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Alterations in Intestinal Microbiota Lead to Production of Interleukin 17 by Intrahepatic \(\gamma\delta\) T-cell Receptor-positive Cells and Pathogenesis of Cholestatic Liver Disease

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Author contributions: D.T. and M.T. conceived and performed the experiments, interpreted the data and co-wrote the manuscript; C.Y.C and E.M.B performed the bacteriological experiments and analysis; P.S. and E.J.E. performed the experiments with D.T. and M.T.; S.G. analyzed the histological slides for scoring, interpretation and provided valuable suggestions; Y.G., M.G. and J. L. performed the deep sequencing analysis for 16S RNA and provided analysis and interpretation; M.M. provided assistance with deep sequencing analysis and interpretation; W.H.K, J.F.M and A.B.A performed transplant surgeries on patients; D.S.W. provided assistance for bacterial identification and analysis, conceived and designed experiments, interpreted the data and provided critical scientific and intellectual input. A.G. conceived and designed the experiments, interpreted the data and co-wrote the manuscript, with all authors contributing to writing and providing feedback.

The authors have no conflicts of interest to disclose.

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Abstract

**Background & Aims**—Variants at the ATP binding cassette subfamily B member 4 gene (*ABCB4* or *MDR2*) locus, which encodes a biliary transport protein, are associated with a spectrum of cholestatic liver diseases. Exacerbation of liver disease has been linked to increased hepatic levels of interleukin 17 (IL17), yet the mechanisms of this increase are not understood. We studied mice with disruption of *Mdr2* to determine how defects in liver and alteration in the microbiota contribute to production of IL17 by intrahepatic γδ T cells.

**Methods**—We performed studies with *Mdr2*−/− and littermate FVB/NJ (control) mice. IL17 was measured in serum samples by an ELISA. Mice were injected with neutralizing antibodies against the γδ T-cell receptor (TCR; anti-γδ TCR) or mouse IL17A (anti-IL17A). Livers were collected and bacteria were identified in homogenates by culture procedures; TCRγδ+ cells were isolated by flow cytometry. Fecal samples were collected from mice and analyzed by 16s ribosomal DNA sequencing. Cells were stimulated with antibodies or bacteria, and cytokine production was measured. We obtained tissues from 10 patients undergoing liver transplantation for primary sclerosing cholangitis or chronic hepatitis C virus (HCV) infection. Tissues were analyzed for cytokine production by γδ TCR+ cells.

**Results**—Mdr2−/− mice had collagen deposition around hepatic bile ducts and periportal-bridging fibrosis with influx of inflammatory cells and increased serum levels of IL17 compared with control mice. Administration of anti-IL17A reduced hepatic fibrosis. Livers from Mdr2−/− mice had increased numbers of IL17A+ γδTCR+ cells—particularly of IL17A+ Vγ6Jγ1 γδ TCR+ cells. Fecal samples from Mdr2−/− mice were enriched in *Lactobacillus*, and liver tissues were enriched in *Lactobacillus gasseri* compared with control mice. Mdr2−/− mice also had increased intestinal permeability. The γδ TCR+ cells isolated from Mdr2−/− livers produced IL17 in response to heat-killed *L gasseri*. Intraperitoneal injection of control mice with *L gasseri* led to increased serum levels of IL17 and liver infiltration by inflammatory cells; injection of these mice with anti-γδ TCR reduced serum level of IL17. Intravenous injections of Mdr2−/− mice with anti-γδ TCR reduced fibrosis; liver levels of IL17, and inflammatory cells; and serum levels of IL17. γδTCR+ cells isolated from livers of patients with primary sclerosing cholangitis, but not HCV infection, produced IL17.

**Conclusions**—In Mdr2−/− mice, we found development of liver fibrosis and inflammation to require hepatic activation of γδ TCR+ cells and production of IL17 production, mediated by exposure to *L gasseri*. This pathway appears to contribute to development of cholestatic liver disease in patients.

**Graphical abstract**
Defects in the \textit{ABCB4} locus, which encodes a biliary transport protein MDR3 in humans and Mdr2 in mice are associated with a spectrum of chronic cholestatic liver diseases\textsuperscript{1}. Patients that lack a functional MDR3 protein cannot transport phospholipids\textsuperscript{2, 3}, resulting into inability to emulsify toxic bile salts, and participate in downstream metabolic processes\textsuperscript{3}. Consequently, this cholestasis - reduction in bile flow causes liver injury via build-up of these toxic bile salts\textsuperscript{1, 2, 4}. Though defects of MDR3 gene expression have been associated with a subtype of progressive familial intrahepatic cholestasis (PFIC), Mdr2 deficiency in mice can progress into fibrosis, primary sclerosing cholangitis (PSC), and hepatocellular carcinoma\textsuperscript{1, 4}. In particular, PSC is a heterogeneous chronic liver disease, that can lead to end-stage cirrhosis in children and adults worldwide\textsuperscript{5–7}, and remain one of the leading indications for liver transplantation\textsuperscript{8, 9}.

PSC is a complex liver disease with etiologies that involves genetic, environmental, immunological, and other potential factors i.e. gut dysbiosis\textsuperscript{7}. An association between PSC and ulcerative colitis in an estimated 75\% of Western PSC patients implicates an etiological role for gut dysbiosis in this process\textsuperscript{10}. It is very likely that alterations in the intrahepatic as well as extrahepatic biliary ducts, and cholangiocytes during cholestasis may promote microbial translocation to liver.

