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Armed CD4⁺ Th1 effector cells and activated macrophages participate in bile duct injury in murine biliary atresia

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Abstract

Biliary atresia (BA) is an inflammatory cholangiopathy of infancy. A proposed mechanism regarding the pathogenesis of BA is that of a virus-induced, immune-mediated injury to bile ducts. The rotavirus (RRV)-induced murine model of BA was utilized to determine the hepatic inflammatory response related to ductal obstruction and if the immune response recapitulated human BA. One week after infection, there was a significant increase in liver CD4⁺ T cells producing IFN- γ and in macrophages producing TNF- α . The intrahepatic pattern of inflammation evolved rapidly from an initial predominant CD4⁺ Th1 cellular response to a subsequent influx of activated macrophages producing TNF- α and iNOS. This immune response persisted despite viral clearance and was representative of the hepatic immune profile present in human BA. Utilization of the murine model of BA yielded mechanistic data that can provide much needed insight into the role played by different arms of the immune system related to the pathogenesis of human BA.

Keywords

Biliary atresia; Armed effector T cells; Macrophage activation; Neonatal cholestasis

Introduction

Biliary atresia (BA) is a progressive, inflammatory, fibrosclerosing cholangiopathy of infancy that results in obstruction of both the extrahepatic and intrahepatic bile ducts [1,2]. Two forms of BA have been proposed: an embryonic/fetal form (approximately 20% of cases) which is associated with other congenital anomalies and may be due to defective morphogenesis of the extrahepatic bile duct and a perinatal/acquired form (80% of cases) in which a normally developed bile duct appears to undergo postnatal injury followed by fibro-obliteration. Infants with the acquired form of BA present with jaundice between 1 and 3 months of age. Despite medical and surgical intervention, the majority of infants with BA will have progressive bile duct injury and fibrosis within the liver, ultimately leading to biliary cirrhosis. Approximately 70–80% of BA patients will eventually require liver transplantation, attesting to the need for a better understanding of the underlying etiology and pathogenesis of this disease [3].

The etiology of BA remains unknown and current theories regarding the pathogenesis of the perinatal/acquired form have included occult viral infection and immune or autoimmune-mediated ductal destruction [4–6]. Two viruses which have been studied in humans as potential

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causes of duct damage are reovirus and rotavirus. Tyler et al. [7] reported finding nested RT-PCR evidence of reovirus in infants with BA and Riepenhoff-Talty et al. [8] found RT-PCR evidence of group C rotavirus (Reoviridae family) in BA hepatobiliary tissue. However, other investigators failed to find presence of these viruses in BA patients [9,10]. It has recently been proposed that the perinatal/acquired form may be caused by a biliary trophic viral infection, leading to an initial bile duct epithelial injury that triggers a persistent immune-mediated sclerosing process resulting in obstruction of extrahepatic bile ducts [4]. In support of this inflammatory hypothesis, we have recently characterized the immunologic phenotype present within the portal tracts of infants with a variety of cholestatic liver diseases at the time of diagnosis [11]. That study demonstrated that the portal tract inflammation which was unique to BA was composed of a CD4⁺ Th1 cell predominant process, characterized by an infiltrate of CD4⁺ and CD8⁺ T cells and macrophages with local cellular production of IL-2, IFN- γ and TNF- α . In order to better understand the kinetics of the pathologic immune events in BA, we are utilizing the rotavirus-induced murine model of BA [12-15].

In the Rhesus group A rotavirus (RRV) murine model of BA, newborn mice are infected with RRV in the first day of life which leads to progressive inflammation and obstruction of the extrahepatic bile duct by 2 weeks of age, closely mimicking the lesion in human BA [12-15]. Furthermore, in the original description of this model, it was noted that the virus was no longer detectable in the liver 2 weeks after infection, yet the inflammatory process was progressive and the mice died of liver failure by 1 month of age [12]. Thus, despite the implication that immune-mediated injury is a key pathogenic component, the triggers and nature of this inflammatory response have not been clearly defined.

