). FXR inactivation also reduced the percentage of dendritic cells (DCs) and the number of CD4 and CD8 effector memory T cells, indicating compromised immunity (Figure 6).

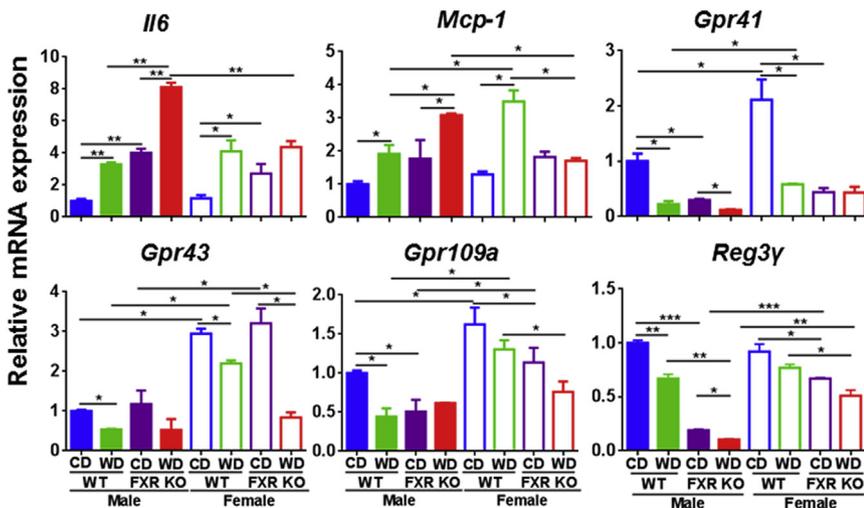


Figure 4 Ileal gene expression in control diet (CD)– and Western diet (WD)–fed wild-type (WT) and FXR knockout (KO) mice of both sexes. Data are expressed as means ± SD. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ [based on a single variable of comparison (ie, diet, genotype, or sex)].

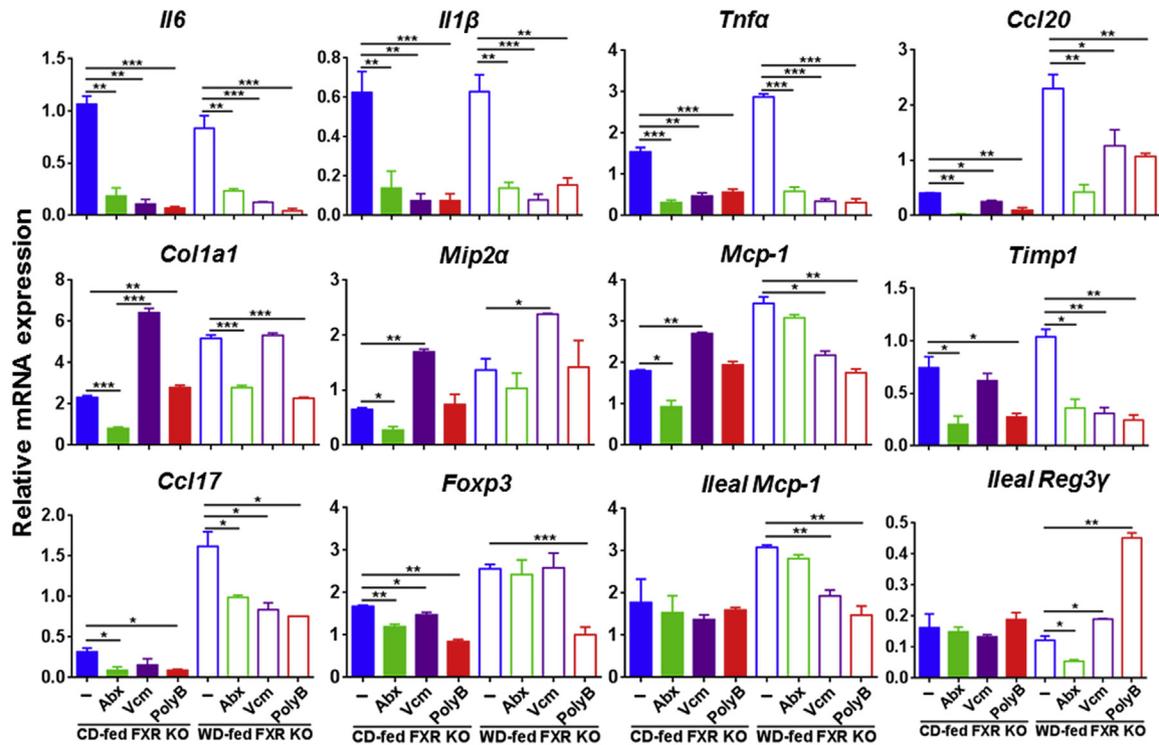


Figure 5 Hepatic and ileal gene expression in Abx-, vancomycin (Vcm)-, and polymyxin B (PolyB)-treated control diet (CD)- and Western diet (WD)-fed FXR knockout (KO) male mice. Data were relative to CD-fed WT male mice. Data are expressed as means \pm SD. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Bile Acid Profiles and Their Relationships with Hepatic Inflammatory Signaling