The liver is an anatomic site that is highly enriched in unconventional T cells including \(\gamma\delta\) T cells\textsuperscript{11}, which are capable of modulating liver injuries through IL-17 production. Mounting evidence demonstrate IL-17+ \(\gamma\delta\) T cells expand in response to inflammation\textsuperscript{12, 13}, particularly important for TCR-mediated recognition of bacterial pathogens invading host tissues\textsuperscript{13–15}. In acute injury setting, such as Concanavalin (Con-A)-induced hepatitis\textsuperscript{16} and experimental hepatectomy regeneration\textsuperscript{17}, this hepatoprotective population is largely

\textbf{Keywords}

PSC; intestinal microbiota; liver disease pathogenesis; immune response
restricted to Vγ4 usage\(^{18}\). However, in chronic models of liver injury, such as high-fat diet\(^{19}\) and biliary atresia\(^{20}\), γδ T cells-derived IL-17 is implicated in perpetuating disease pathogenesis; Vγ-chain usage has yet to be elucidated in this context. Interestingly, IL-17 has also been demonstrated to hypersensitize hepatic stellate cells (HSCs), a sentinel cell types in hepatic fibrosis, to TGF-β; addition of IL-17 to HSC cultures \textit{in vitro} permits a robust response to sub-optimal concentrations of TGF-β\(^{21}\). While this is advantageous in acute liver wound healing, perhaps prolonged hypersensitivity to profibrotic mediators encourages pathology during chronic liver disease. Therefore, we hypothesize that IL-17+ γδ T cells could potentially expand in respond to inappropriately localized commensal bacteria during cholestasis mechanisms. However, the contributions of these mechanisms in the pathogenic progression of cholestatic liver disease remain largely unknown.

Here we used the multidrug resistance gene 2 knockout (\textit{Mdr2}−/−) mouse model, genetic homolog of human MDR3, to determine a functional link between gut dysbiosis and the cholestatic liver disease. Our findings reveal a novel mechanism in which cholestasis-driven gut dysbiosis intersects with a selective expansion of an invariant population of IL-17+ Vγ6Jγ1 γδ T cells to pathogenically propagate liver disease. Expansion of IL-17+ γδ T cells correlates with an enrichment of gut bacteria \textit{Lactobacillus gasseri} in \textit{Mdr2}−/− livers. Most importantly, antibody-mediated blockade of γδTCR or IL-17A attenuated liver fibrosis via targeting Vγ chains of IL-17 producing γδ T cells populations. Moreover, γδ T cells isolated from diseased livers of PSC patients, but not chronic HCV, were capable of producing IL-17. These findings implicate γδ T cells as a promising tool for immunotherapies against cholestatic liver disease.

**Methods**

**Animal Experiments**

\textit{Mdr2}−/+ (FVB.129P2-Abcb4\(^{TM}\)Bor/J) heterozygotes and wild-type (WT) FVB/NJ (001800) mice obtained from the Jackson Laboratory (ME, USA) were housed in specific pathogen-free environment per Emory University, NIH and IACUC guidelines. \textit{Mdr2}−/−, \textit{Mdr2}−/+ and WT littermate controls were generated by backcrossing littermates until desired true breeding lines were established, and confirmed by PCR. Where applicable, anti-mouse IL-17A (clone: 17F3, BioXcell, USA) administered as 250μg in sterile PBS I.P. every 3 days or 500μg α-TCRγδ (clone: UC7-13D5) intravenously (I.V.) every 7–10 days starting at 8 weeks; continued until 25 weeks of age.

**Human Subjects**

A total of 10 patients undergoing orthotopic liver transplantation for either primary sclerosing cholangitis (PSC; \(n=5\)) or Hepatitis C Virus (HCV; \(n=5\)) related end-stage liver disease at The Emory Transplant Center of Emory University Hospital were enrolled in the study in accordance with the Emory University Institutional Review Board (IRB) approval (IRB#00006248). Patient characteristics with clinical information and relevant biological variables are summarized in Supplementary Table 2. Written informed consent was obtained from each patient and IRB #00006248 conforms to the guidelines of the 1975 Declaration of Helsinki (revised 2013).
Histology and Immunohistochemistry

Upon animal sacrifice, liver sections were immediately fixed in 10% formalin overnight and paraffin embedded. Sirius Red and Hematoxylin & Eosin (H&E) staining were performed by the Pathology Core, Yerkes National Primate Research Center, Emory University. Quantification of liver fibrosis was scored by a senior pathologist at Yerkes based on the criteria outlined by Ishak et al.\(^{22}\)

Isolation of Liver Cells and Flow Cytometry

Liver tissues were processed by enzymatic digestion with 2mg/mL type IV collagenase (Worthington, NY). In some experiments, cells were stimulated with 50ng/mL Phorbol 12-Myristate 13-Acetate (PMA; LC Labs, USA) and 1μg/mL Ionomycin (Enzo Life sciences, USA) for 4 hours in the presence of Golgi Stop/Golgi Plug (BD Biosciences). The following antibodies were used: anti-CD3 (17A2), CD8 (53–6.7), CD4 (RM4-5), CD11c (N418), GR1 (RB6-8C5), I/A-I/E (M5/114.15.2), TCRβ (H57-597), CD45.1 (A20), IFNγ (XMG1.2) (Tonbo Biosciences, USA). Anti-Ly6C (HK.14), anti-CD11b (M1/70), TCRγδ (GL3), goat anti-hamster IgG (Poly4055), anti-TCRVγ1.1+1.2 (4B2.9), TCRVγ2 (UC3-10A6), TCRVγ3 (536), and anti-IL-17A (TC11-18H10.1) were purchased from Biolegend (USA). Anti-mouse TCRVγ4 (49.2) and TCRVγ7 (F2.64) were the kind gift from Dr. Pablo Pereira-Esteva (Pasteur Institute, France). Cells were analyzed using BD LSRII and FlowJo9.6.4 (TreeStar, Oregon, USA).

