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The etiology and pathogenesis of bile duct obstruction in children with biliary atresia are largely unknown. We have previously reported that, despite phenotypic heterogeneity, genomic signatures of livers from patients display a proinflammatory phenotype. Here, we address the hypothesis that production of IFN-γ is a key pathogenic mechanism of disease using a mouse model of rotavirus-induced biliary atresia. We found that rotavirus infection of neonatal mice has a unique tropism to bile duct cells, and it triggers a hepatobiliary inflammation by IFN-γ-producing CD4+ and CD8+ lymphocytes. The inflammation is tissue specific, resulting in progressive jaundice, growth failure, and greater than 90% mortality due to obstruction of extrahepatic bile ducts. In this model, the genetic loss of IFN-γ did not alter the onset of jaundice, but it remarkably suppressed the tissue-specific targeting of T lymphocytes and completely prevented the inflammatory and fibrosing obstruction of extrahepatic bile ducts. As a consequence, jaundice resolved, and long-term survival improved to greater than 80%. Notably, administration of recombinant IFN-γ led to recurrence of bile duct obstruction following rotavirus infection of IFN-γ-deficient mice. Thus, IFN-γ-driven obstruction of bile ducts is a key pathogenic mechanism of disease and may constitute a therapeutic target to block disease progression in patients with biliary atresia.

Introduction
Biliary atresia is the most common cause of chronic liver disease in children and the prime indication for pediatric liver transplantation worldwide (1). The disease begins in the first few weeks of life with impaired bile flow due to the progressive obliteration of extrahepatic bile ducts; the onset of jaundice, acholic stools, and hepatosplenomegaly in otherwise healthy looking neonates are hallmarks of the disease. Although surgical creation of a biliary conduit has the potential to improve bile drainage, ongoing cholestasis and cirrhosis are frequent outcomes, at which time liver transplantation is the only hope for long-term survival. The development of nontransplant therapeutic modalities for children with biliary atresia has been hampered by limited knowledge of etiology and presumed multifactorial pathogenesis of disease (1–3). Despite phenotypic heterogeneity, the development of disease in the early postnatal period and the inflammatory injury of extrahepatic bile ducts occur uniformly in all patients and are consistent with a common biological process. Using liver biopsy samples from infants at different stages of disease, we found unique genomic signatures of differential lymphocyte function at the time of diagnosis (4). The signatures displayed the overexpression of IFN-γ and other T lymphocyte–enriched genes, even when inflammatory infiltrates were similar to diseased controls, implying differential activation states of similar cell types.

Direct proof of a cause and effect relationship between a Th1-like, proinflammatory circuit and the hepatobiliary injury has met remarkable experimental challenges as a result of the inability to study human samples prior to or at the time of bile duct obstruction and the obvious ethical barriers to obtain normative data from livers of normal age-matched infants. Therefore, we used a mouse model of biliary atresia to test the hypothesis that IFN-γ plays a key regulatory role in the pathogenesis of bile duct injury and obstruction in biliary atresia. We found that hepatic lymphocytes undergo Th1 commitment at the time of biliary injury and obstruction. More notably, loss of IFN-γ expression completely prevented the inflammatory and fibrosing obstruction of bile ducts, which resulted in resolution of symptoms and improved long-term survival.

