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

Celine S Lages¹, Julia Simmons¹, Avery Maddox¹, Keaton Jones², Rebekah Karns¹, Rachel Sheridan³, Shiva Kumar Shanmukhappa³, Sujit Mohanty⁴, Matthew Kofron⁵, Pierre Russo⁶, Yui-Hsi Wang⁷, Claire Chougnet⁸, and Alexander G Miethke¹

¹Division of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

²University of Cincinnati, College of Medicine, Internal Medicine Residency Program, Cincinnati, Ohio, USA

³Division of Pathology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

⁴Division of Pediatric Surgery, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

⁵Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

⁶Division of Pathology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA

⁷Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

⁸Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

Abstract

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

Corresponding author: Alexander G Miethke, MD, Mail location 2010, 3333 Burnet Avenue, Cincinnati, Ohio, 45229-3026, Tel: 1-513-636-9078, Fax: 1-513-636-7805, Alexander.Miethke@cchmc.org.

Celine S Lages: Celine.Silva-Lages@cchmc.org

Julia Simmons: julierpratt@yahoo.com

Avery Maddox: Avery.Maddox@cchmc.org

Keaton Jones: keatjone@iu.edu

Rebekah Karns: Rebekah.Karns@cchmc.org

Rachel Sheridan: Rachel.Sheridan@cchmc.org

Shiva Kumar Shanmukhappa: shiva.shanmukhappa@novartis.com

Sujit Mohanty: Sujit.Mohanty@cchmc.org

Matthew Kofron: Matthew.Kofron@cchmc.org

Pierre Russo: RUSSO@email.chop.edu

Yui-Hsi Wang: Yui_Hsi.Wang@cchmc.org

Claire Chougnet: Claire.Chougnet@cchmc.org

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.

Keywords

interleukin 17A; CCL2; CCR2; bile duct paucity; inflammatory monocyte-derived macrophages

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; age 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 1.5×10^6 focus forming units of RRV in 20 μ l 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 analyses were performed 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 TCR $\gamma\delta$

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 TCR β and CD4 but not TCR $\gamma\delta$, 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⁺MHCII⁺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-1 β 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 (iM Φ ; Ly6C^{hi}F4/80+CD11b+Gr1-) in the liver (**Figure 4B**). The immune cells expressing CCR2 were mainly Ly6C^{hi} iM Φ , 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 iM Φ 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+iM Φ in the liver and accumulation of F4/80+ M Φ 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 M Φ (**Supplemental Figure 4A**). The cells were restimulated *in vitro* with rIL-17A for 6 h, which resulted in increased expression of *Il-6* and *Il-23p19* (**Supplemental Figure 4B**), especially in hepatic CD11b+ cells containing a higher proportion of iM Φ .

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 Figure4C**).

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 $\gamma\delta$ 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 TCR $\alpha\beta$ + lymphocytes, and not $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 iM Φ . Blockade of the CCL2/CCR2 interaction attenuates infiltration of the liver with iM Φ , and loss of IL17A signaling results in downregulation of hepatic CCL2 expression, reduced recruitment of iM Φ and improved survival. Ly6C^{hi} iM Φ produce high concentrations of TNF α and other pro-inflammatory cytokines and contribute to toxin-induced liver inflammation and fibrosis (22). During acute cholestasis in the bile duct ligation model, TNF α 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^{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+ M Φ express IL-17RA in BA patient at time of diagnosis and the latter persist until LTx, suggesting that both, BECs and M Φ , 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+ iM Φ 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+ iM Φ 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+ iM Φ 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 iM Φ , 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BA	Biliary atresia
KPE	Kasai portoenterostomy
LTx	Liver transplantation
BECs	Biliary epithelial cells
RRV	Rhesus rotavirus
pDCs	Plasmacytoid dendritic cells
mDCs	Myeloid dendritic cells
DTR	Diphtheria toxin receptor
IL-17RA	IL-17 receptor A
NS	Normal saline
IHC	Immunohistochemistry
IF	Immunofluorescence
MNCs	Mononuclear cells
rIL-17A	Recombinant IL-17A
d4/8/12pNS/RRV	Day 4/8/12 post NS/RRV injection
DT	Diphtheria toxin
iMΦ	Inflammatory macrophages

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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+), TCR $\gamma\delta$ (TCR $\gamma\delta$ +TCR β -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 μ g/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 TCR β (TCR $\gamma\delta$ -TCR β +), CD4 (CD4+TCR β +) and TCR $\gamma\delta$ (TCR β -TCR $\gamma\delta$ +) 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 *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+MHCII+CD3-B220-) and mDCs (CD11b+CD8-GFP+MHCII+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 μ m if not otherwise indicated. **(B)** Frequency of DCs (CD11c^{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 *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 *in vitro* with 10 μ g/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 μ m. 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^{hi}CD11b+F4/80+Gr1^{-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^{-int} monocytes (CD11b+F4/80-Ly6G-CD3-CD19-), Ly6C^{hi} monocytes, Ly6C^{-int} monocyte-derived macrophages (MΦ, CD11b+F4/80+ Ly6G-CD3-CD19-), and Ly6C^{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^{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^{hi}CD11b+F4/80+Gr1^{-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 μ m. 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 μ m. 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 μ m. (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 TCR γ δ + (TCR γ δ +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.