For human experiments, liver interstitial mononuclear cells (LIMC) were isolated as described.\(^{23}\) Cryopreserved cells were thawed, and purified by Ficoll-Paque (GE) gradient. Cells were cultured overnight with PMA/Ionomycin. Cells were first stained with purified mouse-anti human TCRγδ (Clone 11F2; BD Biosciences). For detection of TCRγδ+, cells were resuspended in 1X PBS containing goat-anti-mouse IgG (H+L) (Fab2)-A488 (Invitrogen) at a 1:200 dilution and incubated at 4°C for 20 minutes. Cells were subsequently washed, and incubated first with anti-human TCRαβ (Clone IP26; Biolegend USA), and after a short incubation the following antibodies were added: anti-CD3 (UCHT1), CD4 (RPA-T4) (Tonbo), CD161 (DX12; BD) and CD8 (RPA-T8; eBioscience). Samples were fixed, permeabilized and intracellularly stained anti-human IL-17A (eBio64CAP17, eBioscience), and anti-human IFNγ (B27; Biolegend), and were analyzed using LSRII and FlowJo9.6.4.

Quantitative Polymerase Chain Reaction

Liver tissue were homogenized in 1mL Trizol (Zymo Research) and processed to isolate mRNA, and reverse-transcription PCR to generate cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystem). PCR reactions were as follows; initial 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec and 72°C for 30 sec and run using 7900HT Fast Real-Time PCR system (Applied Biosystem). Primer sequences are provided in Supplementary Table 1. Gene expressions were normalized to housekeeping genes GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.
Determination of Serum IL-17A Concentration

Serum was obtained from animals at indicated time point via submandibular bleed, and/or at conclusion of the experiment via cardiac puncture in a serum separator microtube and processed per manufacturer’s instruction. IL-17A levels were assessed using mouse IL-17A ELISA MAX Deluxe kit (Biolegend) per manufacturer’s instruction.

Vγ-chain Usage Identification of Intrahepatic γδ T Cells

Animals were administered 500μg of anti-γδ TCR intravenously or appropriate isotype for gating control. The following day, animals were sacrificed and bulk liver lymphocytes were isolated from 25-weeks-old Mdr2−/− and FVB/N animals (n=3/group). Following staining procedures, in vivo-labeled γδ T Cells were sorted using a FACSaria II directly into buffer RLT (Qiagen) supplemented with 1% 2-mercapto-ethanol (Fisher). Sorted samples were stored in −80°C until shipment to iRepertoire, Inc (Alabama, USA) for sequencing and identification of Vγ-chain usage of in vivo-labeled γδ T cell populations.

Isolation and Functional Analysis of γδ T Cells

Liver/splenic lymphocytes were isolated from 8-10-week-old Mdr2−/− mice. Bulk T cells were isolated using negative selection (STEMCELL), and seeded in 96 well plates at (2×10^5/well) in complete RPMI media supplemented with 50U/mL of recombinant human IL-2 (Roche). Cells were incubated overnight either in media alone, in the presence of 50ng/mL PMA plus 1μg/mL ionomycin, LPS (2μg/mL, Sigma), Pam3CSK4 (1μg/mL, Invivogen) or with 10^6/mL of indicated heat killed (95°C for 1h) microbes isolated from 25-week-old Mdr2−/− livers in presence of Golgi Plug/Golgi Stop. Cells were washed, blocked and stained with the appropriate combinations of antibodies, analyzed as described.

Isolation and Detection of Bacteria in Liver Homogenates

Following sacrifice, the whole liver was harvested, homogenized, plated for colony forming unit (CFU) on blood agar (TSA with 5% sheep blood) (Thermo Scientific™, Remel™). Bacteria colonies isolated from the mice liver homogenates were identified by VITEK® MS (Clinical Microbiology Laboratory, Emory University Hospital).

Deep Sequencing of Mouse Fecal Pellets and Analysis

Mouse fecal pellets were collected from 8- and 25-weeks-old (corresponding mice) Mdr2−/− and FVB/N mice using sterile technique. DNA from fecal samples was isolated using a ZymoBIOMICS DNA MiniPrep kit (Zymo Research, USA). The V4-V5 region of 16S ribosomal DNA was PCR amplified using primers as described previously. The libraries were quantified by a KAPA Library Quantification kit (Kapa Biosystems, USA) and sequenced on an Illumina MiSeq using a MiSeq Reagent Kit V2 (Illumina, USA). Sequence analyses were performed using QIIME v.1.9.0. Briefly, after checking quality of sequenced reads, 8 nucleotides barcodes were extracted and the forward and reverse reads were joined. Sequence libraries were filtered and split based on their corresponding barcodes. An open reference operational taxonomic unit (OTU) picking strategy was used to select OTU, and taxonomy was assigned based on the Greengenes reference database. Subsequently, a taxonomic table for each taxonomic level was generated, and significantly differential
features at each level were identified using linear discriminant analysis (LDA) along with effect size measurements (LEfSe)\textsuperscript{25}. The taxons were filtered by LDA Effect Size (LEfSe) analyses with default criteria ($P < 0.05$ by Kruskal-Wallis test; LDA score > 2).

**Statistical analysis**

All statistical data was obtained using a two-tailed Mann Whitney U test, and two-way ANOVA analysis of variance using Graph Pad Prism 4 software (GraphPad). The CFU values from mice livers homogenates were analyzed using the two-tailed paired Student’s $t$-test. For LDA Effect Size analyses, Kruskal-Wallis test was used.