Results
Rotavirus infection in the immediate neonatal period results in bile duct obstruction. Infection of WT Balb/c mice with rhesus rotavirus (RRV) in the first 24 hours of life results in biliary obstruction and recapitulates the dramatic phenotype of biliary atresia, with generalized jaundice, acholic stools, and bilirubinuria developing in the neonatal period (5, 6). To identify dominant biological processes in this model, we inoculated 1.5 × 106 fluorescence-forming units (ffu) of RRV intraperitoneally into 25 WT Balb/c mice within 24 hours of birth. Jaundice developed in approximately 80% of mice by 7 days of age, at which time the livers displayed expansion of portal triads by lymphocytic infiltrates and bile duct proliferation; the extrahepatic bile ducts appeared atretic, with lumenal obstruction by inflammatory cells (Figure 1, A–D), which was present in more than 90% of mice with jaundice. Focal stenosis of the common bile duct with variable degrees of proximal or distal cystic dila-
RRV infection induces biliary inflammation and growth failure in neonatal mice. WT Balb/c mice were injected with normal saline (control) or RRV within 24 hours of birth, and the hepatobiliary system was examined 7 days later. (A) While livers of control mice had normal appearance of the portal tracts, RRV challenge resulted in the expansion of portal spaces by inflammatory cells and proliferating bile duct cells. (B). (C) Cross section of the extrahepatic bile duct of a control mouse revealed normal epithelium and unobstructed lumen (arrows). (D) In contrast, injection of RRV produced lumenal obstruction of extrahepatic bile ducts (arrows). Tissue sections were stained with H&E. Magnification of ×400 for A and B, ×200 for C and D. Single asterisks denote neighboring arteries in C and D. (E) It can be seen that RRV injection also led to poor growth during the suckling period. **P < 0.01 when compared with controls at days 7–16; n = 25 mice in the beginning of the experiment. Expression of mRNA encoding RRV nonstructural (NSP3) and structural (VP6) proteins was high at day 7 but (F) undetectable at day 14. **P < 0.01; n = 4–7 mice per group at each time point.

Figure 1

RRV infection induces biliary inflammation and growth failure in neonatal mice. WT Balb/c mice were injected with normal saline (control) or RRV within 24 hours of birth, and the hepatobiliary system was examined 7 days later. (A) While livers of control mice had normal appearance of the portal tracts, RRV challenge resulted in the expansion of portal spaces by inflammatory cells and proliferating bile duct cells. (B). (C) Cross section of the extrahepatic bile duct of a control mouse revealed normal epithelium and unobstructed lumen (arrows). (D) In contrast, injection of RRV produced lumenal obstruction of extrahepatic bile ducts (arrows). Tissue sections were stained with H&E. Magnification of ×400 for A and B, ×200 for C and D. Single asterisks denote neighboring arteries in C and D. (E) It can be seen that RRV injection also led to poor growth during the suckling period. **P < 0.01 when compared with controls at days 7–16; n = 25 mice in the beginning of the experiment. Expression of mRNA encoding RRV nonstructural (NSP3) and structural (VP6) proteins was high at day 7 but (F) undetectable at day 14. **P < 0.01; n = 4–7 mice per group at each time point.

Figure 2

RRV targets the biliary epithelium in neonatal mice. Fluorescence immunostaining shows specific signal for RRV (red, A) and the cholangiocyte cytoskeletal filament CK7 (green, B) in the epithelium of extrahepatic bile ducts 3 days after RRV inoculation, with colocalization of RRV and CK7 (yellow, C).
RRV infection results in Th1 polarization of hepatic lymphocytes. Hepatic cell surface staining by flow cytometry for CD3 (A) and CD19 (B) shows that the lymphocytic infiltrate in portal tracts is predominantly composed of CD3⁺ cells beginning 7 days after RRV inoculation. CD3⁺ cells also showed staining with CD4⁺ (C) or CD8⁺ (D). (E) Functional polarization of T lymphocytes is demonstrated 7 days after RRV challenge by an increase in mRNA expression for IFN-γ and IL-12p40. (F) The mRNA expression for Th2 cytokines also increases above the levels of controls at 7 and 14 days, but at lower levels when compared with Th1 cytokines. *P < 0.05 when the RRV group is compared with controls; n = 4–7 mice per group at each time point. NS, normal saline.

of the Th1 cytokines IFN-γ and IL-12p40, with a high level at 7 days and a return to baseline levels by 14 days (Figure 3E), while the increase in the Th2 cytokines IL-4 and IL-5 was milder and temporally delayed until 14 days for IL-5 (Figure 3F).