Based on the significance of FXR in regulating hepatic inflammation, hepatic BAs were quantified (Figure 7A). WD-fed FXR KO male mice, which had the greatest hepatic inflammatory signaling, had elevated hepatic taurine-conjugated lithocholic acid (TLCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), hydoxycholic acid, and α -muricholic acid and β -muricholic acid (α -MCA and β -MCA, respectively) (Figure 7A). β -MCA had the biggest fold induction in response to WD intake and FXR

inactivation in male mice. Moreover, the increase of hepatic GDCA, which is a known IL-6 inducer and tumor promoter, was FXR KO specific.³² In addition, FXR inactivation reduced hepatic chenodeoxycholic acid (CDCA), glycohydoxycholic acid, ursodeoxycholic acid, and taurine- α , β -MCA in one or both sexes (Figure 7A).

All three antibiotic treatments reduced TLCA, DCA, GDCA, and hydoxycholic acid, indicating gut microbiota association with the production of those secondary BAs (Figure 7B). Abx, which totally eliminated neutrophils and lymphocytes in CD-fed mice, increased CDCA, glycohydoxycholic acid, and taurine- α , β -MCA, suggesting

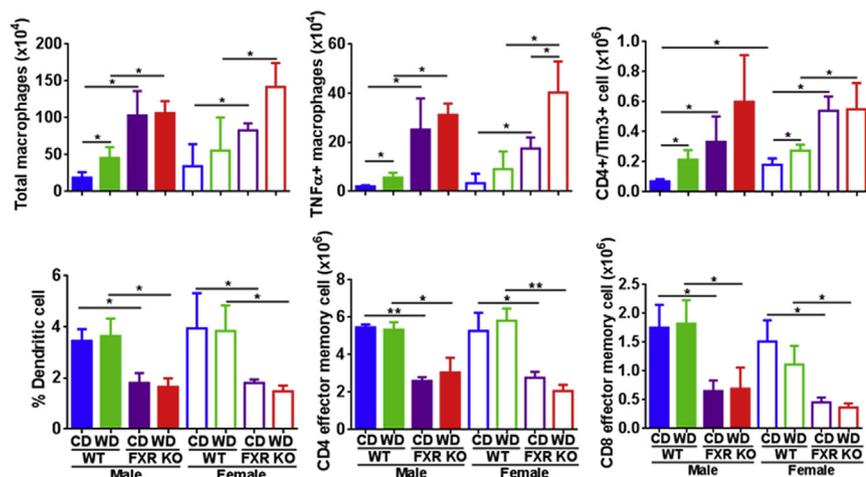


Figure 6 Flow-cytometric phenotyping of spleen cells in control diet (CD)- and Western diet (WD)-fed wild-type (WT) and FXR knockout (KO) mice of both sexes. Data are expressed as means \pm SD. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$ [based on a single variable of comparison (ie, diet, genotype, or sex)]. TNF- α , tumor necrosis factor- α .

