The dendritic cell-Th17-macrophage axis controls cholangiocyte injury and disease progression in murine and human biliary atresia

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Abbreviations:
Biliary atresia (BA)
Kasai portoenterostomy (KPE)
Liver transplantation (LTx)
Biliary epithelial cells (BECs)
Rhesus rotavirus (RRV)
Plasmacytoid dendritic cells (pDCs)
Myeloid dendritic cells (mDCs)
Diphtheria toxin receptor (DTR)
IL-17 receptor A (IL-17RA)
Normal saline (NS)
Immunohistochemistry (IHC)
Immunofluorescence (IF)

Mononuclear cells (MNCs)

Recombinant IL-17A (rIL-17A)

Day 4/8/12 post NS/RRV injection (d4/8/12pNS/RRV)

Diphtheria toxin (DT)

Inflammatory macrophages (iMΦ)

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Biliary atresia is a fibroinflammatory obstruction of the extrahepatic biliary tree in neonates. While intrahepatic bile duct proliferation is universal at diagnosis, bile duct paucity develops later. We hypothesized that polarized T helper lymphocyte responses orchestrate progression of intrahepatic biliary injury in this disease. IL-17A-GFP, CD11c/DTR and IL17RA-/- mice were used to examine T-lymphocyte polarization, inflammatory leukocyte recruitment and biliary injury in rhesus-rotavirus induced biliary atresia. Multiparameter flow cytometry and automated image analysis of immunostaining were applied to liver tissue samples from infants with biliary atresia. In the mouse model, activated CD4+ lymphocytes started to emerge in the liver on day 8 after viral challenge while innate immune responses were waning. Plasma IL-17A levels rose concomitant with hepatic accumulation of Th17 lymphocytes and myeloid dendritic cells. Targeted depletion of CD11c+ dendritic cells diminished hepatic IL-17A production and ameliorated intrahepatic bile duct injury. Recombinant IL17A induced expression of CCL2 in neonatal cholangiocytes in vitro, and blockade of the corresponding chemokine receptor CCR2 reduced recruitment of inflammatory macrophages to the liver in vivo. Genetic disruption of IL-17A signaling was associated with downregulation of hepatic Ccl2/Ccr2 mRNA expression, reduced infiltration of the liver with inflammatory Ly6C^hi macrophages and improved survival. In the liver of infants with biliary atresia, cholangiocytes were found to express IL-17 receptor A and the prevalence of IL-17A+ cells was positively correlated with the degree of CD68+ macrophage infiltration at diagnosis. Hepatic CD4+ lymphocytes were chief producers of IL-17A in patients with progressive disease undergoing liver transplantation. Conclusion: Our findings identify the dendritic cell-Th17-macrophage axis as target for the development of strategies to block progression of intrahepatic bile duct injury in patients with biliary atresia.
Introduction

Biliary atresia (BA) is a devastating condition uniquely restricted to the neonatal period in which fibroinflammatory obstruction of the extrahepatic biliary tree leads to progressive jaundice and rapid fibrosis. Despite attempts to restore bile drainage via Kasai portoenterostomy (KPE) surgery at the time of diagnosis, biliary fibrosis progresses in up to 75% of affected children; thus BA is the most common indication for pediatric liver transplantation (LTx) worldwide. More than 1800 children underwent a first LTx for BA in the US between 2002 and 2012. Of those transplanted before 2 years of age the outcome was inferior compared with those transplanted at a later age, indicating the need to develop adjuvant therapies to prolong transplant free survival (1).

The etiology of BA is multifactorial. Potential causes include dysregulation of biliary development, or exposure to environmental triggers such as toxins or viruses, as recently reviewed (2). Immune-mediated injury to the bile duct epithelial cells (BECs) appears to be the common final pathway leading to the BA phenotype regardless of the initial trigger. Several studies using human samples and the well-established model of experimental BA have furthered our understanding of the immunopathogenesis of BA. Inoculation of neonatal BALB/c mice with rhesus rotavirus (RRV) leads to inflammatory extrahepatic bile duct obstruction by 7 days of life, and death in the majority of infected animals after 14 days. In this model, initiation of injury has been linked to 1) early innate responses involving plasmacytoid dendritic cells (pDCs)-dependent NK cell activation with NKG2D and IFN-γ regulating BEC injury (3, 4), and 2) adaptive regulatory T cells modulating susceptibility to duct obstruction and ability of myeloid (m)DCs to activate cytotoxic CD8+ T lymphocytes (5, 6).
In infants with BA, hepatic gene expression for Th1 and Th2 cytokines correlated with clinical phenotypes of BA (7, 8). Oligoclonal expansion of hepatic CD4+ and CD8+ T lymphocytes, indicating antigen-specific immune responses (9), and altered suppressor function of regulatory T cells (10), were reported. Natural history studies of patients with BA document progressive intrahepatic bile duct loss in the majority of the patients by 400 days of life, despite bile duct proliferation noticed at the time of diagnosis (11). While the mechanism of injury to BECs in BA causing progressive loss of intrahepatic bile ducts is unknown, studies in primary biliary cirrhosis (PBC) linked CD4+ lymphocytes with immune mediated injury against intrahepatic BECs. For instance, increased number of intrahepatic CD4+ Th17 cells and elevated plasma levels of Th17-associated cytokines correlated with serum levels of gamma glutamyl transpeptidase (GGT), a biochemical markers of small duct injury (12). The role of CD4+ lymphocytes in the pathogenesis of BA is largely undefined. We hypothesized that polarized T helper lymphocyte responses orchestrate progression of biliary injury in the later stage of BA pathogenesis.
Material and Methods

**Human Samples.** Fresh liver samples were obtained by wedge biopsy from infants with BA at the time of diagnosis by intraoperative cholangiogram (n=6; median age: 2 months) or at the time of LTx (n=7; median age: 9 months) at Cincinnati Children’s Hospital Medical Center (CCHMC). Archived paraffin embedded liver sections from de-identified patients with BA were obtained by collaboration with pathologists (R.S. and P.R.) at CCHMC/BioBank and at Children’s Hospital of Philadelphia. Archived liver sections from background, non-neoplastic liver from infants who underwent resection of benign neoplasms served as controls (n=4; ages≤12 months). Blood was collected at CCHMC from infants with BA at diagnosis/KPE (n=3; median age: 2 months) and at the time of LTx (n=4; median age: 11 months), and from infants without clinical diagnosis of liver disease and normal serum liver biochemistries (n=4; median age: 9 months) or with Alagille Syndrome (n=1; 44 months). Human study protocols conformed to the ethical guidelines of the 1975 declaration of Helsinki and were approved by the Institutional Review Board.

**Mice.** Wild type BALB/c and CD11c/GFP/diphtheria toxin receptor (DTR) were purchased from Charles River (Wilmington, MA) and The Jackson Laboratories (Bar Harbor, ME), respectively. IL-17A-GFP and IL-17 receptor A (IL-17RA) KO mice were obtained from Biocytogen LLC (Worcester, MA) and from Amgen Pharmaceuticals (Thousand Oaks, CA), respectively, and backcrossed into the BALB/c strain at the CCHMC animal facility for 10 generations. The transgenic mice were described previously (13-15). Mice were kept in conventional conditions (25°C, 12h day light, ad libitum autoclaved chow). IL-17RA KO mice...
and their controls were maintained on 5053 LabTest diet w/ 275 ppm sulfamethoxazole and 1365 ppm trimethoprim (LabDiet, Richmond, IN). The Animal Care and Use Committee at CCHMC approved all experiments involving laboratory animals.