To determine the main inflammatory cells producing dominant Th1 cytokines, we used surface and intracellular labeling–based flow-cytometric analysis, and found that IFN-γ expression increased more than 40-fold in CD3⁺CD4⁺ lymphocytes and approximately 18-fold in CD3⁺CD8⁺ lymphocytes at 7 days (Supplemental Figure 1, A and B). The expression of IL-5 by CD3⁺, CD4⁺, and CD8⁺ cells was low in RRV-infected livers (with highest levels of only 2- to 4.5-fold at 7 and 14 days [Supplemental Figure 1, C and D]). These data demonstrated a striking temporospatial infiltration of the hepatobiliary system by T lymphocytes, with polarization to a Th1 phenotype at the onset of symptoms in infected mice.

Loss of IFN-γ prevents bile duct obstruction and promotes long-term survival. IFN-γ is an important effector of the Th1 phenotype and has been implicated in the pathogenesis of autoimmune disorders (8). In this context, the overexpression of IFN-γ in livers of RRV-inoculated mice and of infants with biliary atresia was consistent with a regulatory role of IFN-γ in the inflammatory injury and obstruction of the bile ducts in biliary atresia. To directly test this claim, we inoculated mice carrying the genetic inactivation of the IFN-γ gene (IFN-γ⁻/⁻) and WT Balb/c mice (9) with RRV. RRV infection resulted in persistent cholestasis in approximately 80% of WT Balb/c mice (Figure 4), and fewer than 10% survived beyond 21 days. In contrast, although jaundice, acholic stools, and bilirubinuria developed in 90–100% of IFN-γ⁻/⁻ mice 3–5 days after RRV challenge in a fashion similar to what occurred in WT Balb/c mice, acholic stools resolved in between 8 and 13 days; this was followed by a complete resolution of jaundice and bilirubinuria (Figure 4) and greater than 80% survival beyond 21 days.

The resolution of jaundice in the absence of IFN-γ suggested that the extrahepatic ductal system was not obstructed. To determine the anatomical basis of jaundice clearance, we examined livers and extrahepatic bile ducts of WT Balb/c and IFN-γ⁻/⁻ mice challenged with RRV (n = 15–20 mice in each group). The hilum of WT Balb/c mice showed typically small, contracted gallbladders and atretic bile ducts (Figure 5, A and C); in stark contrast, gallbladders of IFN-γ⁻/⁻ mice contained bile, and bile ducts were unobstructed and maintained lumenal continuity with the duodenum (Figure 5, B and D). Histologically, extrahepatic bile ducts were obstructed by inflammatory cells (at 7 days) and extracellular matrix (at 14 days) in WT Balb/c mice (Figure 5, E and G), while the lumen of ducts of IFN-γ⁻/⁻ mice remained unobstructed (Figure 5, F and H). To further demonstrate the key role of IFN-γ in duct obstruction, we administered recombinant IFN-γ intraperitoneally to 18 IFN-γ⁻/⁻ mice every day after RRV inoculation until 14 days (time of killing) and examined the development of symptoms and obstruction of extrahepatic bile ducts. Restoration of the IFN-γ-sufficient state resulted in the timely development of cholestasis in more than 80% of the mice challenged with RRV, and the extrahepatic bile duct displayed recurrence of the obstruction in a fashion indistinguishable from the duct injury observed in WT Balb/c mice (Figure 6).