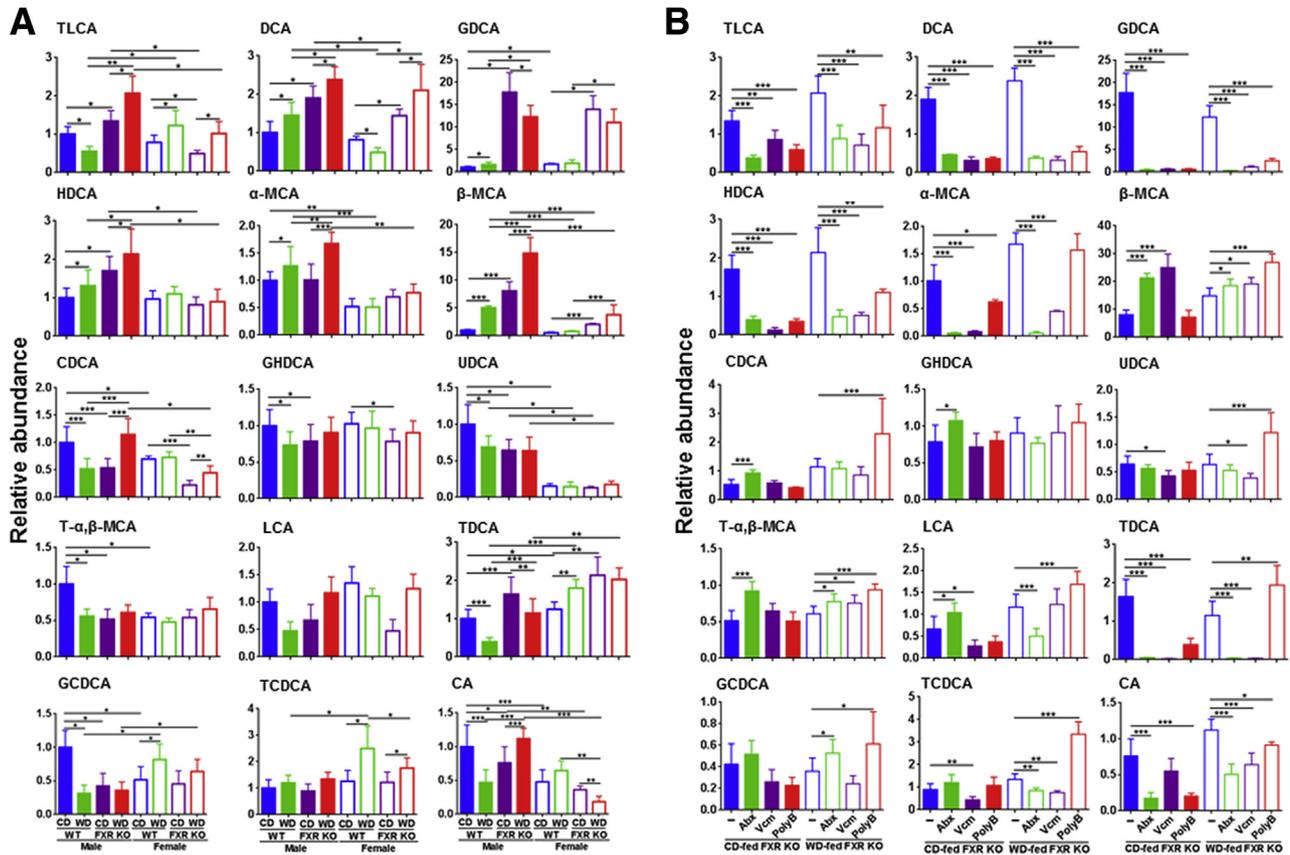


Figure 7 Hepatic bile acid profile. **A:** Relative concentration of hepatic bile acid profile in control diet (CD)— and Western diet (WD)—fed wild-type (WT) and FXR KO mice of both sexes. **B:** Relative concentration of hepatic bile acid profile in Abx-, Vcm-, and PolyB-treated CD- and WD-fed FXR KO mice (data were relative to CD-fed WT male mice). Data are expressed as means \pm SD. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. CA, cholic acid; GCDCA, glycochenodeoxycholic acid; GHGCA, glycohyodeoxycholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; T- α , taurine- α ; TCDCa, taurine-conjugated chenodeoxycholic acid; TDCA, taurodeoxycholic acid; UDCA, ursodeoxycholic acid.

their anti-inflammatory role (Figure 7B). In WD-fed groups, PolyB-reduced hepatic lymphocytes were accompanied by induction of CDCA, ursodeoxycholic acid, and taurine-conjugated chenodeoxycholic acid, and those changes were not found in Abx- and Vcm-treated groups (Figure 7B). Increased CDCA and its conjugated forms, such as glycochenodeoxycholic acid and taurine-conjugated chenodeoxycholic acid, as well as reduced TLCA, DCA, GDCA, hyodeoxycholic acid, and cholic acid, were commonly found in Abx-treated CD-fed or PolyB-treated WD-fed FXR KO mice, of which both showed reduced hepatic inflammation.

To understand the pathobiological effects of those specific BA profiles, we have studied the expression of >150 genes in liver, ileum, and adipose tissues that have roles in inflammation and metabolism. Spearman correlation analysis revealed that the hepatic concentrations of TLCA, β -MCA, DCA, and free primary and secondary BAs, which clustered together, were positively associated with the expression of inflammation genes, such as hepatic *Cd11c*, *Colla1*, *Timp1*, *Saa1*, *Foxp3*, *Ccl17*, *Tlr4*, *Ccl20*, *Il4*, and ileal *Il6* (Supplemental Figure S1). In addition, their concentrations were negatively associated with hepatic

metabolism genes, including hepatic *Cyp4a14*, *Sirt1*, *Pepck*, *Bsep*, *Cyp27a1*, *Srebp1c*, and *Glp1*, ileal *Claudin 1*, *Jam*, *Il18*, *Reg3 γ* , and *Pyy*, and adipose *Fgf21* and *Srebp1c* (Supplemental Figure S1). Moreover, hepatic GDCA concentrations were positively associated with hepatic *Ccna2*, α -*sma*, *Foxp3*, *Ccl17*, *Gpr132*, and *Il22*, and ileal *Il23*, *Il6*, and *Mcp-1*, but negatively associated with hepatic mRNA levels of hepatic *G6pase*, *Pepck*, and *Fgf21*, and ileal *Reg3 γ* , *Glp1*, and *Pyy*, and adipose *Fgf21* and *Fgfr1* genes (Supplemental Figure S1).