**Murine model of BA.** For experimental BA, pups were injected intraperitoneally with $1.5 \times 10^6$ focus forming units of RRV in 20 µl of 0.9% normal saline (NS) within 24 h of birth, as performed by our group before (5, 6). Details for DC depletion in CD11c/GFP/DTR mice and CCR2 blockade are available in Supplemental Data.

**Immunohistochemistry (IHC), immunofluorescence (IF) and automated image analysis.** IHC and IF were performed according to routine protocols. Details are available in Supplemental Data. For image analysis, entire sections were tile-scanned using an upright Nikon 90i motorized microscope. Automated quantification of IL-17A+ cells was performed on scanned images with NIS Nikon Elements software using RGB general analysis. Images were subdivided for improved software performance. Positive cells were defined by manually choosing DAB+ brown cells of proper size. Portal tracts were selected and enumerated as regions of interest (ROI). RGB General Analysis was utilized in HSI (Hue, Saturation, Intensity) mode to identify DAB+ cells and nuclei stained with hematoxylin, resulting in counts of DAB+ cells, all nuclei for each ROI and for the entire subdivided area. All subdivided regions for each slide were averaged to report DAB+ cells/portal tracts.
**Flow cytometry, co-culture experiments and real-time RT-PCR.** Mononuclear cells (MNCs) and intrahepatic cholangiocytes were isolated from neonatal mice and used in flow cytometry studies and co-culture experiments according to protocols reported by us before (5, 6, 16). Quantitative hepatic gene expression studies on cDNA samples were performed using TaqMan Mouse Immune Array and run on a 7900HT Fast Real-Time PCR System (applied Biosystems) according to the manufacturer’s protocol. Details are provided in Supplemental Data.

**Statistics.** Statistical analyzes were perform with GraphPad Prism software. Statistical significance was determined by paired or unpaired t-test (as indicated in figure legends), with a significance set at p<0.05. Statistical differences between survival curves were calculated by Log-rank (Mantel-Cox) test. Linear regression analysis was applied to relative mRNA concentrations from cholangiocytes exposed to various concentrations of recombinant IL-17A (rIL-17A).
**Results**

*Th17 lymphocytes populate the liver during the late phase of experimental BA.* To delineate the predominant immune responses during the phases of experimental BA, we analyzed the composition of putative effector lymphocytes at 4, 8 and 12 days post NS or RRV injection (d4/8/12pNS/RRV) in the target tissue of injury (liver) and in a secondary lymphoid organ (spleen). The proportions of innate NK, NKT cells and TCRγδ lymphocytes increased in the liver during the early phase of BA and diminished thereafter to frequencies similar to or lower than that found in control pups (*Figure 1A*). In contrast, the frequencies of adaptive CD4+ and CD8+ T cells increased significantly at later time points (d8/12pRRV). Their expansion was specific for the liver whereas their frequencies diminished in the spleen (*Supplemental Figure 1*). A majority of intrahepatic CD4+ and CD8+ T cells expressed the activation marker CD69, while CD8+ lymphocytes expressed also high levels of NKG2D, previously implicated in bile duct epithelial injury (*Figure 1B*). Whereas the plasma concentrations of IFN-γ and IL-13, the signature Th1 and Th2 cytokines, respectively, were comparable between RRV- and NS-treated mice at the later time points, IL-17A concentration was significantly higher on day 12 in RRV-infected mice (*Figure 1C*). When MNCs from both groups of mice were restimulated with anti-CD3 *in vitro*, significant production of IL-17A occurred in cultures from hepatic MNCs of RRV-infected mice, and not from non-infected mice or from splenic MNCs (*Figure 1D*). In order to identify unequivocally the cellular source for IL-17A production in the liver, we challenged neonatal IL-17A-GFP reporter mice with RRV at birth. GFP+(IL-17A+) cells were mainly CD3+ at d12pRRV (*Figure 1E*). The frequency of hepatic GFP+(IL-17A+)CD3+ T cells tripled at d12pRRV and predominantly expressed TCRβ and CD4 but not TCRγδ, suggesting a Th17 origin of IL-17A production.
IL-17A production is dependent on DCs in vitro and in vivo, and is associated with intrahepatic bile duct epithelial injury. To determine the role of CD11c+ DCs in CD4+ T cell polarization in experimental BA we examined infiltration of the neonatal liver with CD11c+ DCs following RRV infection in reporter mice, in which the transgene GFP/DTR is expressed under the promoter of CD11c (13). The frequency of CD11c+ DCs (GFP+MHCI-Lineage-) increased more than 2-fold in liver and spleen at d12pRRV (Figure 2A/B). Most of the hepatic DCs belonged to the myeloid DC subset (CD11b+CD8-; mDCs). Hepatic DCs isolated from d12pRRV mice induced naïve splenic CD4+ T cells to secrete IL-17A (Figure 2C). To validate the role of CD11c+ DCs in vivo, neonatal CD11c/GFP/DTR mice were injected with diphtheria toxin (DT) every other day between d3 and d12pRRV. DT-treatment decreased the accumulation of DCs in the portal triads and the frequency of splenic DCs (Figure 3A/B) which was accompanied by diminished intrahepatic accumulation of activated CD69+CD4+ lymphocytes and hepatic Il-17a mRNA expression (Figure 3C). Restimulated MNCs from DT-treated pups produced lower levels of IL-17A than MNCs from control pups in vitro (Figure 3C). Importantly, DT-induced reduction of CD11c+ DC significantly lowered serum total bilirubin levels, a biomarker for cholestasis and bile duct injury (Figure 3D).

Histomorphological analysis of liver sections subjected to CK19 IHC, identifying BECs, revealed preserved interlobular bile ducts with normal lumen in more than 50% of portal triads in DT-treated animals, while livers from vehicle-treated mice predominantly showed dystrophic bile duct profiles indicating bile duct paucity (Figure 3E).

IL-17A signaling controls recruitment of inflammatory macrophages to the site of epithelial injury in BA. IL-17RA forms a heterodimer with IL-17RC, which serves as the receptor for IL-17A and IL-17F. IL-17A signaling is disrupted in IL-17RA/- mice. Following post-natal RRV infection, genes encoding Cd40,
Endothelin 1, and Ifn-γ were up-, and those encoding Cd45, Ccr2, Il-1β and Icos were downregulated in IL-17RA/- compared with IL17RA+/+ mice (Figure 4A). Candidate qPCR studies confirmed significant downregulation of hepatic mRNA expression for Ccr2 and its ligand Ccl2. Examining the role of the CCL2/CCR2 axis in RRV-infected wt mice, we found that plasma CCL2 levels were elevated at d12pRRV coinciding with emergence of inflammatory macrophages (iMΦ; Ly6C hi F4/80+CD11b+Gr1-) in the liver (Figure 4B). The immune cells expressing CCR2 were mainly Ly6C hi iMΦ, whereas T and B lymphocytes or monocytes were less prevalent within the hepatic CCR2+ leukocyte compartment during the late phase of BA. Importantly, treatment with the specific small molecule CCR2 antagonist RS504393 on d8/9pRRV decreased the number of CCR2+ immune cell and of iMΦ in the liver on d10pRRV (Figure 4C).

When probing further whether down-regulation of CCL2 expression in mice lacking IL17A signaling had consequences for leukocyte recruitment, we found by flow cytometric and IHC analyses that the frequency of Ly6C+iMΦ in the liver and accumulation of F4/80+ MΦ around interlobular bile ducts were diminished in IL-17RA/- compared with wt mice on d10pRRV (Figure 4D/E). Importantly, these changes were associated with improved survival of RRV infected IL-17RA/- mice (Figure 4F).