Detailed microscopic surveys of livers of both groups revealed lobular and portal inflammation with periductal infiltration by neutrophils at 3 days (Figure 7, A and B). Thereafter, these inflammatory changes were associated with portal expansion due to bile duct proliferation and inflammation at 7 days (Figure 7, C–F), which suggested that portal inflammation was an important influence in the development of cholestasis in infected mice of both genotypes. Taken together, these findings demonstrated that the onset of cholestasis in IFN-γ⁻/⁻ mice resulted from intrahepatic inflammation and cholangitis after RRV challenge. While this inflammatory
Loss of IFN-γ prevents obstruction of extrahepatic bile ducts. Anatomical view of the hilum (A–D) of WT Balb/c mice displayed small, edematous gallbladders (*) at 7 and 14 days after RRV challenge, with long- (7 days) or short- (14 days) segment atresia of extrahepatic bile ducts (thin arrows). In contrast, IFN-γ–/– mice displayed gallbladders distended with bile (**) and unobstructed bile ducts (thick arrows). Arrowheads point to arterial vessels that follow extrahepatic bile ducts. Microscopically (E–H), bile ducts of WT Balb/c mice demonstrated luminal obstruction by inflammatory cells (7 days) and extracellular matrix (14 days). In IFN-γ–/– mice, extrahepatic bile ducts had periductal inflammation and mild epithelial injury, but the lumen remained patent and without accumulation of matrix substrates at 7–14 days. Sections were stained with H&E; magnification, ×200; arrows in (E) and (G) denote obstructed bile ducts.

Discussion

These data demonstrate that IFN-γ plays a pivotal regulatory role in the obstruction of extrahepatic bile ducts in a mouse model of biliary atresia. In this model, the inflammatory and fibrosing obstruction of bile ducts temporally restricted to early postnatal development following RRV challenge results in progressive cholestasis and liver fibrosis.

Figure 6
Administration of recombinant IFN-γ results in obstruction of extrahepatic bile ducts in IFN-γ–/– mice. H&E staining of transverse sections along the extrahepatic bile duct of an IFN-γ–/– mouse that received daily intraperitoneal injections of 2,000 U of recombinant IFN-γ per gram body weight following RRV challenge. (A) A patent proximal segment of the duct. (B) A narrow lumen with increasing periductal inflammation (approximately 700 μm from the section in A). (C) Section approximately 100 μm from the section in B; the inflammation completely occludes the extrahepatic bile duct. Magnification ×200.
shares striking similarities with clinicopathological findings of biliary atresia in children. Although no viral agent has been consistently associated with biliary atresia in humans (1–3), the ability of RRV to target murine cholangiocytes and induce a tissue-specific inflammatory injury proves the principle that infectious agents may target neonatal cholangiocytes and trigger an undesired inflammatory response that results in occlusion of extrahepatic bile ducts. The hepatobiliary system of suckling mice has also been shown to be targeted by reovirus type 3, but the inflammatory response has not been shown to cause obstruction of extrahepatic bile ducts (17–20). In contrast, RRV challenge triggers an immediate infiltration of the hepatobiliary system by neutrophils, followed by predominantly Th1-committed, IFN-γ–producing T lymphocytes at the time of obstruction of extrahepatic bile ducts.

The pivotal role of lymphocytes and IFN-γ in duct obstruction became evident in mice lacking IFN-γ. Without IFN-γ, the sequential switch to a lymphocyte-based hepatic inflammation did not occur, duct obstruction was completely prevented, and extrahepatic bile ducts maintained lumenal continuity with the duodenum. In keeping with a central role for IFN-γ in duct obstruction, administration of recombinant IFN-γ following RRV infection resulted in recurrence of biliary atresia in IFN-γ–deficient mice. This outcome was in contrast to previous reports in which administration of IFN-α prevented the development of biliary obstruction following RRV infection of neonatal WT Balb/c mice (11, 12). Although these studies did not address the mechanisms used by IFN-α to prevent biliary obstruction, it is possible that IFN-α may interfere with early phases of biliary injury by a direct antiviral effect, by interfering with the interaction between RRV and cholangiocytes, or by blocking the inflammatory response to viral challenge. Our studies did not formally investigate how IFN-α regulates biliary obstruction following RRV challenge, but the findings of a similar increase in mRNA expression for both IFN-α and IFN-β after RRV challenge in WT mice (with biliary obstruction) and IFN-γ–deficient mice (without biliary obstruction) do not support a direct regulatory role for IFN-α in duct obstruction. Collectively, these data provide direct evidence for a singular role of IFN-γ in duct obstruction by lymphocytes in neonatal mice. In light of the shared phenotypic and molecular features between this model and children with biliary atresia (4), we propose that IFN-γ may play a central role in pathogenic mechanisms of disease.