The objectives of this study were: (1) to determine the temporal evolution of the hepatic inflammatory response to RRV infection related to the development of ductal obstruction in the murine model of BA and (2) to determine if the hepatic immune response in the murine model recapitulates that found in human BA. It is essential to identify similarities and differences between the murine and human disease in order to validate the murine model as a legitimate tool in which to study the pathogenesis of human BA.

Methods

RRV infection of neonatal mice

All animals received human care in accordance with criteria from NIH "Guide for Care and Use of Laboratory Animals" (publication #86-25 1985). Timed-pregnant female BALB/c mice (11–14 days post conception) were purchased from rotavirus-free colonies of Jackson Laboratory (Bar Harbor, ME). Average litter size was 3–7 pups and minimum of 5 litters per experiment were analyzed. Forty microliters of a Hanke's Balanced Salt Solution (BSS) with or without (control) 1.0×10^6 pfu/ml of RRV was injected intraperitoneally at 12–18 h of life. The RRV strain MMU 18006, a serotype 3 rotavirus, was kindly provided by M. Riepenhoff-Talty (The State University of New York, Buffalo, NY) and grown in MA-104 rhesus monkey kidney cells (provided by Richard Ward, Cincinnati, OH). Mice were examined post infection (p.i.) for appearance of jaundice in non-fur-covered areas and the development of acholic stools. Weight of animals was determined at day 1, 4, 7, 11 and 14 of life. Animals were sacrificed on day 7 or 14 of life and gross examination of the liver and biliary system was carried out with a dissecting microscope. Blood was collected from the renal artery at time of sacrifice, allowed to clot and centrifuged to obtain serum. Serum direct bilirubin was determined using the Direct Bilirubin Test from Diagnostic Chemicals Ltd. (PEI, CA). Liver and biliary tissues were removed at sacrifice and processed for histology and for RNA, protein and immunocyte isolation. Mouse liver specimens were pooled for all experiments. Tissue was fixed in formalin, embedded in paraffin, sectioned with a cryotome and 6 μ m sections were stained with hematoxylin and eosin.

Fluorescent immunohistochemistry

Liver tissue removed at 7 and 14 days of life was snap frozen in OCT (Miles Laboratories; Elkhart, IN) on dry ice. 6µm thick sections were stained using Tyramide Signal Amplification (TSA) Direct Kit (NEN Life Science Products, Boston, MA) according to manufacturer's instructions. Non-specific staining was blocked with anti-CD16/32 (FcR III/II receptor, 2.4G2; Pharmingen; San Diego, CA) in addition to the blocking reagent provided by the TSA kit. Slides were incubated with FITC-conjugated antibodies to CD4 (L3T4, GK1.5), CD8a (Ly-2, 53-6.7), CD11b, CD45, anti-smooth muscle actin-Cy3 conjugated or isotype matched controls (BDPharmingen; San Diego, CA) for 60 min at room temperature and counterstained with nuclear stain DAPI for 1 min (Sigma, St. Louis, MO). The slides were sealed with VectaShield (Vector Labs, Burlingame, CA) and visualized under 200× magnification with the Zeiss Axioplan2 microscope (Thornwood, NY). Portal tracts were identified based on hepatic artery staining for smooth muscle actin. Digital photographs were obtained with autocalibration of exposure time through the Slidebook analysis program (Denver, CO).

Flow cytometry of hepatic immune cells

Liver tissue was homogenized over wire mesh, hepatocytes pelleted at 300 rpm for 3 min and remaining cells subjected to RBC lysis with Tris-NH₄Cl. Cells were resuspended in FACS buffer (PBS/5% FBS) blocked with purified anti-CD16/32 and incubated with PE-conjugated CD45, APC-conjugated CD3 and either FITC-conjugated CD4 (L3T4, GK1.5), CD8a (Ly-2, 53-6.7), CD19 (B cell) or CD11b for 60 min at room temperature. As a control for non-specific antibody binding, parallel populations of cells were incubated in the presence of irrelevant, isotype-matched mAb. Expression of cell surface markers was determined on a FASCaliber flow cytometer (Becton-Dickinson, Mountain View, CA) using CellQuest software for analysis. Leukocyte populations were identified for study by gating on the CD45⁺ population. For each sample 50,000 cells were analyzed.