**Results**

*γδ T Cells Are a Predominant Source of IL-17A During Cholestatic Liver Disease*

In this study, our aim was to identify the mechanistic contributions of IL-17A in pathogenesis of cholestatic liver disease in Mdr2\textsuperscript{−/−} mice. In agreement with other characterizations of this model\textsuperscript{2,4}, Mdr2\textsuperscript{−/−} mice exhibited characteristic collagen deposition around hepatic bile duct, moderate to severe periportal to bridging fibrosis with marked influx of inflammatory infiltrate at 25-weeks of age (Figure 1A). Compared to age-matched WT control, Mdr2\textsuperscript{−/−} mice demonstrated elevated levels of serum IL-17A (Figure 1B). Administration of IL-17A neutralizing antibody to Mdr2\textsuperscript{−/−} animals from 8-weeks to 25-weeks of age was sufficient to reduce hepatic fibrosis score (Supplementary Figure 1A–B). Consistent with this finding, we found reduced expression of collagen-1a and alpha-smooth muscle actin ($\alpha$-SMA) in liver, and a marked reduction in infiltrating inflammatory populations such as monocytes (CD11b\textsuperscript{+}Ly6c\textsuperscript{hi}) and neutrophils (CD11b\textsuperscript{+}Gr1\textsuperscript{+}) in animals receiving IL-17A-neutralizing treatment (Supplementary Figure 1C–F). These data implicated a critical role for IL-17A in pathogenesis of cholestatic liver fibrosis in Mdr2\textsuperscript{−/−} mice.

To identify source(s) of IL-17A in Mdr2\textsuperscript{−/−} mice, we performed intra-cellular cytokine staining analysis on PMA/Ionomycin stimulated intrahepatic lymphocytes isolated from 25-week-old Mdr2\textsuperscript{−/−} mice. Mdr2\textsuperscript{−/−} animals demonstrated a marked increase in frequency of CD3\textsuperscript{+}IL-17\textsuperscript{+} T cells in comparison to WT (Figure 1C). There were no appreciable changes in splenic frequency of T cell derived IL-17A despite significant changes in the liver (Figure 1C, bottom). A closer examination of intrahepatic CD4\textsuperscript{+} T cell and γδ-T cell IL-17A production demonstrated a subtle increase in the frequency of CD4\textsuperscript{+}IL-17A\textsuperscript{+}, while a dramatic increase in the frequency of IL-17A\textsuperscript{+} γδ-T cells in Mdr2\textsuperscript{−/−} mice (Figure 1D). Phenotypic characterization further revealed that IL-17A\textsuperscript{+} γδ T cells exhibit CD27\textsuperscript{−} RoRγT\textsuperscript{hi} CD44\textsuperscript{+} phenotype, which have been attributed to IL-17-producing γδ T cells subset (Supplementary Figure 2)\textsuperscript{26}. Thus, elevated serum IL-17A in fibrotic Mdr2\textsuperscript{−/−} mice may be attributed to intrahepatic γδ T cell compartment.

**Liver-specific Alterations of γδ T Cell Compartment During Cholestatic Liver Disease**

Anatomic compartmentalization and functions of γδ T cells, such as propensity to make IL-17, are dictated by composition of the TCR\textsuperscript{27}. To examine Vγ-chain usage in Mdr2\textsuperscript{−/−} livers, we utilized a novel approach of in vivo administration of anti-γδTCR (Clone:
UC7-13D5) and detection with anti-hamster IgG (Supplementary Figure 3). We performed a FACS analysis using commercially available antibodies directed against Vγ1.1+1.2, Vγ2, Vγ3 in combination with Vγ4 and Vγ7 within TCRβ-CD3+ gate. This approach revealed significant alterations within the composition of the intrahepatic γδ-T cell compartment in Mdr2−/− animals in comparison to WT controls (Figure 2A). The most dramatic increase in fibrotic livers was the accumulation of in vivo-labeled anti-γδTCR+ T cells (Figure 2A, C). This was liver-specific, as the splenic γδ T cell compartment was not appreciably changed in fibrotic animals (Figure 2B, D). IL-17A was primarily derived from in vivo-labeled γδ T cell with Vγ2 and Vγ4 comprising the remainder (Figure 2C). Overall, these data indicate that the fibrotic liver γδ T cell compartment is altered in favor of IL-17A γδ T cell subsets.

**Cholestasis Drives Intrahepatic Expansion of IL-17A+ Invariant Vγ6Jγ1 γδ T Cells**

To determine Vγ-chains that are targeted by in vivo administration of anti-γδTCR, we sorted in vivo-labeled cells from WT versus Mdr2−/− livers (Figure 3A). The purified population was sequenced to identify VγJγ usage as well as determine the CDR3 diversity. In WT mice, in vivo labeling targeted predominantly Vγ4, Vγ2 and Vγ1 populations (Figure 3B,C). Mdr2−/− mice, on the other hand, revealed that in vivo labeling targeted predominantly Vγ6 bearing γδ-T cells (Figure 3B–D). Examination of the overall CDR3 region diversity indicated that the Vγ6 population is invariant (Figure 3D). Consistent with this analysis, in vivo labeled γδ T cells from knockout livers demonstrated a substantial reduction in the number of CDR3 sequences present in the sample (Figure 3E). Analysis of peptide sequences of the most prevalent CDR3 indicates a massive expansion of Vγ6Jγ1, an invariant population of γδ T cells in the livers of Mdr2−/− animals, comprising approximately 76% of the total in vivo-labeled population (Figure 3F). The same analysis of WT livers demonstrates that 2 out of 3 mice have Vγ6Jγ1 as the most prevalent population, whereas Vγ4Jγ1 was the most prevalent in the remaining control mouse (Figure 3F). Altogether, these data indicate that liver fibrosis drives expansion of IL-17A+ invariant Vγ6Jγ1 γδ T cells, which is predominantly targeted by in vivo administration of anti-γδ-TCR.