Our findings also provide strong evidence that the pathogenetic mechanisms of biliary atresia conform to a biological continuum previously not recognized (1, 2, 21). The initiating events of this continuum, namely the insult by a viral agent and the immediate neutrophil-based inflammatory response, were not affected by IFN-γ. In contrast, the progression to ductal obliteration by inflammatory cells was prevented by loss of IFN-γ, either through

Figure 7
Persistent infiltration of portal space by neutrophils in IFN-γ–/– mice. RRV challenge induces a neutrophil-based pericholangitis within 3 days in WT and IFN-γ–/– mice (A and B). In WT mice, inflammatory cells switch to lymphocytes in expanded portal spaces 7–14 days after challenge (C and E). This switch is incomplete in IFN-γ–/– mice, which continue to display portal neutrophils (D and F). Arrows point to neutrophils.

Figure 8
Hepatic population of T lymphocytes and cytokine expression in IFN-γ–/– mice. Flow cytometry shows a decreased population in the livers of IFN-γ–/– mice by CD3+CD4+ and CD3+CD8+ lymphocytes after RRV challenge (A–C). Despite the loss of IFN-γ, expression of hepatic mRNA expression for IL-2p40, IL-4, and IL-5 in response to RRV challenge does not change (D–F). n = 4–7 mice per group at each time point. *P < 0.04.
a direct interference with the tropism of T lymphocytes to the hepatobiliary system or indirectly through the suppression of downstream targets, such as Mig (15, 16, 22). The final step of ductal fibrosis, in which inflammatory cells are replaced by the concentric deposition of matrix substrates, was also prevented by loss of IFN-γ. On the basis of the antifibrogenic properties of IFN-γ in the liver (23, 24), it is improbable that the lack of fibrosis is a direct consequence of the decrease in IFN-γ production. Instead, lack of progression to ductal fibrosis probably represents an indirect consequence of the lack of intra ductal inflammation induced by the loss of IFN-γ, which allows for restoration of epithelial integrity.

The argument for a biological continuum in the pathogenesis of biliary atresia does not rule out an important role of predisposing variables in disease susceptibility. The existence of such variables is supported by the variable degrees of biliary injury induced by RRV in different mouse strains (12). Furthermore, animal- and patient-based studies implicate specific genes in the pathogenesis of a subgroup of patients with biliary atresia who also display defects in laterality (2, 25–27). The presence of mutations in laterality genes in patients with extrahepatic malformations, but not consistently in patients who also have biliary atresia (27, 28), points to a modification in human biliary atresia. In this context, the identification of IFN-γ will have broad implications for the development of novel therapeutic targets to block progression of disease and foster long-term survival with the native liver in children with biliary atresia.

**Methods**

Infection of neonatal mice with RRV. Balb/c mice were maintained in a specific pathogen-free vivarium and housed in a room with a 12-hour dark-light cycle. WT Balb/c and IFN-γ−/− mice were injected with 0.9% saline solution (controls) or 1.5 × 10^6 ffu of RRV intraperitoneally within 24 hours of birth. The strain of RRV used was generously provided by Marie Riepenhoff-Talty (University at Buffalo, The State University of New York, Buffalo, New York, USA) (5) and titered in MA104 cells by a fluorescence focus assay prior to injection as described previously (32). Infected mice that died within the first 2 days or that were not fed by their mothers after infection were excluded from further analysis. All mice were weighed daily and examined for the development of icterus of the skin not covered with fur, of acholic stools, and of bilirubinuria (using Multistix 10SG strips; Bayer Corp., Elkhart, Indiana, USA). Mice were killed at 3, 7, 14, and 21 days after saline or RRV injection. At these time points, gross appearance of livers and bile ducts was recorded, and organs were harvested for RNA isolation and histological analyses; whole livers were isolated from separate groups of mice and used for isolation of messenger RNA.