Intracellular cytokine analysis by flow cytometry

For ex vivo intracellular cytokine staining, hepatic immune cells were incubated for 2 h in PBS with GolgiPlug (BD Pharmingen, San Diego, CA), washed, blocked with purified anti-CD16/32 and incubated with APC-conjugated CD3 and either FITC-conjugated CD4, CD8a or CD11b for 60 min at room temperature. Cells were then washed 2× in PBS, fixed in 4% paraformaldehyde overnight at 4°C and permeabilized with PBS/0.1% saponin for 30 min at 37°C, blocked again with purified anti-CD16/32 and incubated for 60 min at 4°C with PE-conjugated antibodies to either IL-2, IFN-γ or TNF-α or isotype antibody controls (1:100 dilutions). Analysis by flow cytometry was performed as above.

Isolation of liver RNA and real-time RT-PCR analysis of cytokine mRNA expression

Liver immune cells were isolated as above and resuspended in TRIZOL buffer (Invitrogen, Carlsbad, CA). RNA was isolated from upper layer of a phenol/chloroform mixture and precipitated with 75% EtOH. The RNA pellet was resuspended in RNase-free water and was incubated with DNase (Sigma, St. Louis, MO) to remove any contaminating genomic DNA. Standard RT-PCR was performed on IL-4, IL-5 (primer pairs, R&D Systems, Minneapolis, MN) and RRV VP-4 (primer sequence—see Supplementary Table 1). Real-time RT-PCR reactions were performed for the following genes: IL-2, IFN-γ, TNF-α and actin (for normalization). Primer sequences and probes for real-time RT-PCR analysis were determined using Primer Express software (Applied Biosystems, Foster City, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are reported in a supplementary table (Supplementary Table 1). One step RT-PCR reactions were performed on an I-Cycler (BioRad) using reagents from SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). Real-time RT-PCR standard curves were generated

for each gene using in vitro transcribed T7 promoter-flanked PCR fragments (Lign'Scribe and MEGAscript kits, Ambion Inc., Austin, TX).

Cytokine protein analysis–Luminex bead assay

Liver tissue was weighed, homogenized in 0.1% weight/volume BSS and subjected to 1 freeze-thaw cycle at -20°C to lyse cells. Following centrifugation to remove cellular debris, supernatant was analyzed on a Luminex 100 instrument (Luminex Corp., Austin, TX) for presence of cytokines using the Th1/Th2 and TNF- α antibody bead kits as per manufacturer's instructions (BioSource International, Camarillo, CA).

Viral plaque assay

Liver tissue from BSS mice and RRV inoculated mice were pooled for analysis. Stock RRV was used for positive control. Tissue was weighed and then homogenized with a glass tissue homogenizer (Bellco, Vineland, NJ) in sterile PBS (0.1% weight/volume). Tissue homogenate was plated on a monolayer of MA-104 cells in duplicate in 6 well plastic tissue culture plates (Nunc, Naperville, IL) at a range of dilutions (10^{-1} – 10^{-5}). After a 30 min incubation at 37°C , 5% CO_2 , the cells were overlaid with warmed 1:1 MSO/0.1% BSA/1% Noble agar solution. After 3 days, cells were overlaid with 0.25% neutral red (Sigma, St. Louis, MO) in $2 \times$ BSS/1% Noble agar and the plates allowed to develop at 37°C . for 3 days. Cultured cells were examined for RRV plaque formation daily. Titers were calculated based on number of plaques present $\times$ dilution factors. All plaque assays were performed in duplicate and repeated three times.