To determine whether IL-17A exerts epithelial injury, we examined its receptor expression on primary neonatal cholangiocytes and the functional responses of these cells to cytokine stimulation in vitro. IL-17RA co-expressed with the cholangiocyte marker CK19, as shown by direct immunofluorescence (IF) (Figure 5A). No difference in survival of primary cholangiocytes cultured for 48 h with rIL-17A was observed in a MTS colorimetric assay (Supplemental Figure 3). While IL-17A was not directly cytopathic to BECs, we found that incubation with rIL-17A for 24 h induced mRNA expression of Ccl2, Cxcl1, Cxcl5 and Il-6 in vitro (Figure 5B).
IL-17A induces expression of Th17 polarizing cytokines in hepatic leukocytes. We hypothesized that IL-17A may not only promote recruitment of inflammatory cells to the bile duct epithelium, but also upregulate expression of pro-inflammatory cytokines in leukocytes. Thus, we isolated CD11b+ myeloid cells from livers and spleens of RRV-infected pups, which contained neutrophils, monocytes and MΦ (Supplemental Figure 4A). The cells were restimulated in vitro with rIL-17A for 6 h, which resulted in increased expression of \textit{Il}-6 and \textit{Il}-23p19 (Supplemental Figure 4B), especially in hepatic CD11b+ cells containing a higher proportion of iMΦ.

\textbf{Cholangiocytes and macrophages express IL-17RA in patients with BA}. In order to investigate whether Th17 lymphocytes are linked to BEC injury in humans with BA, we performed dual IF on liver sections for IL-17RA and AE1/AE3, a marker for cholangiocytes. The two proteins co-expressed in a neonate without cholestasis and in an infant with BA at time of diagnosis (Figure 5C). Furthermore, hepatic iMΦ, identified by the surface marker CD68, expressed IL-17RA in patients with BA at diagnosis and at the time of LTx (Supplemental Figure 4C).

\textbf{Activated CD4+ lymphocytes and myeloid DCs are present in the livers of infants with BA}. Multi-parameter flow cytometry on fresh liver tissue samples from patients with BA at diagnosis identified NK, CD4+ and CD8+ cells as prominent lymphocyte populations, with lower frequency of NKT cells (Figure 6A/B). Activated CD4+ and CD8+ T lymphocytes (expressing low levels of CD127) were more frequent in liver than in blood of BA patients (Figure 6C). Analysis of DC populations demonstrated predominance of
CD11c+ mDCs over CD123+ pDCs (Figure 6D/E). Similar to experimental BA, hepatic CD11c+ DCs were localized in the portal triad in close proximity to the interlobular bile ducts, as detected by IHC against CD11c (Figure 6F).

**Prevalence of IL-17A+ cells is associated with hepatic infiltration of CD68+ cells in infants with BA.** IHC against IL-17A showed that hepatic IL-17A+ cells localize to the periportal areas (Figure 7A). Automated image analysis revealed that the number of periportal IL-17A+ cells was higher in subjects with BA compared to non-cholestatic controls. Using CD68 IHC on liver sections, subjects with BA at diagnosis were qualitatively divided into two groups: those with absent or low number of CD68+ cells, and those with high prevalence of periportal CD68+ MΦ (Figure 7B). Notably, the frequency of periportal IL-17A+ cells was significantly higher in the second group. In this small cohort, demographics, and short and long-term outcomes were similar between both groups (Supplemental Table 1).

**Hepatic Th17 responses persist in subject with BA and rapidly progressive disease.** IL-17A+ cells were highly prevalent in the portal triads in subjects with BA and progressive disease requiring LTx at less than 2 years of age, whereas in conditions like Alagille Syndrome hepatic IL17A+ cells were inconspicuous at the time of LTx (Figure 7C). MNCs from fresh tissue of explanted livers were stimulated with PMA and ionomycin and subjected to intracellular cytokine staining which identified CD4+ and CD8+ lymphocytes as the predominant producers of IL-17A and IFN-γ, respectively (Figure 7D). Circulating CD4+ T cells in these patients displayed an activated phenotype with absent expression of CD127, compared with age-matched healthy controls (Figure 7E). These data suggest that IL-17A lymphocyte infiltration is
associated with recruitment of CD68+ iMΦ to the site of injury in human BA, and that Th17 polarization of hepatic CD4+ lymphocytes persists in patients with progressive disease requiring LTx.
Discussion

Our studies in experimental BA show that following post-natal RRV infection DCs are critical in polarizing Th17 lymphocytes orchestrating the post-obstructive phase of intrahepatic bile duct injury. IL-17A is capable of inducing production of the chemokine ligand CCL2 by neonatal cholangiocytes which then attracts CCR2+Ly6C+ iMΦ to the site of epithelial injury (Figure 8).

Elevated plasma concentrations for IL-17A and infiltration of the liver with IL-17A+CD4+ at d12pRRV demonstrate emergence of Th17 responses during the late phase of BA. These findings are consistent with a recent report showing upregulation of Th17-associated genes in hepatic leukocytes from RRV-infected neonatal mice (17). However, based on ex-vivo stimulation of purified hepatic leukocytes, this study concluded that γδ T cells represented the primary cellular source for IL-17A production in experimental BA. In contrast, our studies using IL-17A-GFP reporter mice identify TCRαβ+ lymphocytes, and not γδ T cells, as the predominant cell type driving hepatic IL-17A responses during the post-obstructive phase of murine BA. Our findings indicate a critical role for Th17 cells in orchestrating hepatobiliary injury in BA which share similarities with the impact of Th17 cells on progression of inflammatory BEC injury in PBC and PSC and in their murine models (12, 18). Based on our studies and literature evidence, we propose a model for the pathogenesis of experimental BA, in which IL-17A+ producing γδ T cells contribute to an early acute inflammatory response to RRV infection, whereas Th17 adaptive immune responses perpetuate intrahepatic BEC injury in the post-obstructive phase of the disease.
We show that hepatic IL-17A responses depend on DCs in experimental BA. *In vitro*, co-culture of RRV-primed DCs and CD4 lymphocytes amplifies production of IL-17A and IL-6. *In vivo*, DC-depletion reduces Th17 polarization and is associated with reduced cholestasis. We show that innate NK and γδ T lymphocytes infiltrate the liver early in the disease course which is potentially linked to pDCs which populate the liver during the first 5 days after viral challenge and provide cytokine support for activation and survival of NK cells (19). Activated CD4+ lymphocytes emerge in the liver during the post-obstructive phase of BA, coinciding with expansion of mDCs. Consequently, disrupting the DC-dependent polarization of Th17 lymphocytes in DT-treated CD11c/GFP/DTR mice or their effector function in IL-17RA/- animals does not prevent ductal obstruction, but attenuates the late BA phenotype, as evidenced by prevention of bile duct paucity and prolongation of survival.