**Increased Gut Permeability and Dysbiosis Favor Microbial Enrichment in Liver During Cholestatic Liver Disease**

Chronic inflammatory conditions such as liver disease can contribute to the imbalances in intestinal flora, loss of intestinal integrity, which can result in translocation of gut commensals to the periphery. To address commensal imbalances, fecal samples were obtained from 25-weeks-old WT (n=5) and Mdr2−/− (n=5) for 16s ribosomal DNA analysis. Interestingly, linear discriminant analysis effect size (LEfSe) predictions for bacterial families found in the fecal DNA implicated a marked enrichment (LDA score) of *Lactobacillus, Bacilli, Turicibacter, Unclassified Streptococcus, and Prevotella* in the intestine of Mdr2−/− mice (red) compared to WT animals (green) (Figure 4A). Likewise, cladogram representation indicated the close phylogenetic relationship among bacterial families identified in the Mdr2−/− mice (Figure 4B). LEfSe predictions also implicated the enrichment of *Lactobacillus* and other bacterial families as young as 8 weeks of age, the beginning of disease onset in Mdr2−/− mice (Supplementary Figure 4). These findings correlated with increased intestinal permeability detected by oral administration of FITC-
dextran assay (Figure 4C). This was even more pronounced at 25-weeks of age, when animals experienced the peak of liver fibrosis (Figure 4C).

In line with these analyses, standard bacteriological culture technique detected Lactobacillus sp. as the predominant bacterial species in Mdr2−/− livers (Figure 4D). The isolated bacterial colonies were subjected to confirmatory diagnosis by using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis. MS profiles and PCR protocol as described previously\textsuperscript{30, 31} identified Lactobacillus gasseri in Mdr2−/− livers (Supplementary Figure 5A–C). To investigate the mechanisms that may favor Lactobacillus enrichment in Mdr2−/− mice liver, we performed in vitro bile salt and pH tolerance assays using isolated strains of L. gasseri. A commensal bacterium Ralstonia picketti isolated from WT intestine was used as control (Supplementary Figure 5A–B). Strikingly, L. gasseri exhibited bile salts (1.5%) and pH (2) resistance after 4 hours of treatment, while R. picketti was highly sensitive to both conditions (Figure 4E–F). Taken together, these data suggest that cholestatic liver disease selectively enriches intestinal flora in Mdr2−/− mice, which corresponds to inappropriately localized L. gasseri in fibrotic liver tissues.

**Intrahepatic γδ T Cells Produce IL-17 in Response to Translocated Gut Microbiota**

Next, we determined if γδ T cells isolated from Mdr2−/− livers could respond to bacteria isolated from Mdr2−/− livers. Indeed, in vitro stimulation of isolated bulk T cells from 25-week-old Mdr2−/− livers with heat-killed L. gasseri resulted in IL-17A production (Figure 5A). Compared to L. gasseri, stimulation with heat-killed R. picketti resulted no IL-17A production by γδ T cells (data not shown). To rule out non-specific stimulation by toll like receptors (TLRs) or other innate mechanisms, we also stimulated control wells with Pam\textsubscript{3}CSK\textsubscript{4}, a TLR2 agonist, and LPS, a TLR4 agonist. IL-17 responses were not elicited against these stimuli (Figure 5A, right), nor any appreciable splenic γδ T cell IL-17A was observed (Figure 5A, bottom). Intrahepatic γδ T cell responses to heat-killed microbes resulted in weak IFN-γ production in comparison to IL-17A (Figure 5B, top). Splenic γδ T cells were not appreciably influenced by microbial stimuli (Figure 5B–D). Thus, these data indicate that intrahepatic γδ T cells can specifically respond to Mdr2−/− liver-derived microbes in vitro.

**Mdr2−/− Liver-derived L. gasseri Induces IL-17 Responses in WT Mice**

To determine the implications of γδ T cells responses on perpetuating hepatic inflammatory processes, we administered 1×10\textsuperscript{6} CFU of liver-derived L. gasseri to WT animals (Supplementary Figure 6A). Intra-peritoneal (I.P.) injection was chosen to ensure that these bacteria were introduced into the periphery rather than colonize the intact intestinal tracts. In fact, I.P. inoculation of FVB/N mice resulted in bacterial colonization of the liver after 18–24 hours as detected by standard culture technique (Supplementary Figure 7). Introduction of Mdr2−/− liver-derived L. gasseri resulted in a marked surge of serum IL-17A in WT animals (Supplementary Figure 6B). This corresponded with an influx of hepatic inflammatory mediators such as CD11b\textsuperscript{+}Gr-1\textsuperscript{+} neutrophils, which was present in a low frequency in the spleens of these animals (Supplementary Figure 6C–D). We treated FVB/N mice with anti-γδTCR or Hamster IgG isotype control one day prior to infection with L.
Following bacterial inoculation, we found that blockade of the γδ TCR resulted in a subtle reduction of serum IL-17 levels (Supplementary Figure 6B). However, a dramatic effect was found in the livers of these mice, with a marked reduction in hepatic inflammatory infiltration (Supplementary Figure 6C–D). Altogether, these data suggest that intrahepatic γδ T cells can mount inflammatory IL-17 responses to inappropriately localized commensal bacteria.