Statistical analysis

All results were based on at least 3 separate experiments of pooled mouse samples (10 mice per experiment) and are reported as mean $\pm$ SEM. Differences between the two experimental groups (BSS vs. RRV) were analyzed statistically with the Student's t test for unpaired samples (InStat program, San Diego, CA). Differences in means were considered significant for P values < 0.05 .

Results

Rotavirus infection causes biliary obstruction and growth failure despite viral clearance

Newborn BALB/c mouse pups were injected with BSS or RRV and followed for signs of cholestasis (jaundice, acholic stools). $60 \pm 4.4\%$ of RRV infected mice developed jaundice and acholic stools by 1 week of life; $14.7 \pm 4.8\%$ failed to develop jaundice. Death occurred by 1 week in $25.3 \pm 1.5\%$ of mice and by 4 weeks in over 90% of jaundiced mice. Decreased weight gain was present in the RRV-diseased group compared to BSS controls, beginning at day of life 7 (Fig. 1a). Cholestasis was confirmed by direct hyperbilirubinemia (Fig. 1b). Histologic examination of sections of the extrahepatic bile duct and liver demonstrated peri-ductular cellular infiltrates in both interlobular and extrahepatic bile ducts, which increased from day 7 of life to day 14. By 2 weeks of life, microscopic dissection and histology showed that the extrahepatic bile ducts were severely narrowed or completely obstructed and were surrounded by an intense inflammatory infiltrate (Fig. 1c).

RT-PCR and standard viral plaque assay techniques were utilized to determine if virus was present within the livers of RRV diseased animals. At 1 week post inoculation, there was both PCR evidence of virus (RRV VP-4) and plaque assay confirmation of infectious virus. However, by 2 weeks of age, there was minimal VP-4 product detected and absent RRV infectious plaque formation (Fig. 1d). This finding was consistent with the original report of

this model by Riepenhoff-Talty et al. [12], in which RRV was cleared in neonatal mice between the 1st and 2nd week post infection.

The requirement of active viral infection for disease onset was determined by administration of UV inactivated virus. 1×10^6 pfu/ml of RRV was placed on ice and exposed to a UV light source (30 cm. from light source for 30 min). Intraperitoneal injection of the UV-irradiated RRV into 10 newborn mouse pups resulted in no clinical disease within the first 2 weeks of life. Measurements of disease (UV-irradiated RRV group and BSS control group) included histology of liver and extrahepatic bile duct, serum direct bilirubin levels and liver immune cell quantification by FACS analysis. Direct bilirubin levels from pooled serum samples revealed no direct hyperbilirubinemia (BSS: <0.05 mg/dL, UV-RRV: 0.05 mg/dL). There was no immune cell infiltration surrounding the ducts in the UV-RRV group and the immune cell profile within the liver was similar in both groups (data not shown). This work implies that active viral infection is necessary for disease onset in the murine model of BA.

CD4⁺ and CD8⁺ T cell and macrophage infiltrate in portal tracts of RRV diseased mice

In human BA, we have reported that the portal tract inflammatory infiltrate is composed primarily of increased CD4⁺ and CD8⁺ T cells as well as a dramatic influx of macrophages surrounding intrahepatic bile ducts [11]. The immunophenotype of the inflammatory cells surrounding the bile ducts in the murine BA model was first determined by immunohistochemistry. Prominent clusters of CD4⁺ and CD8⁺ T cells and CD11b⁺ cells (macrophages) were observed within the portal tracts of RRV diseased mice compared to BSS controls (Fig. 2). Flow cytometry was then utilized to quantitate temporal changes in the number of liver immune cells. Immune cells were stained with CD45 (to identify all cells of hematopoietic origin) and either CD3/CD4, CD3/CD8, CD19 or CD11b. FACS analysis was gated on CD45⁺ cells and expressed as the percent of CD45⁺ cells that were also positive for the specific cell surface markers (Fig. 3). At 1 week post RRV infection, there was a significant increase in CD4⁺ T cells and macrophages within RRV diseased livers compared to controls (CD4: BSS: $6.3 \pm 1.2\%$ vs. RRV: $10.0 \pm 0.9\%$; CD11b: BSS: $29.2 \pm 3.3\%$ vs. RRV: $43.2 \pm 3.7\%$). At 2 weeks post infection, the CD4⁺ T cells continued to increase, and the rise in the number of CD11b⁺ cells within RRV diseased liver was even more striking and was accompanied by an increase in CD8⁺ T cells (CD11b: BSS: $13.9 \pm 1.5\%$ vs. RRV: $54.4 \pm 4.5\%$; CD8: BSS: $5.4 \pm 0.9\%$ vs. RRV: $11.6 \pm 1.9\%$). There was no significant difference in the percentage of CD19⁺ cells (B cells) at either time point (data not shown). Furthermore, the total number of immune cells present within the livers of RRV diseased mice was markedly greater than that found in BSS controls, substantially augmenting the percentage increases noted above (Table 1).