The effects of IL-17A on neonatal bile duct epithelium have not been studied to date. Co-expression of IL-17RA with the BEC marker CK19 was detected on intrahepatic cholangiocytes from neonatal mice. BECs are sensitive to cytolysis induced by pro-inflammatory cytokines and immune mediated injury (20, 21). In our hands, rIL-17A does not kill neonatal BECs *in vitro*, but it induces expression of chemokines, like Ccl2. In experimental BA, elevated plasma concentration of CCL2 at d12pRRV coincides with population of the liver with Ly6C iMΦ. Blockade of the CCL2/CCR2 interaction attenuates infiltration of the liver with iMΦ, and loss of IL17A signaling results in downregulation of hepatic CCL2 expression, reduced recruitment of iMΦ and improved survival. Ly6C^hi iMΦ produce high concentrations of TNFα and other pro-inflammatory cytokines and contribute to toxin-induced liver inflammation and fibrosis.
During acute cholestasis in the bile duct ligation model, TNFα induces apoptosis and inhibits regeneration of BECs (23). This leads to paucity of interlobular bile ducts forming a lumen and to histopathological changes similar to those of dystrophic bile duct profiles we observed in experimental BA. IL-17A not only exerts effects on BECs, but also upregulates expression of Il-6 and Il-23p19 in CD11b+ leukocytes from RRV-infected mice, which may help to maintain Th17 responses in the liver. Studies in carbon tetrachloride-induced liver injury reported IL-17A to stimulate expression of pro-inflammatory cytokines in Kupffer cells and to activate hepatic stellate cells promoting hepatic fibrosis (24).

In validation studies on archived and fresh liver tissues from subjects with BA we show that liver infiltration with conventional CD11c+HLA-DR+CD14-CD123- mDCs is temporarily associated with accumulation of activated CD127\textsuperscript{low} CD4+ and CD8+ T lymphocytes in the liver at the time of diagnosis. The frequency of hepatic pDCs, which are critical for initiation of murine BA (19), is low in infants at diagnosis supporting the notion that infants with BA are diagnosed at the late stage of their disease. By performing short term stimulation and intracellular cytokine staining on hepatic MNCs from explanted livers of patients with progressive disease we ascribe IL-17A production to CD4+CD3+ lymphocytes, while IFN-γ is primarily generated by CD8+ T lymphocytes. Concordant with the murine studies, AE1AE3+ cholangiocytes and CD68+ MΦ express IL-17RA in BA patient at time of diagnosis and the latter persist until LTx, suggesting that both, BECs and MΦ, are cellular targets of IL-17A in neonates. Previously, CCL2 was shown to be expressed by BECs and scar margin hepatocytes in infants with BA and was linked to activation of stellate cells propagating biliary fibrosis (25). Of note, abundance of periductal IL-17A+ cells is associated with periportal infiltration of CD68+ iMΦ and these portal IL17A+ cells persist in the liver of
patient with progressive disease. An increased number of periductal IL-17A+ cells was previously linked to worse surgical outcome of KPE in Europe (26) and elevated plasma levels of IL-17A associated cytokines were determined in an Asian cohort of infants with BA (27). Whether infiltration of the liver with Th17 lymphocytes and CD68+ iMΦ at the time of diagnosis is linked to progressive intrahepatic bile duct injury following KPE in North America will require further investigations.

Collectively, our studies in the murine model and on human tissue samples suggest that CD11c+ DC-dependent polarization of CD4+ T cells into Th17 cells is a key immunologic event during the post-obstructive phase of BA. It governs CCL2 production by BECs and recruitment of CCR2+ iMΦ to the intrahepatic bile ducts. Disruption of DC-dependent Th17 polarization of CD4+ lymphocytes, of IL-17A signaling or of the CCR2/CCL2 pathway decreases hepatic recruitment of iMΦ, prevents progressive destruction of interlobular bile ducts, alleviates cholestasis and prolongs survival in experimental BA. Therefore, we have identified DCs, Th17 cells and the CCL2/CCR2 axis as potential targets for adjuvant therapy in patients with BA.
References


Author names in bold designate shared co-first authorship.
Figure legends

Figure 1: Th17 lymphocytes infiltrate the liver during the post-obstructive phase of experimental BA.

Blood, liver and spleen were collected on d4, d8 and d12 after NS/RRV challenge. (A) Frequencies of NK (CD49b+CD3-), NKT (CD49b+CD3+), TCRγδ (TCRγδ+TCRβ-CD3+), CD4 (CD4+CD8-CD3+CD49b-) and CD8 (CD8+CD4-CD3+CD49b-) cells in hepatic 7AAD- lymphocytes were determined by flow cytometry. (B) Frequencies of CD69+ and NKG2D+ cells in hepatic CD4 and CD8 7AAD- MNCs. (C) Plasma concentrations of candidate cytokines were measured by Luminex and ELISA. (D) IL-17A concentrations were measured in the supernatants of at least two independent cultures of hepatic and splenic MNCs from d12pNS/RRV mice restimulated with 10 μg/ml anti-CD3 for 3 days. (E) Representative flow cytometric plots of GFP+(IL-17A+)CD3+ in hepatic CD45+7AAD- MNCs from d12pNS/RRV IL-17A-GFP reporter mice. Right panels: frequencies of GFP+(IL-17A+)CD3+ in hepatic CD45+7AAD- MNCs and of TCRβ (TCRγδ-TCRβ+), CD4 (CD4+TCRβ+) and TCRγδ (TCRβ-TCRγδ+) cells in hepatic GFP+(IL-17A+)CD3+CD45+7AAD- MNCs. Bar graphs are means + SEM. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 was determined by unpaired t-test. Each dot represents results from an individually treated animal.

Figure 2: Myeloid DCs emerge in the liver during the post-obstructive phase of BA and promote Th17 polarization \textit{in vitro}.

Livers and spleens from CD11c+/GFP/DTR transgenic mice were collected on d8 and d12pNS/RRV. (A) Representative flow cytometric plots for the characterization of DCs (GFP+MHCI+CD3-B220-) and mDCs (CD11b+CD8-GFP+MHCI+CD3-B220-) in hepatic 7AAD- MNCs from neonatal mice at d12pNS/RRV. (B) Left panels: frequencies of DCs in MNCs, and of CD11b+ cells in 7AAD- DCs. Right panels: numbers of
DCs and mDCs per 100 mg of tissues at d12pNS/RRV. (C) IL-17A and IL-6 concentrations were measured by ELISA in the supernatants of 4 independent cultures of hepatic CD11c+ DCs from d12pRRV mice, splenic CD4+ cells from non-infected neonatal mice, and co-culture of CD4:DCs at a 2:1 ratio in the presence of anti-CD3. Bar graphs are means + SEM with * p<0.05, ** p<0.01, and *** p<0.001 and **** p<0.0001 as determined by unpaired t-test.

Figure 3: Depletion of CD11c+ DCs diminishes Th17 responses and intrahepatic bile duct injury in experimental BA.

CD11c+/GFP/DTR neonatal mice were treated with diphtheria toxin (DT) or water as vehicle (veh.) between d3 and 12pRRV. Blood, livers and spleens were collected on d12pRRV. (A) Representative photomicrographs of GFP(CD11c) IHC on liver sections. Arrows denote interlobular bile ducts. Bar = 100 µm if not otherwise indicated. (B) Frequency of DCs (CD11c\textsuperscript{hi}GFP+MHCII+B220-) in splenic 7AAD- MNCs. (C) Left panel: frequency of activated CD4 (CD69+CD4+CD3+) cells in the lymphocyte population. Middle panel: hepatic \textit{Il-17a} mRNA expression quantitated by SYBR green qPCR. Right panel: IL-17A concentration in supernatants from at least four independent cultures of hepatic and splenic MNCs restimulated \textit{in vitro} with 10 µg/ml anti-CD3 for 3 days. (D) Plasma total bilirubin concentrations measured by colorimetric assay. (E) Left panels: representative photomicrographs of CK19 IHC on liver sections. Arrows denote clusters of CK19+ cells without a lumen (dystrophic bile ducts [BD]) in veh.-treated mice and those with preserved lumen in DT-treated mice. Bar = 50 µm. Right panel: frequency of mice/group with less than 50% of portal triads [PT] containing patent interlobular BD defining BD paucity (n pups/group≥16) in gray, and those without paucity in black. Bar graphs are means + SEM. * p<0.05 and ** p<0.01 was determined by unpaired t-test (B, C and D) or by Fisher’s exact test (E) between groups of veh.- and DT-treated mice.
Figure 4: IL-17A controls recruitment of inflammatory macrophages to the liver via CCL2/CCR2 pathway.