Gut Microbiota Profiles and Their Relationships with Hepatic Inflammatory Signaling

16S rRNA gene sequencing of genomic DNA from cecal samples revealed that FXR inactivation substantially reduced Firmicutes and increased Proteobacteria relative abundance in both sexes (Figure 8A and Supplemental Table S1). The WD-fed FXR KO mice had the highest relative abundance of Proteobacteria and Deferribacteres among the eight studied groups (Figure 8A). However, Abx treatment of CD-fed FXR KO mice, which completely eliminated hepatic neutrophils and lymphocytes, had

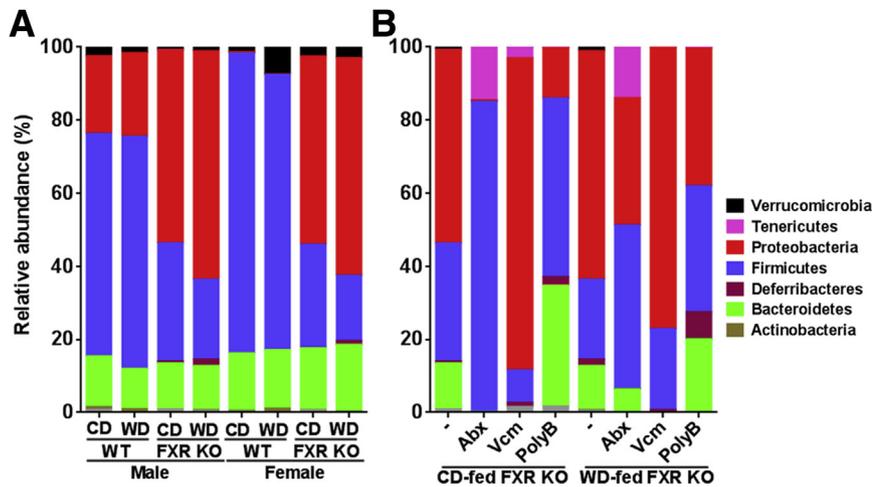


Figure 8 Relative abundance of cecal microbiota at phylum level of control diet (CD)- and Western diet (WD)-fed wild-type (WT) and FXR knockout (KO) mice of both sexes (A) and antibiotic-treated CD- and WD-fed FXR KO male mice (B). Data are given as means. PolyB, polymyxin B; Vcm, vancomycin.

opposite effects (Figure 8B). The relative abundance of Firmicutes and Tenericutes increased in both CD- and WD-fed FXR KO mice treated with Abx (Figure 8B and Supplemental Table S2). In addition, Proteobacteria and Bacteroidetes persisted after Abx treatment, which were accompanied by the persistent inflammation found in WD-fed FXR KO mice. Vcm increased the relative abundance of Proteobacteria in both CD- and WD-fed mice. In contrast, PolyB, which reduced hepatic lymphocytes in WD-fed mice, substantially decreased Proteobacteria and increased Firmicutes relative abundance in WD-fed FXR KO mice (Figure 8B). The sex difference in hepatic inflammation was apparent. Accordingly, FXR inactivation reduced the relative abundance of Verrucomicrobia phylum in males, but increased it in CD-fed WT females. In addition, Deferribacteres were more abundant compared with other microbes in male FXR KO than the female counterparts (Figure 8A and Supplemental Table S1).

Under Firmicutes, the relative abundance of *Erysipelotrichaceae* and *Clostridiaceae* was substantially reduced because of FXR inactivation (Figure 9A). In addition, the reduction of *Lachnospiraceae*, *Streptococcaceae*, and *Christensenellaceae* in FXR KO was more apparent in females than males (Figure 9A). Furthermore, in CD-fed mice, Abx substantially increased relative abundance of *Streptococcaceae*, which might account for reduced inflammation (Figure 9B). Moreover, PolyB-reduced inflammation was accompanied by increased relative abundance of *Christensenellaceae* in WD-fed FXR KO mice (Figure 9B).

Under the Proteobacteria phylum, the relative abundance of *Helicobacteraceae* and *Desulfovibrionaceae* substantially increased because of FXR inactivation (Figure 9A). Consistently, Abx and PolyB reduced them in both CD- and WD-fed FXR KO mice (Figure 9B).

Under Bacteroidetes, the relative abundance of *Bacteroidaceae*, *Porphyromonadaceae*, *Paraprevotellaceae*, and *Odoribacteriaceae* families was increased in FXR KO mice

(Figure 9A), and Abx was able to eliminate them in CD-fed FXR KO mice (Figure 9B). The bacteria that account for antibiotic-reduced hepatic inflammation might not be the same bacteria that caused hepatic inflammation in FXR KO mice. For example, the relative abundance of *Deferribacteraceae* (Deferribacteres phylum) was increased by FXR KO, and was further increased by WD, but PolyB, which reduced hepatic lymphocytes, also increased the relative abundance of *Deferribacteraceae* in WD-fed FXR KO mice (Figure 9B).