CD4⁺ Th1 cell-mediated immune response predominates at 1 week post infection

In human BA, we have shown that increased mRNA expression of all Th1 cytokines (IL-2, IFN- γ , IL12, TNF- α) was present at the time of diagnosis and that this expression was localized to cells residing in the portal tracts [11]. To determine if a similar CD4⁺ Th1 cellular profile of cytokines was present in the murine BA model, we determined cytokine protein and message levels. At 1 week after RRV infection, there was a significant increase in the number of CD4⁺ T cells producing IFN- γ and in macrophages producing TNF- α from RRV diseased mice (Fig. 4a). A similar trend was seen with measurement of cytokine proteins within liver homogenates and mRNA cytokine expression (Figs. 4b,c). Increased mRNA expression of IFN- γ and TNF- α was observed in liver tissue from RRV diseased mice. IL-2 protein levels were not different between the two groups and IL-2 mRNA expression was undetectable in both groups (data not shown). IL-2 protein clearance is rapid and IL-2 mRNA has a high turnover rate, leading to potentially lower levels of detection at a given time point [16,17]. Investigation for the presence of Th2 cytokines (IL-4, IL-5) was performed with RT-PCR and

Luminex bead assays. No statistical differences in Th2 cytokine levels were found between the two groups (data not shown).

Abundant production of TNF- α from effector cells at 2 weeks post infection

Our studies of human BA revealed increased mRNA expression of TNF- α in the livers of patients at the time of diagnosis [11]. This predominance of TNF- α was also observed at 2 weeks post infection in the murine model of BA. Intracellular cytokine staining revealed that CD4⁺ T cells and macrophages from the RRV diseased group had increased levels of TNF- α (Fig. 5a). The IFN- γ production by the CD4⁺ T cells from the RRV groups was greatly reduced at 2 weeks compared to the 1 week time point (Figs. 4a and 5a). Furthermore, measurement of cytokine protein levels within RRV liver tissue confirmed the significant increase in TNF- α compared to BSS controls (Fig. 5b). The predominance of TNF- α mRNA expression and down-regulation of IFN- γ was also demonstrated by real-time PCR (Fig. 5c). No statistical differences in Th2 cytokine levels were found between groups at this time point (data not shown).

Significant increase in inducible nitric oxide synthase (iNOS) mRNA expression in RRV diseased mice

To further elucidate the potential role of the portal tract macrophage infiltrate in the pathogenesis of bile duct injury, we next examined the regulation of another downstream effector molecule produced by macrophages, nitric oxide. This was examined because up-regulation of TNF- α , which accompanied the macrophage infiltrate, can directly stimulate nitric oxide synthesis by macrophages. mRNA expression of iNOS, the enzyme responsible for macrophage nitric oxide production, was measured in 1 and 2 week old mice. Real-time PCR analysis of liver immune cells showed that there was no detectable iNOS expression in BSS control mice, however, iNOS was highly expressed in RRV diseased mice at both time points. Thus, the macrophages appeared to be activated and participating in immune-mediated bile duct injury (Fig. 6).