In summary, we used a mouse model of biliary atresia to explore the claim from human-based studies that expression of IFN-γ may be an important pathogenic mechanism of disease (4). Notably, the absence of IFN-γ expression in vivo produced no obvious interference with the host’s susceptibility to bile duct injury following viral challenge or with the initial response orchestrating an acute inflammatory cholangitis. As a consequence, jaundice and acholic stools developed in a timely fashion, but the surprising resolution of symptoms implied paucity of the biliary system. Within the liver, IFN-γ deficiency minimized the expansion of periductal inflammation by T lymphocytes at later phases of injury. More notably, extrahepatic bile ducts displayed orderly lining by epithelial cells and were free of obstruction, maintaining luminal continuity and bile flow between the liver and the duodenum. These data do not necessarily prove that IFN-γ regulates biliary obstruction in humans; however, the remarkable similarities in onset of disease restricted to early postnatal stage (30), histopathological features (5, 31), and molecular signatures toward proinflammatory cytokines shared by this murine model and infants with biliary atresia underscore shared pathogenic mechanisms of disease. Therefore, new patient-based studies addressing genetic heterogeneity for IFN-γ and counter-regulatory cytokines will be logical steps to validate a functional role of IFN-γ in disease pathogenesis in humans. In this context, the identification of IFN-γ will have broad implications for the development of novel therapeutic targets to block progression of disease and foster long-term survival with the native liver in children with biliary atresia.
To perform two-color flow-cytometric analyses, mononuclear cells from the liver and spleen were resuspended in a concentration of 1 × 10^7–1 × 10^8 cells/ml in FACS buffer (PBS containing 0.1% [w/v] sodium azide and 2% [v/v] FCS) and added to a V-bottom, 96-well microtiter plate (Corning Inc., Corning, New York, USA) at a volume of 200 μl/well. Cell staining for flow cytometry was conducted as described previously (40). In brief, cells were preincubated with 1:200 dilution of anti-mouse FcγRIII receptor mAb CD16/CD32 (2.4G2) for 15 minutes at 4°C in the dark to block nonspecific adherence of mAbs to Fc receptors. The cells were surface stained by incubating with fluorochrome-conjugated mAbs at a concentration of 10 μg/ml, for 30 minutes at 4°C in the dark. The following antibodies were used: FITC- or phycoerythrin-conjugated (PE-conjugated) anti-mouse anti-CD3 (17A2, IgG2b), anti-mouse CD4 (RM4-4, IgG2a), anti-mouse CD8a (53-6.7, IgG2b), and anti-mouse CD19 (1D3, IgG1), all purchased from BD Biosciences (San Jose, California, USA). Background fluorescence was evaluated by staining the cells at optimal concentrations with isotype control antibodies. Cells were then analyzed using a FACSCalibur dual-laser flow cytometer (BD Biosciences), with excitation at 488 and 633 nm. Data were analyzed using CellQuest software (BD Biosciences). For each sample, 20,000 events were analyzed.

Intracellular cytokine staining. Intracellular cytokine staining was performed using the Cytofix/Cytoperm (with GolgiPlug) kit according to the manufacturer’s instruction (BD Biosciences). In brief, intrahepatic mononuclear cells were incubated with 1 μl/ml GolgiPlug (Brefeldin A) for 4 hours at 37°C, followed by incubation with antibodies that recognize the FcγRII/III receptors (2.4G2), FITC-conjugated anti-mouse CD4 (RM4-4, IgG2a), and anti-mouse CD8a (53-6.7, IgG2b). The cells were washed twice with staining buffer, then fixed and permeabilized by incubating in 100 μl of BD Cytofix/Cytoperm solution for 20 minutes at 4°C. Cells were then stained with PE-conjugated anti–IFN-γ (XMG1.2, IgG1), anti–IL-5 (TRFK5, IgG1), or isotype control (PE-conjugated rat IgG1). Stained cells were analyzed using a FACSCalibur flow cytometer.

Statistical analysis. Values are expressed as mean ± SD, and statistical significance was determined by unpaired t test, with a significance level of P < 0.05.

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