At the genus level, the lack of FXR increased the relative abundance of genera *Bacteroides* and *Parabacteroides* (Bacteroidetes phylum), which were also reduced by Abx in CD-fed FXR KO mice, suggesting their role in hepatic inflammation (Figure 10, A and B). In addition, Abx dramatically increased *Lactococcus* genus (Firmicutes phylum). Moreover, PolyB also increased the relative abundance of *Lactococcus* in WD-fed FXR KO mice. Furthermore, PolyB substantially increased the relative abundance of genera *Lactobacillus* and *Coprococcus* (Firmicutes phylum) in WD-fed FXR KO, suggesting the protective effects of those bacteria (Figure 10B). Furthermore, the relative abundance of genera *Desulfovibrionaceae_g* and *Helicobacteraceae_g* (Proteobacteria phylum) was significantly increased because of FXR inactivation, and both Abx and PolyB substantially reduced them (Figure 10, A and B). Under Tenericutes phylum, genus *Mycoplasmataceae_g*, which increased because of FXR deficiency in males, was further increased by Abx in both CD- and WD-fed FXR KO mice (Figure 10, A and B).

Functional Analysis of Gut Microbiota

FXR deficiency had a higher impact than diet or sex in shifting microbiota function based on the phylogenetic investigation of communities by reconstruction of unobserved states analysis (Supplemental Table S3). In addition, there were 49 pathways with a significant difference

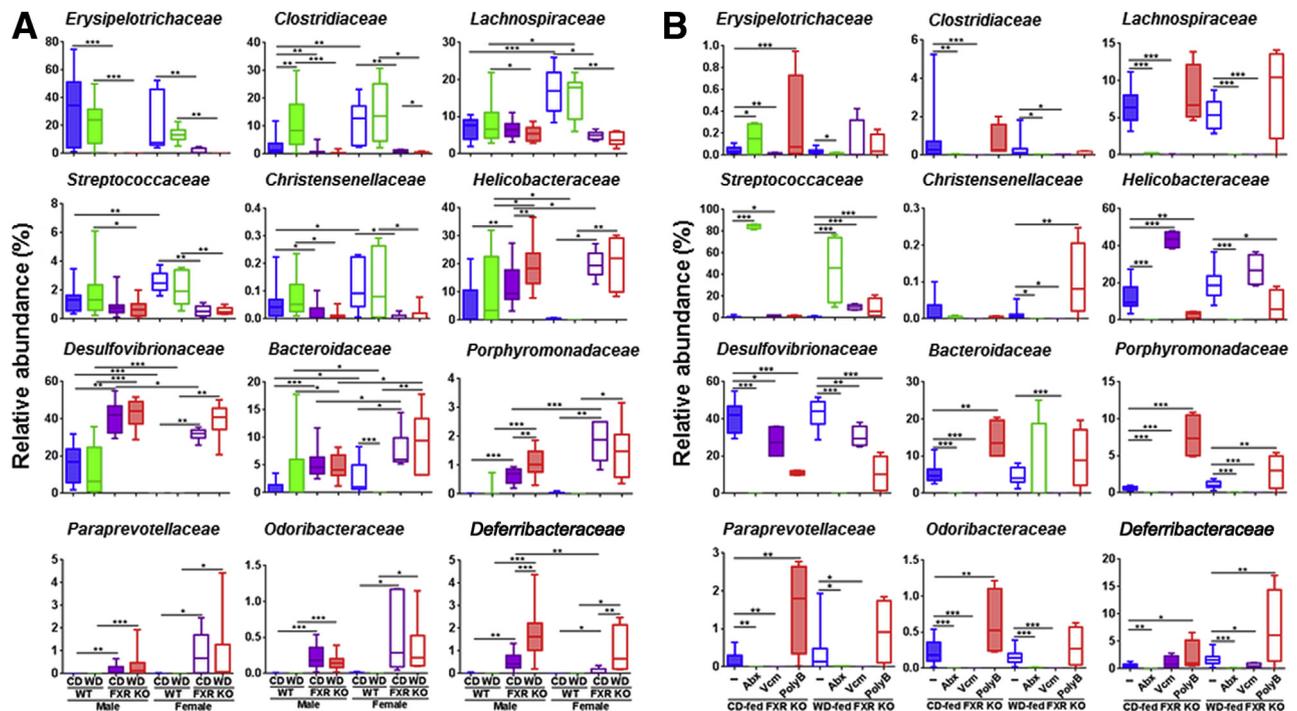


Figure 9 Relative abundance of cecal microbiota at family level of control diet (CD)– and Western diet (WD)–fed wild-type (WT) and FXR knockout (KO) mice of both sexes (A) and antibiotic-treated CD- and WD-fed FXR KO male mice (B). Box plots display the median, 25th percentile, and 75th percentile; whiskers display minimum and maximum values. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. PolyB, polymyxin B; Vcm, vancomycin.

between WT and FXR KO mice (Supplemental Figure S2A). WD-fed FXR KO male mice, which had the most hepatic lymphocyte infiltration, had enriched pathways in Alzheimer disease, cardiac muscle contraction, biotin metabolism, and betalain biosynthesis. These pathways were reduced by Abx in CD-fed mice and PolyB in WD-fed mice (Supplemental Figure S2). In addition, Abx reduced bacterial motility proteins, LPS biosynthesis, and bacterial chemotaxis in CD-fed FXR KO mice. Moreover, PolyB reduced inflammation in WD-fed FXR KO mice, which was accompanied by increased tryptophan, carbohydrate, and fructose metabolism as well as reduced fatty acid biosynthesis pathways (Supplemental Figure S2B).