Discussion

Biliary atresia is a devastating liver disease in infants which, without surgical intervention or liver transplantation, would result in biliary cirrhosis and death within the first 2 years of life. It has been proposed that acquired cases of BA may be caused by perinatal acquisition of a cholangiotropic virus that initiates a secondary immune response leading to bile duct epithelial injury and eventual obstruction and fibrosis of the extrahepatic bile duct [4]. Thus, the immune response potentially is more important in producing this lesion than is the initial viral infection. In order to begin testing this hypothesis, it was important to determine if the hepatic immune profile within the RRV-induced murine model of BA was similar to that found in human BA as well as to clearly define the sequence of immune events associated with bile duct injury.

In the murine model, the mice became jaundiced at 1 week of age, had growth failure by 2 weeks and died of biliary cirrhosis by 4 weeks. This clinical picture is similar to that of an infant with BA, with jaundice observed in the first few months of life, growth failure due to the liver disease and death by 2 years of life if left untreated. Furthermore, the histology of the extrahepatic bile duct lesion in the mouse is strikingly similar to that found in humans; a severely narrowed or obliterated duct surrounded by inflammation and fibrosis. The intrahepatic ductal lesions in the mouse are similar to those found in humans with intense periductular infiltrates and bile duct epithelial damage. The human lesion is also composed of “bile plugs” within the bile ducts and reactive bile duct proliferation. Bile plugs were not observed in the murine model and bile duct proliferation was mild (2–4 ducts per portal tract).

The results from this study showed that the bile duct injury in the murine model was associated with an initial armed CD4⁺ Th1 effector cell response that, through release of IFN- γ , activated macrophages to produce TNF- α and nitric oxide. This immune response persisted despite viral clearance and is representative of the hepatic immune profile present in human BA at the time of diagnosis [11]. The intrahepatic bile duct infiltrates in human BA are composed of CD4⁺ and CD8⁺ T cells and macrophages with local portal tract cellular production of IL-2, IFN- γ and TNF- α . These results validate the use of the RRV-induced murine model as a powerful tool to study the pathogenesis of human BA.

A Th1 pathway predominated at the 1 week time point within the livers of RRV infected mice, with portal tract infiltrates containing IFN- γ -producing CD4⁺ T cells. CD4⁺ T cells are clearly present within the portal tracts of human BA at the time of diagnosis [11,18,19]. Furthermore, Shivakumar et al. [20] recently reported the outcome of RRV infection to IFN- γ knockout mice compared to wildtype BALB/c mice. The IFN- γ knockout mice had histological recovery from biliary obstruction and increased survival compared to wildtype controls, indicating the important role played by IFN- γ in bile duct injury. That study correlated with our findings of increased CD4⁺ T cells and IFN- γ production at 1 week of life in the wildtype RRV diseased mice. Our study further extends these observations by not only analyzing other Th1 cytokines involved in the immune response, but also investigating the role of activated macrophages in bile duct injury.

The pattern of inflammation in the murine model of BA evolved rapidly by the second week of life from a CD4⁺ Th1 cell predominant infiltrate to an influx of activated macrophages secreting TNF- α . Macrophages require two signals for activation; one of which is provided by IFN- γ and the other is provided by a variety of means including CD40/CD40 ligand interactions. Armed Th1 cells can deliver both signals [21]. The abundant IFN- γ production by CD4⁺ T cells at 1 week may be key to the subsequent macrophage activation. TNF- α is a pro-inflammatory cytokine that has many effector functions, including interference with mitochondrial respiration that may cause direct necrosis of cells, stimulation of nitric oxide production by macrophages and induction of apoptosis through direct interactions with TNF-receptor p55 or indirectly by enhancing Fas expression [22,23]. We showed that one of these downstream effects, nitric oxide production, was up-regulated at the iNOS mRNA level in RRV diseased mice at 2 weeks.