**Antibody-mediated Blockade of the γδ TCR Attenuates Fibrosis**

γδ T cells have been implicated in perpetuating liver injuries through interaction with inflammatory populations and IL-17A signaling\(^\text{32, 33}\). We took an antibody-mediated approach to target the γδ TCR and examine whether γδ-T cells are required for the pathogenesis of liver fibrosis in our model. Due to limited commercial availability of FVB/N background for specific-cell population knockouts, an antibody-mediated approach by infusing Mdr2−/− animals with monoclonal antibody directed against the γδ TCR (Clone: UC7-13D5) I.V. every 10 days from 8 to 25 weeks-of-age was adopted\(^\text{32}\). Compared to untreated Mdr2−/− mice (average score 3), anti-γδ TCR treated animals exhibited reduced fibrosis lesion (average score 2) and a significant reduction in serum IL-17A levels (Figure 6A–C). This corresponded with reduced expression of fibrosis-associated genes: Collagen-1a, α-SMA and TNF-α in liver (Figure 6D). We determined the efficiency of antibody-mediated blockade by measuring reduced RoRγT expression by γδ T cells, reduced gene expressions of IL-17A and IL-23 within the liver tissues, and an increase in serum IL-6 cytokine levels (Supplementary Figure 8). A trend of reduced serum ALT was found that did not reach statistical significance (Figure 6E). In addition, reduced serum IL-17A concentration observed in antibody-treated Mdr2−/− corresponded to a marked decrease in frequency of intrahepatic inflammatory monocyte and neutrophil populations in comparison to untreated Mdr2−/− animals (Figure 6F, Supplementary Figure 9). These data indicate that intrahepatic γδ T cells augment the pathogenesis of cholestatic liver disease via IL-17 response.

**Intrahepatic γδ T Cells Isolated from PSC Patient Livers are Capable of Producing IL-17**

Our findings in the Mdr2−/− mouse model implicate γδ-T cell-derived IL-17 responses as key mediator of pathogenic processes during cholestatic liver disease. An elevation in the frequency and number of γδ T cells has been demonstrated in the peripheral blood and livers of PSC patients\(^\text{34}\). To determine the clinical significance, we obtained intrahepatic lymphocytes from human patients undergoing orthotopic liver transplant for either PSC (n=5) or HCV (n=5) related end-stage liver diseases (ESLD). Relevant biologic and clinical variables of these patients have been summarized in Supplementary Table 2. To determine IL-17 production capability of intrahepatic γδ T cells, bulk lymphocyte cultures were stimulated over night with PMA/Ionomycin. PMA-stimulated γδ T cells from PSC patients produced IL-17, whereas HCV patient γδ T cells were not capable of producing IL-17 (Figure 7A). Although γδ TCR+ composed a higher proportion of the intrahepatic γδ T cell population in HCV patients, γδ T cells from both PSC and chronic HCV patients comparably produced IFN-γ (Figure 7B–C; Supplementary Figure 10). Phenotypic analysis further revealed that higher fraction of IL-17A+ γδ T cells expressed CD161, a marker of...
human IL-17 producing γδ T cells (Figure 7D)\textsuperscript{35}. Together, these findings indicate that PSC drives selective enrichment of γδ T cell subset capable of IL-17 production.

**Discussion**

Anatomic sequestration of commensal flora relies on intact barriers to ensure that microbes serve their respective functions in discrete locations. Disruption of intestinal barriers is associated with various etiologies of chronic liver diseases\textsuperscript{2,28,36,37}. Alterations in the liver microenvironment, biliary ducts, and cholangiocytes during cholestatic liver disease can promote microbial translocation to biliary portals and liver tissues. Particularly, toxic bile acids associated with cholestasis contribute to increased oxidative stress, hepatocyte death, inflammation and suppression of Kupffer cell (KC) phagocytic functions\textsuperscript{38}. The effects of bile acids on KC functions contribute to the both ineffective clearance of fibrotic tissues\textsuperscript{39} and permission of intrahepatic microbial accumulation\textsuperscript{38}. Our results demonstrate biochemical processes of cholestasis in Mdr2\textsuperscript{−/−} mice selectively enriched intestinal microflora for bacterial species such as L. gasseri. This is consistent with PSC patients, whom Lactobacillus and other related bacterial families have been reported regardless of treatment regimens or co-existent intestinal disorders\textsuperscript{40}.

Intrahepatic IL-17+ γδ T cells have dichotomous roles in modulating liver injury depending on the acute vs. chronic settings. It can be protective in acute injury settings such as Con-A induced hepatitis\textsuperscript{16} and experimental hepatectomy\textsuperscript{17}, while this population is implicated in perpetuating disease pathogenesis in chronic models such as high-fat diet and biliary atresia\textsuperscript{20,21}. In our model, we observed an attenuation of fibrosis in anti-γδTCR-treated Mdr2\textsuperscript{−/−} animals with a substantial reduction in serum IL-17A levels. Paradoxical outcomes of these studies suggest that duration and persistence of injury dictate the therapeutic versus pathogenic effect of IL-17A, and make γδ-T cell compartment an attractive therapeutic target for liver diseases.