Discussion

Our novel data revealed there is an apparent sex difference in hepatic inflammation. The amount of hepatic lymphocytes, serum endotoxin levels, and ALT levels were higher in males than females in several study groups. The expression levels of proinflammatory genes, such as hepatic *Saa1*, *Foxp3*, *Ccl17*, and *Ccl20*, as well as ileal *Il6* were higher in male than female WD-fed FXR KO mice. All those cytokines are implicated in hepatic inflammation, as shown in different models. For example, serum amyloid a1 has a known effect of inducing the migration, adhesion, and tissue infiltration of monocytes and neutrophils in humans.^{33–35} In addition, *Foxp3* is induced because of

hepatitis C virus infection.³⁶ *Ccl17* is closely associated with intestinal inflammation and hepatitis C virus infection.^{37–40} Moreover, *Ccl20* is highly induced in alcoholic patients and LPS-induced liver injury.⁴¹ In addition, our data revealed that the substantial induction of those hepatic cytokine genes was accompanied by elevated hepatic *Colla1* in a male-predominant manner, suggesting males are more susceptible to the development of steatohepatitis or fibrosis. Furthermore, WD-fed male FXR KO mice had reduced ileal *Reg3 γ* , which has a protective effect against the infection of Gram-positive bacteria.⁴² Consistently, increased *Reg3 γ* found in PolyB-treated WD-fed FXR KO might account for reduced hepatic inflammation. Moreover, although Abx reduced almost all of the proinflammatory signaling in CD-fed FXR KO mice, WD-fed FXR KO mice still had elevated hepatic *Ccl17* and reduced ileal *Reg3 γ* as well as hepatic lymphocytes after Abx treatment. These findings clearly indicate the protective role of regenerating islet-derived protein 3 γ in inflammation.

A sex difference in microbiota was also noted. *Lachnospiraceae*, *Clostridiaceae*, *Streptococcaceae*, and *Christensenellaceae* are likely to have a protective effect against hepatic inflammation because their relative abundance is increased in the female mice. This scenario is in part supported by the finding that *Lachnospiraceae*-generated SCFAs have anti-inflammatory and histone deacetylation inhibitory properties.^{43–45} Consistently, the expression level of ileal SCFA receptors (*Gpr41*, *Gpr43*, and *Gpr109A*) was