Several human BA studies have documented the influx of macrophages [11,24-26] into portal tracts and we have recently reported up-regulation of TNF- α mRNA expression in livers of BA infants [11]. The relationship of macrophages with bile duct injury has also been shown in other human biliary diseases. Cameron et al. reported persistent activation of macrophages in livers of patients with primary sclerosing cholangitis, which participate in the chronic release of TGF- γ and contribute to the chronic inflammation and fibrosis [27]. Primary biliary cirrhosis is associated with Kupffer cell portal tract infiltrates that express monocyte chemotactic proteins, resulting in persistent recruitment of monocytes surrounding bile ducts [28].

Furthermore, Wu et al. demonstrated that portal tract infiltration of CD68 (Kupffer cell) and myeloperoxidase positive cells were associated with increased 3-nitrotyrosine levels (nitric oxide and reactive oxygen species) and bile duct destruction in early stages of primary biliary cirrhosis [29]. Further investigation of the role of macrophages in bile duct injury is warranted in both human BA as well as the murine model.

In summary, utilization of the RRV-induced murine model of BA will yield mechanistic data that have the potential to provide much needed insight into the pathogenesis of human BA, specifically the role played by different arms of the immune system in the initiation and progression of disease. Understanding the pathophysiology of BA is the first step in the

development of new, potentially effective treatment strategies (e.g. immunomodulatory therapies) geared at halting the bile duct injury and obstruction, preventing biliary cirrhosis and delaying or avoiding the need for liver transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.clim.2005.01.012.

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Fig 1.

RRV-induced murine model of BA. (a) Growth retardation in RRV infected mice. Mice were inoculated with BSS or RRV at 18 h of life and sacrificed at 16 days of life (10 mice in each group). RRV diseased mice had diminished weight gain compared to BSS controls, beginning at day 7 of life. (b) Direct hyperbilirubinemia in RRV infected mice. Serum was isolated from 1 and 2 week old mice and assayed for direct bilirubin levels. (c) Extensive inflammatory infiltrates found in extrahepatic bile duct and portal tracts in RRV infected 2 week old mice. Formalin fixed, paraffin embedded specimens stained with H&E from 2 week old mice previously inoculated with BSS or RRV. Histology of the extrahepatic ducts (arrow) showed severely narrowed or obliterated ductal lumen of RRV infected mice with abundant surrounding immune cell infiltrates and edema. The portal tracts from RRV infected mice had extensive immune infiltrates surrounding intrahepatic bile ducts (arrows) compared to BSS controls. (d) Viral clearance by 2 weeks of age. RT-PCR for the RRV-specific viral gene VP-4 was performed on liver RNA from 1 and 2 week old mice. Strong expression of VP-4 was found at 1 week and faint expression by 2 weeks. Quantification of actively infectious virus was determined by viral titer plaque assay. Infectious virus was present at the 1 week time point but was undetectable by 2 weeks of life.

Fig 2.

Portal tract infiltrates in RRV infected mice. 6 μ m sections of frozen liver tissue from 2 week old mice previously inoculated with BSS or RRV were stained with smooth muscle actin (red) to identify hepatic arteries present within portal tracts and double stained with either CD4, CD8 or CD11b (green) followed by DAPI nuclear counterstain (blue). Infiltrates of CD4⁺ and CD8⁺ T cells and abundant influx of macrophages were seen in the portal tracts of RRV diseased mice compared to BSS controls.

Fig 3.

Increased CD4⁺ and CD8⁺ T cells and macrophages in livers of RRV-diseased mice. (a) Newborn mouse pups inoculated with BSS (control) or RRV were sacrificed at 1 and 2 weeks of age. Liver immune cells were isolated from pooled livers ($n = 10$) and stained with CD45 and either CD3/CD4, CD/CD8 or CD11b and analyzed by flow cytometry. Quantification of each cell type is expressed as the percent of CD45⁺ cells that were also positive for the specific cell surface markers. Shown are the mean $\pm$ SEM from 4 separate experiments at the 1 week time point and 7 experiments at 2 weeks. Significant increases in CD4⁺ T cells and macrophages (CD11b⁺) at 1 week and in CD4⁺ and CD8⁺ T cells and macrophages at 2 weeks were found in the RRV diseased animals compared to BSS controls. (b) Representative FACS analysis dot plot of CD45/CD11b positive cells from the 2 week time point. Shown here is the significant infiltration of macrophages within livers of RRV diseased animals. * $P < 0.05$.