(A) Loss of IL17A signaling diminishes hepatic CCL2/CCR2 expression. Neonatal IL-17RA+/+ and -/- mice were challenged with RRV postnatally, livers were harvested on d10pRRV, and hepatic expression of 88 genes encoding cytokines and chemokines was quantitated using Taqman low-density arrays. Heatmap, representing the hierarchical clustering of Hprt-normalized mRNA expression, depicts the genes with p<0.2 for FC difference between the groups. FC and * p<0.05 was indicated. Expression of mRNA encoding Ccr2 and Ccl2 was validated by SYBR green qPCR (n pups/group>3) (B) The CCL2/CCR2 axis controls recruitment of inflammatory MΦ to the liver in experimental BA. Plasma CCL2 concentrations from mice at d12pNS/RRV were measured by ELISA. Kinetic of emergence of iMΦ (Ly6C\text{hi} CD11b+F4/80+Gr1\text{-int} CD3-CD19-) in the liver was enumerated by flow cytometry. Representative plot for the expression of Ly6C and CCR2 in hepatic MΦ (staining CD11b+F4/80+Ly6G-CD3-CD19-CD45+7AAD-). Frequencies of T and B lymphocytes (CD3+CD19+), neutrophils (Ly6G+CD3-CD19-), Ly6C\text{-int} monocytes (CD11b+F4/80-Ly6G-CD3-CD19-), Ly6C\text{hi} monocytes, Ly6C\text{-int} monocyte-derived macrophages (MΦ, CD11b+F4/80+ Ly6G-CD3-CD19-), and Ly6C\text{hi} iMΦ within the CCR2+ compartment of immune cells (7AAD- CD45+ MNCs) populating the liver at d10pRRV. (C) Neonatal mice were treated with the CCR2 antagonist RS504393 at d8/9pRRV. Livers were collected at d10pRRV. The numbers of CCR2+CD45+7AAD- cells and of Ly6C\text{hi}CD11b+F4/80+Ly6G-CD3-CD19-CD45+7AAD- (iMΦ) per 100 mg of liver were determined by cytometry. (D-F) Loss of IL17A signaling reduces recruitment of iMΦ and prolongs survival. Representative flow cytometric plots for the characterization of Ly6C\text{hi}CD11b+F4/80+Gr1\text{-int} CD3-CD19- iMΦ in hepatic CD45+ MNCs. Right panel: frequency of iMΦ in hepatic CD45+ MNCs. (E) Representative photomicrographs of IHC for F4/80 on liver sections. Arrows
denote interlobular bile ducts. Bar = 50 µm. (F) Neonatal IL-17RA+/+ and -/- mice injected with RRV were monitored for 3 weeks and survival between the groups was compared applying the Log-rank (Mantel-Cox) test (n pups/group>22; *p<0.05). Bar graphs are means + SEM and unpaired t-test was applied to test for significance with *p<0.05, **p<0.01 and ****p<0.0001.

Figure 5: Cholangiocytes express IL-17RA and respond to IL-17A with production of chemokines

For (A) and (B), primary intrahepatic cholangiocytes were isolated from 2-day-old wild type BALB/c mice and passed in culture. (A) Representative photomicrographs of IF against IL-17RA and CK19 on primary cholangiocytes. Bar = 30µm. (B) mRNA expression for candidate genes in cholangiocytes after 24 h treatment with various doses of rIL-17A compared to not treated cells (n=2 independent experiments/dose). Bar graphs are means + SEM. ** p <0.01, *** p<0.001 and **** p<0.0001 was determined by linear regression between mRNA expression and amount of rIL-17A in vitro. (C) Representative photomicrographs of IF against IL-17RA and the epithelial marker AE1/AE3 on cryosections from livers from a neonate without cholestatic liver disease and from a subject with BA at the time of diagnosis. Arrows denote interlobular bile ducts. Bar = 50 µm.

Figure 6: Livers are infiltrated with activated T lymphocytes and CD11c+ dendritic cells in subjects with BA at the time of diagnosis.

Blood and fresh liver samples were collected from BA patients at diagnosis. (A) Representative flow cytometric plots for the characterization of lymphocyte populations in hepatic CD45+ MNCs. (B) Frequencies of NK (CD56+CD3-), CD4 (CD4+CD3+), CD8 (CD8+CD3+) and NKT (CD56+CD3+) in hepatic CD45+ lymphocytes. (C) Frequencies of activated CD127-CD25- cells in CD4+ (CD4+CD3+) and CD8+
(CD8+CD3+) CD45+ T cells in hepatic MNCs and blood. (D) Representative flow cytometric plots for the characterization of pDCs (CD123+CD11c-HLA-DR+CD14-CD3-CD19-) and mDCs (CD11c+CD123-HLA-DR+CD14-CD3-CD19-) in hepatic 7AAD- MNCs. (E) Frequencies of pDCs (CD123+CD11c-) and mDCs (CD11c+CD123-) in hepatic HLA-DR+CD14-CD3-CD19-CD45+7AAD- MNCs (n=4 patients). (F) Representative photomicrographs of IHC against CD11c on sections from the liver of a patient with BA at diagnosis and from lymph node as positive control. Arrow denotes an interlobular bile duct. Bar = 50 µm. Bar graphs are means + SEM. *p<0.05, ***p<0.001 and ****p<0.0001 was determined by paired t-tests.

Figure 7: IL-17A is linked with liver infiltration of CD68+ macrophages and is produced by hepatic CD4+ lymphocytes in patients with progressive disease.

(A) Left panels: representative photomicrographs of IL-17A IHC on liver sections from a neonate without cholestasis (control) and from a BA patient at diagnosis. Arrows denote interlobular bile ducts. Bar = 100 µm. Right panel: average number of IL-17A+ cells in PT enumerated by automated image analysis of digitalized slides. (B) Left panels: representative photomicrographs of IL-17A and CD68 IHC on livers from two BA patients at diagnosis, stratified by abundance of CD68+ cells. Arrows denote interlobular bile ducts. Bar = 100 µm. Right panel: average number of IL-17A+ cells in portal tracts in the two groups of BA patients. (C) Representative photomicrographs of IL-17A IHC on liver sections from a patient with Alagille Syndrome and from subjects with BA at the time of LTx. Bar = 100 µm. (D) Left panels: representative flow cytometric plots for the characterization of IL-17A+ and IFN-γ+ cells in hepatic CD45+ MNCs and for enumeration of CD4+CD3+ and CD8+CD3+ T cells in IL-17A+ and IFN-γ+ cells, respectively, from a BA patient at LTx. Right panels: frequencies of NK (CD56+CD3-), CD4 (CD4+CD8-CD3+CD56-), CD8 (CD8+CD4-CD3+CD56-), NKT (CD3+CD56+) and TCRγδ+ (TCRγδ+CD3+) cells in IL-17A+
(IFN-γ-IL-17A+) and IFN-γ+ (IFN-γ+IL-17A-) hepatic CD45+ MNCs from BA subjects at LTx. (E) Left panels: representative flow cytometric plots of activated CD127-CD25- cells in CD4+CD3+ and CD8+CD3+CD45+ lymphocytes in blood from a BA patient at LTx. Right panel: frequencies of circulating activated CD25-CD127- cells in CD4+ (CD4+CD3+CD45+) and in CD8+ (CD8+CD3+CD45+) cells in healthy control [HC] and BA patients at LTx. Bar graphs are means + SEM. **p<0.01 was determined by unpaired t-test.