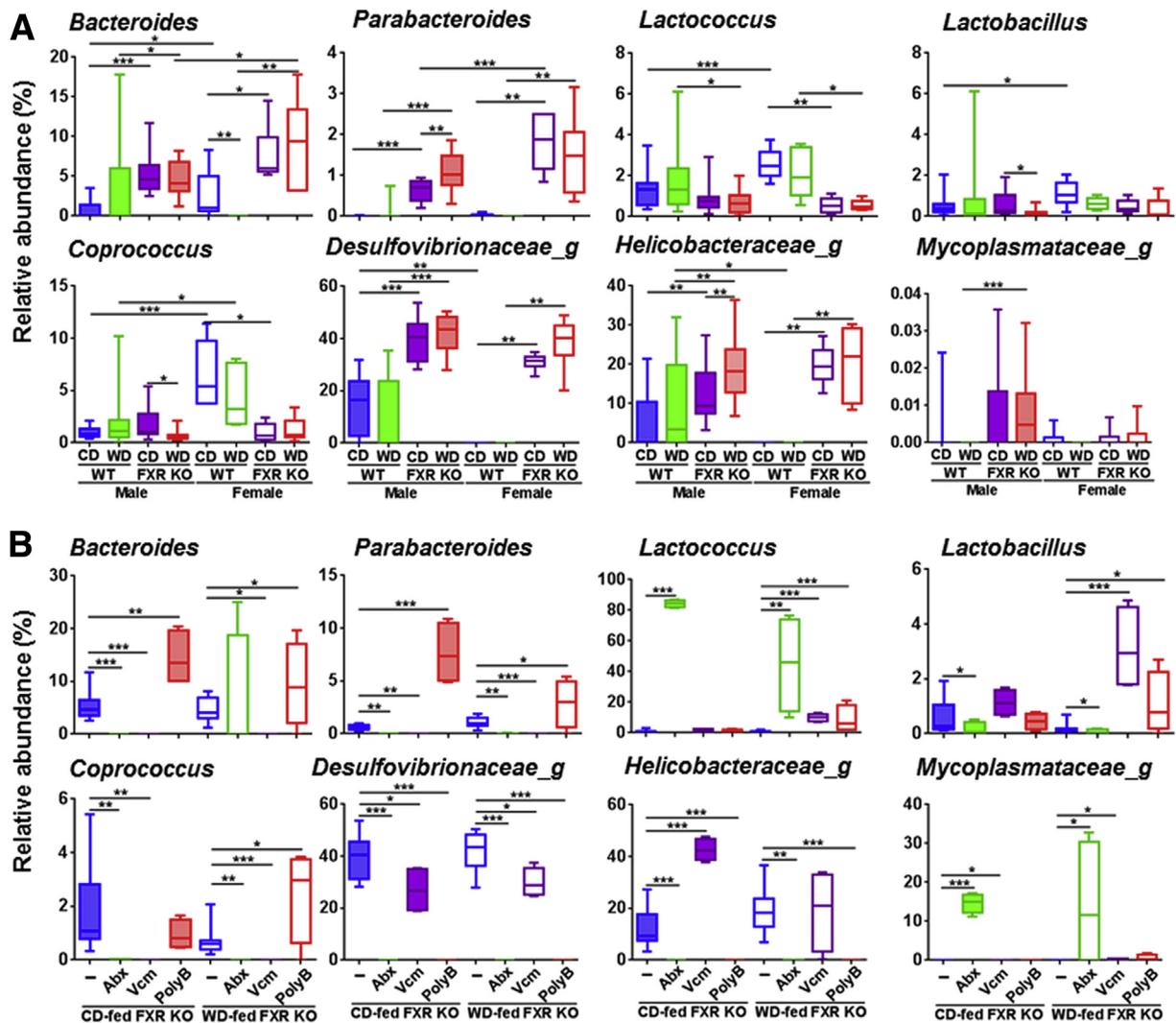


Figure 10 Relative abundance of cecal microbiota at genus level of control diet (CD)– and Western diet (WD)–fed wild-type (WT) and FXR knockout (KO) mice of both sexes (A) and antibiotic-treated CD- and WD-fed FXR KO male mice (B). Box plots display the median, 25th percentile, and 75th percentile; whiskers display minimum and maximum values. $n \geq 6$ per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. PolyB, polymyxin B; Vcm, vancomycin.

higher in female than male WT mice. Among those three receptors, GPR109A is butyrate specific, and its mRNA reduction folds were greater in males than females because of WD intake or FXR activation. Moreover, ileal mRNA levels of *Gpr43*, which can be bound by propionate and butyrate, were higher in females than in males as well, with the exception of WD-fed FXR KO groups. *Christensenellaceae* also generate SCFAs and are enriched in lean individuals.^{46,47} Moreover, *Clostridiaceae* and *Streptococcaceae* also have many known health benefits.^{48,49} Together, the sex difference in those bacteria may in part explain the sex disparity of liver disease, including cancer.

FXR deactivation increased hepatic lymphocytes and neutrophils, indicating the presence of both chronic and acute inflammation. Neutrophils are usually the first responders to the acute phase of inflammation, especially in the case of bacterial infection.⁵⁰ Through chemotaxis controlled by cytokines, neutrophils migrate through the

blood vessels, reaching the interstitial tissue and then the liver. In the spleen, FXR KO mice had increased total macrophages as well as tumor necrosis factor- α^+ macrophage and CD4⁺/Tim3⁺ cells, but reduced DCs. It has been shown that elevated Tim3 causes T-cell disruption and exhaustion, leading to inflammation and compromised immunity.^{51,52} In addition, the significance of FXR in regulating inflammation through DCs has been revealed in the dextran sulfate sodium–induced colitis model and other intestinal models.^{53,54} Furthermore, activation of FXR by obeticholic acid can retain DCs and have an anti-inflammatory effect.⁵³ The consistent reduction of DCs in the liver and intestinal inflammation clearly indicates their protective effect, which is regulated by FXR.

The beneficial and detrimental effects of BAs depend on their hydrophobicity, concentration, and exposure time.^{3,6} Our published data showed that BAs can induce and dictate the intracellular location of NUR77 to regulate

proliferation or apoptosis of liver and colon cancer cells.⁵⁵ Moreover, a long-term exposure to high concentrations of hydrophobic secondary BAs generates BA-resistant cells that are highly proliferative and have tumorigenic potential because of nuclear NUR77 overexpression.⁵⁵ In the current study, our data revealed many free as well as conjugated secondary BAs, such as TLCA, DCA, GDCA, and hyodeoxycholic acid, that were enriched because of FXR inactivation and WD intake. Moreover, their hepatic concentrations were decreased because of reduced inflammation as a result of antibiotic treatment. These findings are in line with the data that show increased GDCA in FXR KO mice is associated with tumor growth and increased inflammatory cytokines.⁵⁶ In contrast, taurine- α , β -MCA, CDCA, glycochenodeoxycholic acid, and ursodeoxycholic acid are likely to have a protective effect against inflammation because they were either reduced by WD or FXR KO in certain groups, and such reductions could be reversed by reduced hepatic inflammation after Abx or PolyB treatment. Consistently, it has been shown that ursodeoxycholic acid activates FXR and induces type 1 helper T-cell response via DCs to suppress airway inflammation.⁵⁷ Together, it is clear that dysbiosis and dysregulated BAs are inseparable, and they jointly contribute to hepatic inflammation via the gut-liver axis. Moreover, in antibiotic-treated mice, the change in BA profile can also be primary as well as secondary to the alterations in gut microbiota because antibiotics can directly eliminate BA-generating bacteria, which, in turn, causes additional changes in BA composition.