Fig 4.

CD4⁺ Th1 cell-mediated immune response predominates at 1 week post infection. Values represent mean $\pm$ SEM from 3 separate experiments at the 1 week time point. (a) Intracellular cytokine staining. Liver immune cells from BSS or RRV treated mice were isolated as previously described and stained with cell surface markers (CD3/CD4, CD3/CD8 or CD11b) followed by intracellular staining of either IL-2, IFN- γ or TNF- α . The percentage of each cell type producing the various cytokines in each group was quantified with FACS analysis. Results are expressed as the fold increase in percent positive cells in RRV diseased mice compared to BSS control. Significant increases in CD4⁺ T cell production of IFN- γ and in CD11b production of TNF- α was found. (b) Luminex bead assay. Liver homogenates were analyzed for cytokine protein expression. Increased protein amounts of IFN- γ and TNF- α were found in RRV diseased mice. (c) Real-time PCR. Quantification of cytokine mRNA transcripts from liver immune cells revealed increased expression of IFN- γ and TNF- α in RRV diseased mice compared to controls. * $P < 0.05$.

Fig 5.

Abundant production of TNF- α from activated macrophages at 2 weeks post RRV infection. Values represent mean $\pm$ SEM from 3 separate experiments at the 2 week time point. (a) Intracellular cytokine staining. Liver immune cells from BSS or RRV treated mice were stained with cell surface markers (CD3/CD4, CD3/CD8 or CD11b) followed by intracellular staining of either IL-2, IFN- γ or TNF- α . The percentage of each cell type producing the various cytokines in each group was quantified by FACS analysis. Results are expressed as the fold increase in percent positive cells in RRV diseased mice compared to BSS control. Significantly elevated CD11b production of TNF- α was found in RRV diseased mice. (b) Luminex bead assay. Liver homogenates were analyzed for cytokine protein expression and showed significantly increased TNF- α protein from RRV diseased mice. (c). Real-time PCR. Quantification of cytokine mRNA transcripts from liver immune cells revealed significantly increased expression of TNF- α in RRV diseased mice compared to controls. * $P < 0.05$.

Fig 6.

Increased mRNA expression of inducible nitric oxide synthase (iNOS) in RRV diseased mice. $\text{TNF-}\alpha$ is a pro-inflammatory cytokine that can stimulate macrophages to release nitric oxide, leading to cellular injury. mRNA expression of iNOS (enzyme involved in nitric oxide production) was measured in 1 and 2 week old mice. Real-time PCR analysis showed that there was no detectable iNOS in BSS control mice, however iNOS was up-regulated in RRV diseased mice at both time points. n.d. = none detected. * $P < 0.05$.

Table 1

Dramatic increase in the total number of liver immune cells in RRV diseased mice at 2 weeks of age

	# cells × 10 ⁴ /mouse		
	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8 ⁺	CD11b ⁺
1 week BSS (<i>n</i> = 10)	6.1	4.1	19.0
1 week RRV (<i>n</i> = 9)	7.1	3.9	46.0
Fold increase over BSS	1.2	0.95	2.4
2 weeks BSS (<i>n</i> = 8)	7.5	4.2	10.8
2 weeks RRV (<i>n</i> = 10)	71.8	36.8	197.0
Fold increase over BSS	9.6	8.8	18.2

8–10 mouse livers were pooled and immune cells isolated as previously described. The number of each cell type per mouse was calculated as follows: yield of cells (hemocytometer count) × % CD45⁺ cells (purity) divided by the number of mice and finally multiplied by the % of either CD4, CD8 or CD11b positive cells. One representative experiment of three performed is shown here.