Figure 8: Proposed mechanisms by which the DC-Th17-CCL2/CCR2 axis perpetuates intrahepatic bile duct injury in BA

Dendritic cells, following priming by RRV or other damage signals, produce IL-6 and promote differentiation of naïve CD4+ into Th17 lymphocytes. Their IL-17A induces production of CCL2-like chemokines by cholangiocytes attracting inflammatory Ly6C+CCR2+ macrophages perpetuating destruction of intrahepatic BECs.
Acknowledgements

Luminex experiments were performed by the Research Flow Cytometry Core. IHC for human CD11c and CD68 performed by the division of pathology at CCHMC. IL-17A IHC quantification was performed at the CCHMC Imaging Core. We would also like to thank Dr. Shivakumar for help with design of experiments, and providing the rhesus rotavirus used in this study as well as Tiffany Shi for technical assistance with the IF. We thank Drs. James Heubi and William Balistreri for carefully reviewing the manuscript. We express our gratitude to families and patients for participating in the translational studies, the clinical research and transplant coordinators for recruiting study subjects into the IRB approved studies, and the pediatric surgeons and pathology staff at CCHMC for facilitating rapid collection of fresh liver tissue specimens.
Figure 1

209x188mm (300 x 300 DPI)
Figure 2

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Figure 7

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Figure 8

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Supplemental Data:

The dendritic cell-Th17-macrophage axis controls cholangiocyte injury and disease progression in murine and human biliary atresia

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Material and Methods

Murine model of BA. For DC depletion, neonatal CD11c/GFP/DTR mice were injected intraperitoneally with 0.01 ng/g of body weight of diphtheria toxin (DT) (Sigma Aldrich; St. Louis, MO) resuspended in water [vehicle] every other day between d3 and d12pRRV. In preliminary studies in adult mice it was found that administration of the conventional doses of 0.5 or 5 ng/g body weight of DT reduced the number of splenic GFP+(CD11c) by more than 50% without affecting the proportion of PDCA-1+ plasmacytoid DCs (pDCs) and was well tolerated (Supplement Figure 2A). However, injection of 5 ng/g of DT into neonatal mice caused hepatocellular injury within 14 h as shown by rise in plasma alanine aminotransferase levels (ALT; Supplement Figure 2B). The lethality for this dose was 100% within 48 hours, a phenomenon ascribed to “leakiness” of DTR expression and general toxicity of DT in newborn
mice (1). Dose finding studies showed that administration of 0.01 ng/g of DT in RRV-infected neonatal mice did not increase mortality or plasma ALT levels (Supplement Figure 2C) compared with vehicle treated mice. Therefore, this attenuated dose was used for CD11c+ depletion studies in experimental BA.

For CCR2 blockade, neonatal mice were injected intraperitoneally with 2 μg/g of body weight of the antagonist RS504393 (Tocris Bioscience; Avonmouth, UK) re-suspended in DMSO (vehicle) at day 8 and day 9 after RRV infection and harvested the next day. Each dot in the murine studies included in Fig 1-4 represents an individually treated animal.

Cell Isolation. For murine MNC isolation, mice were anesthetized, the portal vein was cannulated and livers were perfused with 10 ml of 1 mg/ml of collagenase D (Roche Diagnostics) in RPMI (Mediatech Inc) before harvest. Livers and spleens were gently minced and digested with 1 mg/ml of collagenase D in RPMI at 37°C for 30 min prior to passage through a 70 μm strainer (Fisher Scientific), centrifugation in 33% Percoll (Sigma-Aldrich) and lysis of red blood cells. CD4+ T cells, CD11c+ DCs and CD11b+ myeloid cells were isolated from MNCs following the manufacturers’ instructions for Dynabeads® FlowComp™ Mouse CD4 (Invitrogen), CD11c and CD11b MicroBeads (Miltenyi Biotec), respectively.

Primary cholangiocytes were harvested from 2-day-old BALB/c pups as described previously (2). Briefly, livers were homogenized, digested in DMEM/F12 containing collagenase, hyaluronidase and DNase I (Sigma-Aldrich), passed through a 40 μm strainer and purified through a Percoll gradient (GE Healthcare). Isolated cells were resuspended in PBS with 0.1% BSA (Gibco) and incubated with an Ep-CAM antibody (Developmental Studies Hybridoma Bank at the University of Iowa) at 4°C for 2 h followed
by 1 h incubation with sheep anti-rat Dynabead IgG antibodies (Invitrogen). The magnetically purified
dynabead-coated cells were plated on collagen-coated culture flasks and incubated at 37°C in 5% CO₂
until 75% confluency. During passaging, cells were regularly assayed for the cholangiocyte markers CK7
and CK19.

MNCs from fragments of excess liver tissue from study subjects with BA were isolated by repeated
injection of 500 µg/ml collagenase D solution (in 250 mg/ml DNaseI, 2% FCS, 0.6% BSA), incubation at
37°C for 30 min, mechanical dissociation using the Medimachine (BD), passage through a 70 µm
FilconCup (BD) and a 70 µm strainer, centrifugation in 33% Percoll (Sigma-Aldrich) and lysis of red blood
cells.

**In vitro Cultures.** MNCs, CD4+ T cells, CD11c+ DCs, CD11b+ myeloid cells and primary cholangiocytes
were cultured in RPMI 1640 supplemented with 10% of decomplemented FCS (Life Technologies). When
indicated, MNCs were stimulated with 10 µg/ml of anti-CD3 (145-2C11; eBioscience) and the
supernatant was collected after 72 h for further analysis. CD4+ cells, DCs (CD11c+ cells), and their
cocultures in 2:1 ratio were stimulated with 50 ng of immobilized anti-CD3 and the supernatant was
collected after 96 h culture for further analysis. Primary cholangiocytes were stimulated for 24 h with
rIL-17A (Peprotech) in concentrations ranging between 1.25 ng/ml and 100 ng/ml or the supernatant
from anti-CD3-stimulated hepatic MNCs from RRV infected mice serving as control for cytokine-induced
cytolysis. CD11b+ cells were stimulated for 6 h with 10 ng/ml of rIL-17A.
**In vitro Cellular Viability and Proliferation Assay.** Cell proliferation was assayed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Primary cholangiocytes were seeded in quintuplet into 96-well plate at a density of 20,000 cells per well coated with 10 μg of collagen I from rat tail (Corning) and cultured for 48 h. Each well was stained with 40 μg of MTS solution for 4 h. The optical density (OD) was determined at 490 nm for cellular viability and at 700 nm for reference wavelength to eliminate background using a BioTek Synergy H1 Plate Reader.