Regarding microbiota, the biggest change due to FXR inactivation was reduction of Firmicutes and increase in Proteobacteria relative abundance in both sexes. Similar changes in Firmicutes and Proteobacteria were found in obesity and nonalcoholic steatohepatitis patients.^{58,59} Proteobacteria are Gram-negative endotoxin producers, which can cause dysbiosis and contribute to chronic liver injury in nonalcoholic fatty liver disease.⁶⁰ In the Firmicutes phylum, *Erysipelotrichaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Streptococcaceae*, and *Christensenellaceae* families were reduced by FXR inactivation, suggesting the specific effect of BA production in reducing those beneficial bacteria. It has been shown that *Erysipelotrichaceae* is involved in BA metabolism and also has anti-inflammatory properties.^{54,61,62} Moreover, patients with Crohn disease and inflammatory bowel disease have reduced *Erysipelotrichaceae* and *Clostridiaceae*.^{61,63} The abundance of genus *Coprococcus* under *Lachnospiraceae* family was enriched in PolyB-treated WD-fed FXR KO mice, revealing their potential beneficial effect. FXR KO mice also had reduced *Streptococcaceae*, which can be increased by probiotic VSL#3.⁶⁴ Moreover, genus *Lactococcus* under *Streptococcaceae* might have the largest percentage increased because of the anti-inflammatory effect of Abx in CD-fed mice (from 3% to 80%). *Lactococcus* have many beneficial effects, ranging from metabolism improvement to anti-inflammation.^{48,65} Moreover, increased *Lactococcus* and

butyrate production were found in mice after vertical sleeve gastrectomy.⁶⁶ However, WD intake substantially reduced the effect of Abx to enrich *Lactococcus*. This outcome might explain the presence of hepatic lymphocytes after Abx treatment in WD-fed mice.

The increase in relative abundance of Proteobacteria phylum in FXR KO mice was mainly because of an increased relative abundance of *Desulfovibrionaceae* and *Helicobacteraceae* families. Under these two families, FXR KO substantially increased *Desulfovibrionaceae_g* and *Helicobacteraceae_g*. Certain *Desulfovibrionaceae* can generate hydrogen sulfide via taurine respiration, which is a potent genotoxin and gut mucosal barrier breaker, leading to acute inflammation.^{67,68} Moreover, mice with impaired glucose tolerance and ulcerative colitis also have increased *Desulfovibrionaceae*.⁶⁹ In addition, different species of *Desulfovibrionaceae* are colonized in oral cavities and gastrointestinal tracts, which can have a systemic effect.^{70–72} Within the *Helicobacteraceae* family, *Helicobacter pylori*, *Helicobacter aurati*, and *Helicobacter hepaticus* are well-known for their roles in infection and carcinogenesis.^{73,74} Moreover, Abx- and PolyB-reduced hepatic inflammation was accompanied by elimination of *Desulfovibrionaceae_g* and *Helicobacteraceae_g*. Furthermore, our novel data showed that Vcm-induced ALT in CD-fed FXR KO was associated with increased relative abundance of *Helicobacteraceae_g*.

Under Bacteroidetes phylum, *Bacteroides* and *Parabacteroides* genera, which are the predominant anaerobes in the gut (25%), increased as a result of FXR deactivation in both sexes. *Bacteroides* species are BA-resistant pathogens, which are found in most anaerobic infections, with a mortality of >19%.⁷⁵ However, the percentage of *Bacteroides* increases due to lack of FXR were higher in females than males. Thus, the resistance to inflammation found in females might be due to having more beneficial microbiota rather than less proinflammatory bacteria. Our data revealed that genus *Bacteroides* was eliminated by Abx in CD-fed FXR KO mice. However, *Bacteroides* that persisted after Abx treatment might lead to persistent hepatic inflammation after Abx treatment. Together, these results suggested that decreased *Bacteroides* and *Parabacteroides* contribute to reduced hepatic inflammation.

In conclusion, using dietary modification and antibiotic treatments, our data unequivocally revealed the role of gut microbiota in control of hepatic inflammation. Moreover, BAs and their receptor FXR play a pivotal role in mediating the inflammatory and anti-inflammatory effects of microbiota in a sex-dependent manner. This scenario is supported by the findings that sex or diet effects are frequently abolished or enhanced because of lack of FXR. For example, the effect of diet on microbiota functions became apparent or was no longer found when FXR was inactivated in male and female mice, respectively. Moreover, there are similarities between the WD intake and FXR KO. This is shown by the reduction of the number of bacterial functional pathways

differentially found between WT and FXR KO because of WD intake. Thus, probiotics and FXR agonists should be effective in the prevention and treatment of hepatic inflammation and progression into advanced liver diseases, such as cancer. The present study does not exclude the role of microbiota in regulating hepatic metabolism. Additional analyses are required to address the effect of diet and antibiotics in hepatic metabolism.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.04.019>.

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