Commensal microbes drive IL-17 responses in the GI tract, which has been implicated in maintaining intestinal integrity and homeostasis\textsuperscript{41,42}. While beneficial in the GI tract, intrahepatic IL-17 responses can perpetuate destruction of biliary networks and hepatic architecture via hyper-sensitization of HSCs to profibrotic mediators\textsuperscript{12}. Since IL-17+ γδ T cells are capable of expanding to inflammation\textsuperscript{12,43}, it is possible that translocation of gut commensals act in concert with other mechanisms to drive the expansion of pathogenic γδ T cells. Furthermore, germ-free mice which lack commensal microflora have a range of immune defects that include ineffective regulatory T cells, and increased Th2 CD4+ T cell differentiation\textsuperscript{44}. In combination, these features of the germ-free mouse T cell compartment would favor perpetuation of liver damage\textsuperscript{45} and may account for the exacerbation of liver injury reported by Tabibian and colleagues\textsuperscript{46}. However, our findings revealed that antibiotic treatment in Mdr2\textsuperscript{−/−} mice from 8-weeks to 25-weeks of age does not alter liver disease or IL-17 production (unpublished data). Moreover, co-housing of Mdr2\textsuperscript{−/−} with WT mice for 8 weeks revealed no alteration in liver disease and IL-17 production by γδ T cells, and a marked enrichment of Lactobacillus was still found in co-housed KO animals (Supplementary Figure 11). These findings indicate that the genetic deficiency leading to cholestasis is critical in inducing gut dysbiosis and the enrichment of Lactobacillus in these
mice; and co-housing is not sufficient to overcome the genetic defect in cholestasis. This is supported by clinical reports that suggest treating PSC patients with metronidazole; an antibiotic which targets anaerobic microbes such as *Lactobacillus* transiently corrects plasma levels of liver enzymes. However this strategy was not curative, as the study participants still required liver transplant.

While both PSC and chronic HCV can cause ESLD, our data suggests that the etiology of disease can differentially alter the intrahepatic γδ T cell compartment. PSC patients with ESLD shared a selective skewing of γδ T cell subset capable of IL-17 production in comparison to HCV patients despite comparable frequencies of intrahepatic γδ T cells capable of IFN-γ production. It must be noted that the PSC patients in our cohort are not likely harboring the complete *MDR3/Mdr2* deletion we have modeled in mice; all patients were into adulthood at the time of transplant. We therefore cannot infer or rule out γδ T cell dynamics during the initiation of cholestasis in these patients. Our findings suggest that specific features of cholestatic liver disease permit enrichment of γδ T cells with ability to produce IL-17.

Overall, these findings provide insight into pathogenesis of cholestatic liver disease, understanding the intricate balance between intestinal microflora and γδ T cells is essential for modulating this disease in clinical setting. For instance, there is a growing body of work that demonstrates IL-17+ γδ T cells are particularly important for TCR-mediated recognition of bacterial pathogens invading host tissues. Our data suggest fibrotic livers have an expansion of IL-17+ γδ T cells, and therefore blockade may inhibit TCR dependent recognition of translocated microbes that reside in the fibrotic liver. WT mice administered with *L. gasseri* corroborate this finding, as acute hepatitis is attenuated when anti-γδTCR is administered prior to inoculation. Although methods for the direct targeting of IL-17+ γδ T cells remain under development, our study provides an important advance in the understanding and a potential target for the treatment of cholestatic liver diseases.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

- α-SMA  alpha-smooth muscle actin
- γδ TCR  gamma delta T cell receptor
- ABCB4  ATP binding cassette subfamily B member 4
- Con-A  Concanavalin
- FVB/N  friend virus B NIH
- GAPDH  glyceraldehyde-3-phosphate dehydrogenase
- HCV  Hepatitis C virus
- HSC  hepatic stellate cell
- IFNγ  interferon gamma
- IL  interleukin
- KC  Kupffer cells
- LDA  linear discriminant analysis
- LEfSe  linear discriminant analysis effect size
- LPS  lipopolysaccharide
- Mdr2/Mdr3  multidrug resistance gene 2/3
- PSC  primary sclerosing cholangitis
- TNF-α  tissue necrosis factor-alpha

References


Figure 1.
Intrahepatic γδ T Cells are Predominant Source of IL-17A During Cholestatic Liver Disease. (A) Liver tissues from 25-wk-old FVB/N and Mdr2−/− mice were sectioned and stained with Sirius red and H&E stain (Mag. 100×). (B) Serum samples were obtained from 25-wk-old Mdr2−/− and FVB/N mice and the levels of IL-17A were detected using a standard ELISA. (C–D) Bulk lymphocytes isolated from livers and spleens were stimulated with PMA/Ionomycin for 4 hours to determine frequency of live, non-autofluorescent CD3+ (T cells) (C) that produce IL-17A from livers and spleens derived from the indicated group. (D) Quantitation of frequencies of IL-17A+ CD4+ T cells (Top) and γδ T cells (bottom) from livers are shown. Representative figures from more than 3 independent experiments are shown. Two-tailed Mann Whitney test, ***$P<.001$. 