**Flow Cytometry.** Single cell suspensions were stained with antibodies against surface molecules. For murine cells, CD3ε (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (6D5), CD45 (30-F11), CD49b (DX5), CD69 (H1.2F3), B220 (RA3-6B2), CCR2 (SA203G11), F4/80 (BM8), Gr1 (RB6-8C5), Ly6C (AL-21), Ly6G (1A8), MHCII (M5/114.15.2), NKG2D (CX5), PDCA-1 (JF05-1C2.4.1), TCRβ (H57-597) and TCRγδ (GL3) were purchased from BioLegend, eBioscience, BD Biosciences or Miltenyi Biotec. For human cells, CD3 (UCHT1, SK3 and OKT3), CD4 (RPA-T4), CD8 (RPAT8), CD11c (S-HCL3), CD14 (M5E2), CD19 (H1B19), CD25 (BC96), CD45 (HI30 and 5B1), CD56 (HCD56), CD123 (6H6), CD127 (eBioRDR5), HLA-DR (L243) and TCRγδ (B1) were purchased from BioLegend, eBioscience or BD Biosciences. 7-AAD (BD Pharmingen) was used to exclude dead cells. For intracellular cytokine staining of human MNCs, cells were stimulated for 5 h with 50 ng/ml PMA (Sigma-Aldrich) and 700 ng/ml ionomycin (Calbiochem) in presence of GolgiStop and GolgiPlug for the last 4 h (BD Biosciences). After surface staining, cells were fixed and permeabilized as manufacturer’s instructions (BD Biosciences), then stained with antibodies against IFN-γ (B27; BD Biosciences) and IL-17A (eBio64DEC17; eBioscience). Cell counts were acquired on a BD Canto (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC).
Quantitative Real-Time RT-PCR. Total RNA was isolated from frozen liver tissue and from primary cholangiocytes using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA from CD11b+ myeloid cells was isolated using the Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems) as recommended by the manufacturer. First strand cDNA was synthetized using SuperScript II reverse transcriptase (Invitrogen) and oligodT 12-18 primer (Life Technology). Quantitative hepatic gene expression studies on cDNA samples from livers of IL-17RA+/+ and IL-17RA−/− mice (d10pRRV, n=4 per group) was performed using TaqMan Mouse Immune Array (Applied Biosystems) with genes encoding 88 cytokines and chemokines and 8 house-keeping genes. 0.1 μg cDNA from total hepatic RNA was loaded into the array's 384 wells and was run on Applied Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer's instruction. Real-time RT-PCR were performed using the SYBR Green PCR Master Mix (Invitrogen) on a Mx3000P (Stratagene). Gene-specific primers were synthesized by eurofins MWG operon with the following sequences: Hprt (for: 5'-TGC CGA GGA TTT GGA AAA AG-3', rev: 5'-CCC CCC TTG AGC ACA CAG-3'); Il-17a (for: 5'-AGC TTT CCC GCA TTG ACA CAG-3', rev: 5'-CTC CAG AAG GCC CTC AGA CTA C-3'); Il-6 (for: 5'-AAG AAA TGA TGG ATG CTA CC-3', rev: 5'-GAG TTT CTG TAT CTC TCT GAA G-3'); Cxcl1 (for: 5'-CAA ACC GAA GTC ATA GCC ACA-3', rev: 5'-TTG GGG ACA CCT TTT AGC ATC-3'); Cxcl2 (for: 5'-TGA ACA AAG GCA AGG CTA ACT G-3', rev: 5'-AAG TGA ACT CTC AGA CAG CGA GG-3'); Cxcl5 (for: 5'-TGG GCA GTG ACA AAA AGA AAG-3', rev: 5'-AAA TCC GTG GGT GGA GAG AAT C-3'); Ccl2 (for: 5'-GTC TGT GCT GAC CCC AAG AA-3', rev: 5'-GTG CTG AAG ACC TTA GGG CA-3'); Ccr2 (for: 5'-TGA GCC TGA TCC TGC CTC TA-3', rev: 5'-AAA GAT GAG CCT CAC AGC CC-3'); Il-23p19 (for: 5'-AAT GTG CCC GTG ATC GAC TGG TTT GCT ATC TCA-3', rev: 5'-GAA GAT GTC AGA GTC AAG CAG GTG-3'); Tnfα (for: 5'-ACC GTC AGC CGA TTT GCT ATC TCA-3', rev: 5'-TGG ACA TGC GCT GAT CCT CCA GTG AAT-3'). The ΔCt values in specific samples were calculated using Ct for the gene of interest - Ct from the housekeeping gene Hprt and the
\[ \Delta \Delta Ct \] value using the \( \Delta Ct \) mean of the reference control group - the \( \Delta Ct \) of the sample. The fold change was calculated as the log 2 of the \( \Delta \Delta Ct \).

**IHC.** IHC was performed on 5 \( \mu \)m paraffin-embedded liver sections. Slides were dewaxed in an oven at 55°C overnight prior to clearing paraffin with histoclear and rehydrating sections with serial washes of decreasing ethanol concentration before antigen retrieval. Endogenous peroxidases was quenched with 3% hydrogen peroxide. 5% normal serum was used for protein blocking prior to avidin/biotin block (Vector laboratories). Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Incubation with secondary antibody occurred for 1 to 2 h at room temperature (RT).

Vectastain *Elite* ABC reagent (Vector laboratories) was applied for 30 min followed by the DAB reagent (Vector laboratories) for detection. Slides were counterstained with hematoxylin and cover-slipped with Permount®.

For GFP IHC, zinc-fixed (BD Pharmingen) murine livers were incubated in 1M Na Citrate for antigen retrieval in a pressure cooker for 15 min, anti-GFP primary antibody was diluted 1:250 (polyclonal antibody; Novus Biologicals; Littleton, CO) and goat anti-rabbit-biotin secondary antibody was diluted 1:500 (Jackson ImmunoResearch). For CK19 IHC, formalin-fixed murine livers were incubated in Tris pH 9.0 buffer for antigen retrieval, anti-CK19 primary antibody was diluted 1:200 (TROMAIII; uiowa.edu) and goat anti-rat-biotin secondary antibody was diluted 1:750 (Jackson ImmunoResearch). Collaborating pathologist (K.S.) who was blinded to the group assignment analyzed CK19+ interlobular bile ducts in DT- and veh.-treated mice after RRV infection for patency. For F4/80 IHC, formalin-fixed murine livers were incubated with proteinase K (DAKO) for 5 min at RT for antigen retrieval, anti-F4/80 primary antibody
was diluted 1:500 (MCA497GA; AbD Serotec) and goat anti-rat-biotin secondary antibody was diluted 1:1000 (Jackson ImmunoResearch).

For CD68 and CD11c IHC, formalin-fixed human livers were incubated and run on a BenchMark ULTRA IHC/ISH Staining module using respectively ultraViewDAB (Roche; cat# 760-091) and iViewDAB (Roche; cat# 760-500). EDTA was used as antigen retrieval, anti-CD68 was undiluted (KP1; Roche) and anti-CD11c was diluted 1:200 (EP1347Y; Novus Biologicals). For IL-17A IHC, formalin-fixed human livers were incubated in 1M Na citrate at pH 9.0 for antigen retrieval and heated three times at 130 watt for 3 min in microwave. Anti-IL-17A primary antibody was diluted 1:25 (polyclonal antibody; R&D Systems) and donkey anti-goat-biotin secondary antibody at 1:250 (Jackson ImmunoResearch).

**Immunofluorescence (IF).** IL-17RA and CK19 IF was performed on primary murine cholangiocytes cytopspined on glass slides. Cells were fixed in 4% PFA and blocked with 5% normal goat serum. Primary antibodies were diluted in blocking solution at 1:100 for CK19 (TROMAIII; uiowa.edu) and for IL-17RA (polyclonal antibody; Abcam) and incubated overnight at 4°C. Donkey anti-rat-AF594 and donkey anti-rabbit-AF488 secondary antibodies (both from Jackson ImmunoResearch) for detection of CK19 and IL-17RA, respectively, were diluted at 1:250 and incubated for 1.5 h at RT. Slides were mounted using permount/DAPI and visualized under a Nikon Eclipse Ti microscope.

IL-17RA, AE1/AE3/PCK26 or CD68 IF were performed on 5 µm cryosections of frozen human liver. Sections were fixed in ice-cold 100% acetone and blocked with 10% donkey serum in PBS/2%BSA/0.1%
milk solution. Primary antibodies were diluted in blocking solution at 1:50 for IL-17RA (polyclonal antibody; Abcam) and AE1/AE3/PCK26 (Pan Keratin, Ventana) and at 1:100 for CD68 (PG-M1; DAKO) and incubated at 4°C overnight. Goat anti-rabbit-AF647 and goat anti-mouse-AF488 (both from Jackson ImmunoResearch) for detection of IL-17RA and AE1/AE3/PCK26 or CD68, respectively, were diluted at 1:500 and 1:300 in PBS/1% BSA and incubated for 2 h at RT. Slides were mounted using a prolong gold anti-fade reagent with DAPI (Life Technologies) and imaged on a Nikon 90i upright wide-field microscope equipped for epifluorescence.

**Cytokine Detection.** Mouse plasma was collected by centrifugation of heparinized blood at 2,300 rpm for 12 min. IFN-γ, IL-13 and IL-17A concentrations on indicated plasma from d4/8/12pNS/RRV in BALB/c pups were determined using Milliplex™ kits (Millipore) according to manufacturer's protocol. Briefly, in a 96-well black plate, 25 μl of sample in duplicate was incubated with 25 μl antibody-coated beads at 4°C overnight. 25 μl of secondary antibody was added and incubated at RT for 1 h, followed by addition of 25 μl of streptavidin-RPE and incubation for 30 min at RT. Plates were then washed and read using luminex technology on the Bio-Plex™ (Bio-Rad). Other concentrations of IL-17A, IL-6 and CCL2 were determined by ELISA according to manufacturer’s instructions (R&D Systems and Biolegend) on plasma or supernatants of indicated cell culture assays. Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml.

**Biochemistry Assay.** Plasma total bilirubin concentrations were measured with Total Bilirubin Reagent Set (Pointe Scientific Inc., Canton, MI) and plasma alanine aminotransferase (ALT) levels with DiscretPak.
reagents (Catachem, Bridgeport, CT) according to the manufacturers' instructions. Photometric absorbance was read on a Synergy H1 Hybrid Reader (BioTek, Winooski, VT) at 555 nm.

**Supplemental Table 1:**

Demographics, short term outcome (Serum Bilirubin levels) and long term outcome (survival with native liver) for subjects with BA enrolled into the IHC study examining the expression patterns of CD68 and IL17A

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<th>Direct Bilirubin at 6 mo in mg/dL</th>
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**Supplemental Figure Legends:**

**Supplemental Figure 1:** Kinetic of T lymphocyte responses in the secondary lymphoid organ spleen following neonatal RRV challenge. Spleens were collected on d4, d8 and d12 after NS/RRV challenge.
(A) Frequencies of NK (CD49b+CD3-), NKT (CD49b+CD3+), TCRγδ (TCRγδ+TCRβ-CD3+), CD4 (CD4+CD3+CD49b-) and CD8 (CD8+CD4-CD3+CD49b-) cells in splenic 7AAD- lymphocytes. (B) Frequencies of CD69+ and NKG2D+ cells in CD4+ and CD8+ splenic 7AAD- MNCs. (C) Frequency of GFP+(IL-17A+)CD3+ in splenic CD45+7AAD- MNCs and of TCRβ (TCRγδ-TCRβ+), CD4 (CD4+TCRβ+) and TCRγδ (TCRβ-TCRγδ+) in splenic GFP+(IL-17A+)CD3+CD45+7AAD- MNCs from IL-17A-GFP reporter mice. Bar graphs are means + SEM. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 was determined by unpaired t-test.

Supplemental Figure 2: Hepatotoxicity of diphtheria toxin in neonatal CD11c/GFP/DTR mice.

CD11c+/GFP/DTR mice were treated with diphtheria toxin (DT) or water [vehicle]. Blood or spleens were collected 14 h or 48 h later. (A) Frequencies of GFP+(CD11c+)PDCA-1- DCs and GFP-PDCA-1+ pDCs in CD3-7AAD- MNCs were enumerated by flow cytometry 48 hours after treatment with DT at 5 ng/g of body weight in 6-week-old mice. (B) Plasma ALT concentration in 6-day-old, non-infected mice 14 hours after treatment with a single dose of DT at 5 ng/g of body weight or vehicle. (C) Plasma ALT concentration in 5-day-old neonatal mice that were challenged with RRV within 24 hours of life and received a single dose of DT ranging between 0 to 0.1 ng/g of body weight on DOL4. Bar graphs are means + SEM. Statistical significance was determined by unpaired t-test (NS: non significant).

Supplemental Figure 3: Recombinant IL-17A does not exert cytolytic effects on intrahepatic neonatal cholangiocytes. Intrahepatic cholangiocytes purified from 2-day-old mice were assessed for viability using a MTS colorimetric assay. Primary cholangiocyte viability 48 h after culture on collagen with various doses of rIL-17A (0, 1.25, 2.5, 5 and 10 ng/ml) (n=4 read/condition). As control served the supernatant of d12pRRV hepatic MNCs restimulated in vitro with 10 μg/ml anti-CD3 for 3 days (sup.
MNCs; red). This supernatant contains high concentrations of IFN-γ (>1.4 ng/ml) which was previously shown to reduce cholangiocyte survival. Bar graph are means + SEM.

Supplemental Figure 4: IL-17A induces the production of cytokines in myeloid inflammatory cells. For (A) and (B), hepatic and splenic MNCs were obtained from neonatal mice at d8pRRV and subjected to bead-separation of CD11b+ cells. (A) Left panel: frequencies of CD11b+ cells in CD45+7AAD- MNCs (black) and in CD11b+ enriched cells (green) from liver (circle) and spleen (square) of pups, demonstrating the purity of the cell separation. . Right panels: proportions of Gr1hiCD11b+ neutrophils (neutroφ, black), CD11b+F4/80-Gri/ Inhibitor monocytes (mono, white), Ly6CinhCD11b+F4/80+Gr1/ Inhibitor inflammatory macrophages (iMΦ, red), Ly6C inh CD11b+F4/80+Gr1/ Inhibitor macrophages (other MΦ, blue) and other subsets (other, gray) in CD11b+ cells. (B) Fold change of relative mRNA expression for Il-6, Il-23p19 and Tnf in CD11b+ cells isolated from liver and spleen of d8pRRV pups and cultures for 6 h with 10 ng/ml of rIL-17A (black bar) or vehicle (white bar). Expression data were normalized on Hprt. Bar graphs are means + SEM. *** p value <0.001 and **** p<0.0001 was determined by unpaired t-test. (C) Representative photomicrographs of dual IF for IL-17RA and CD68 and counterstain with DAPI on cryosections from livers from a BA patient at diagnosis and at LTx. Arrows denote cells coexpressing IL-17RA and CD68. Bar = 100 μm.

References

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