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Figure 2.
Fibrotic Liver γδ T Cell Compartment is Altered in Favor of IL-17 Producing Subsets. (A–B) Mdr2−/− and FVB/N mice were administered 500μg of anti-γδ TCR (Clone UC7-13D5) or appropriate Hamster-IgG isotype control intravenously and sacrificed one day following treatment. Livers and spleens were harvested; and bulk lymphocytes were first stained with anti-hamster IgG, followed by TCRVγ1.1–1.2, Vγ2, Vγ3, and for Vγ4, Vγ7 in combination with appropriate surface antibodies. Frequencies were determined based on the percentage of the indicated Vγ-chain within the live non-autofluorescent TCRβ− CD3+ gate (A–B). “Other” refers to in vivo bound and ex vivo detectable UC7-13D5 (A, top; B, Top). (C–D) Lymphocytes were stimulated with PMA/ionomycin in the presence of Golgi Plug/ Golgi Stop for 4 hours at 37°C, and subsequently stained for intracellular IL-17A. Statistical
significance was determined by a two-tailed Mann-Whitney Test, *$P<.05$, **$P<.005$, ***$P<.0001$. All data are representative of 3 independent experiments ($n=3–5$ per group).
Figure 3.
Cholestasis Drives Expansion of IL-17A+ Invariant V\textgamma6J\gamma1 \gamma6 T Cells. (A) Three FVB/N and three Mdr2−/− animals were administered 500\mu g of anti-\gamma6TCR intravenously and sacrificed one day following treatment. One WT and one Mdr2−/− animal was administered appropriate hamster-IgG isotype control for gating control. Lymphocytes were stained as described and sorted based on positivity for bound UC7-13D5 in live non-autofluorescent CD3+ TCR\beta- anti-Hamster IgG+. (B–C) Post-sort purity was >95% in all animals, and in vivo bound antibody+ populations were sequenced to identify V\gamma-chains usage. (D) V\gamma chain usage of sorted population bound by in vivo administration of anti-\gamma6TCR, and diversity diagram of the sorted populations based prevalence of V\gamma-chain using a unique CDR3. (E) Total unique CDR3 sequences identified in antibody bound populations of individual mice. (F) In 2 out of 3 WT animals; invariant V\textgamma6J\gamma1 chain was the most prevalent population identified by in vivo administration of anti-\gamma6TCR (F, top), while this population was the most prevalent in 3 out of 3 Mdr2−/− animals (F, bottom).
Figure 4.
Selective Enrichment of *Lactobacillus gasseri* in the Gut and Liver of *Mdr2−/−* mice. (A) Linear discriminant analysis size effect (LEfSe) predictions for bacterial families found in fecal pellets of 25-wk-old *Mdr2−/−* (red) and FVB/N (green) mice are shown. Linear discriminant analysis (LDA) score represents log changes in bacterial families shown. (B) The Cladogram representing the phylogenetic relationship between bacterial families (red = *Mdr2−/−* and green = FVB/N) is shown. (C) Gut permeability was determined in 8-wk-old and 25-wk-old *Mdr2−/−* and FVB/N animals by using FITC-Dextran oral feeding assay. (D)
Whole liver homogenates were plated on blood agar plates and 24 hours following plating, colonies were quantified as colony forming units per gram (CFU/gram) of tissue in 25-wk-old Mdr2−/− and WT controls. (E–F). Bile salt and pH tolerance assays for L. gasseri indicated the possible mechanism by which L. gasseri was selectively enriched in the Mdr2−/− livers. Statistical analysis was performed by one-way analysis of variance (ANOVA) for C. *P<.05, **P<.005, ***P<.0001. Error bars reflect the standard error of mean (SEM).
Figure 5.
Intrahepatic γδ T Cells Produce IL-17 in Response to Translocated Gut Microbiota. (A-B) Bulk T cells were isolated from Mdr2−/− livers and spleens via negative selection. Cells were seeded at 2×10^5 cells per well and stimulated for 24 hrs in the presence of media (complete RPMI +20U/mL IL-2), or indicated heat-killed microbial (10^6/mL) species isolated from Mdr2−/− livers. T cells were analyzed for IL-17A (A–B; top panels) and IFNγ (A–B; bottom panels) responses. To rule out non-specific TLR pathways, control wells were stimulated with 2μg/mL LPS, or 1μg/mL Pam3CSK4; PMA/Ionomycin stimulation served as
a positive control (A–B; far right). (C–D) Frequency of IL-17A (C) and IFNγ (D) production by TCRβ-γδTCR+ were compared in the liver versus spleen. Numbers on FACS plots represent the proportion of the TCRβ-γδTCR+ population producing the indicate cytokine. Statistical significance was determined by a two-tailed Mann-Whitney Test, *P<.05, **P<.005, ***P<.0001.
Figure 6.
Antibody-mediated Blockade of the γδTCR Attenuates Hepatic Fibrosis. *Mdr2−/−* mice were treated I.V. with 500μg anti-γδTCR (UC7-13D5) every 10 days from 8 to 25 weeks. (A–B) Histological analyses of representative liver samples for H&E, Sirius red and polarized Sirius red staining (Mag. 100×) (A) and fibrosis scores (B) are shown. (C) IL-17A ELISA analysis of serum samples following animal sacrifice from the indicated 25-wk-old animals; (D) Fibrosis gene expression in total liver tissues by RT-PCR analysis; and (E) Serum ALT values from treated and control *Mdr2−/−* animals; are shown. (F) Analysis of

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the frequency of infiltrating intrahepatic inflammatory myeloid populations; monocytes (top, MHC class II-ve, CD11b+, Ly6C+), and neutrophils (bottom, MHC class II-ve, CD11b+, GR-1+). Statistical significance was determined by a two-tailed Mann-Whitney Test, *P<.05, **P<.005, ***P<.0001.
Figure 7. Intrahepatic γδ T Cells Isolated from PSC Patient Livers are Capable of Producing IL-17. (A–B) Bulk liver lymphocytes from human subjects with ESLD as a result of PSC (n=5) or HCV (n=5) were stimulated overnight with PMA/Ionomycin as described. γδ T cells from PSC patients were capable of IL-17 production (A, left; *P<.05) whereas γδ T cells from HCV patients were not (A, right), despite comparable frequency of IFNγ production (NS, P>.05) (B). (C) HCV patients demonstrated an increased frequency γδTCR+ CD3+ T cells (*P<.05) in comparison to PSC patients. (D) Frequencies showing the CD161+ γδ T cells
from PSC and HCV patients expressing IL-17A and IFN-γ. Frequencies represent live, non-autofluorescent, CD3+ γδTCR+ expressing the indicated cytokine. Statistical significance was determined by a two-tailed Mann-Whitney Test, *P<.05.