Published in final edited form as:

J Hepatol. 2013 October ; 59(4): . doi:10.1016/j.jhep.2013.05.010.

Regulatory T Cells Inhibit Th1 Cell-Mediated Bile Duct Injury in Murine Biliary Atresia

Rebecca M. Tucker*, Amy G. Feldman[†], Erika K. Fenner*, and Cara L. Mack*,[†]

*University of Colorado, Denver

[†]Children's Hospital Colorado, Denver, CO 80262

Abstract

Background and Aims—Biliary atresia (BA) is a pediatric inflammatory disease of the biliary system which leads to cirrhosis and the need for liver transplantation. One theory regarding etiology is that bile duct injury is due to virus-induced autoreactive T cell-mediated inflammation. Regulatory T cells (Treg) abnormalities in BA could result in unchecked bystander inflammation and autoimmunity targeting bile ducts. The aim of this study was to determine if Tregs are dysfunctional in the rotavirus-induced mouse model of BA (murine BA).

Methods and Results—Murine BA resulted from infection of BALB/c neonates with Rhesus rotavirus (RRV). Liver Tregs from BA mice were decreased in number, activation marker expression and suppressive function. Adoptive transfer studies revealed that RRV-infected mice that received Tregs had significantly increased survival (84%) compared to controls (12.5%). In addition, ablation of Tregs in older mice, followed by RRV infection, resulted in increased bile duct injury.

Conclusion—These studies demonstrate that dysregulation of Tregs is present in murine BA and that diminished Treg function may be implicated in the pathogenesis of human BA.

Introduction

Biliary atresia (BA) is a pediatric liver disease characterized by progressive inflammation and fibrosis of both the extrahepatic and intrahepatic bile ducts. As a result, approximately 80% of patients with BA will require liver transplantation, accounting for half of all pediatric liver transplants [1]. The etiology of BA is unknown and theories of pathogenesis include viral infection [2][3], autoimmune-mediated bile duct destruction [4][5] and abnormalities in bile duct development [6]. A current view of the pathogenesis of BA is that it may involve both a primary perinatal hepatobiliary virus infection and a secondary generation of an exaggerated inflammatory or autoimmune-mediated bile duct injury. The current study uses the Rhesus group A rotavirus (RRV)-induced murine model of BA, which entails a virus-induced, progressive inflammatory destruction of bile ducts leading to extrahepatic bile duct obliteration, mimicking the human disease. [3,7–9]. Two groups have demonstrated that this inflammation is composed, in part, of autoreactive T cells specific to bile duct epithelia [9,10]. Liver T cells from RRV-induced BA mice generated IFN- γ in response to self-bile duct epithelial antigens [9] and adoptive transfer of these liver T cells from BA mice into naïve immunodeficient recipients led to bile duct-specific inflammation

Corresponding Author: Rebecca M. Tucker, Phone: 303-724-7207, Fax: 303-724-7212, rebecca.tucker@ucdenver.edu.

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(9,10). In addition, evidence for humoral autoimmunity exists: sera from BA mice contained antibodies reactive to multiple proteins (i.e. enolase) within bile duct epithelial homogenate, suggesting the presence of autoantibodies specific to bile duct epithelia [11].

One potential mechanism to explain the abnormal autoimmune response is loss of functional regulatory T cells (Tregs). Importantly, RRV infection must take place in the first 24–48 hours of life in order to induce BA. Incidence of disease is highest when virus is administered in the first 12–24 hours of life and, conversely, virus infection of mice >1 week of age does not result in any biliary disease (“BA-resistant”) [2]. The necessity of early age at infection to produce disease leads to the hypothesis that early infection could alter the release of Tregs from the thymus or decrease their regulatory capacity in the periphery, thus allowing for pathogenic autoreactive T cells and generalized inflammation to flourish. It is important to note that functional Tregs are not present in the periphery of mice prior to day 3 of life [12,13]. In addition, alterations in the number or function of these neonatal Tregs can give rise to a variety of autoimmune conditions. [14–16]. To address this hypothesis, the goals of this study were (1) to determine if Tregs are altered either in number or function in BA mice as compared to controls; (2) if the biliary inflammation can be abrogated by supplementation with functional Tregs; and (3) if depletion of Tregs from older, BA-resistant mice could allow for RRV-induction of biliary disease.

Materials and Methods

Mice

Timed-pregnant female BALB/c mice were purchased from rotavirus-free colonies of Harlan Laboratories (Indianapolis, IN). Foxp3-GFP mice on the BALB/c background were supplied by Jackson Laboratory (Bar Harbor, ME). Mice were injected IP at 12–18 hours of life with either 1.5×10^6 pfu/ml of virus in balanced salt solution (BSS) or BSS alone (control mice). Mouse whole liver specimens were pooled (n=3–5 livers/pool) and results reflect 3 pools for all experiments. All animals were housed and handled in accordance with the UC Denver Office of Laboratory Animal Medicine.

Virus culture/titering

Rhesus rotavirus (RRV) strain MMU 18006 was grown in MA-104 African green monkey kidney cells (ATCC) and assayed for concentration by infectious plaque assay as previously described [9].

Serum Bilirubin Assay

Blood was collected from the renal artery of mice (n=3–5 mice/pool) at time of sacrifice. Direct bilirubin was determined using the Direct Bilirubin Assay from Sekisui Diagnostics (Oxford, CT).

Suppression Assay

Regulatory function of Tregs from either RRV-infected BA mice (n=7) or BSS control mice (n=8) was determined by purification of CD4⁺Foxp3⁺ cells from 2 week old Foxp3GFP⁺ mice with the FACSARIA cell sorter. Adult BALB/c CD4⁺ cells were purified using MACS magnetic beads (Miltenyi, Auburn CA) (responder T cells- 5×10^4 cells/well). Mitomycin-C treated adult BALB/c splenocytes were used as antigen presenting cells (5×10^4 /well). 0.2 µg/ml anti-CD3 (clone 2C11, eBiosciences, San Diego, CA) and Treg/T responder ratios of 1:2 to 1:16 were added. Cells were cultured for 3 days, [³H] thymidine was added and 18 hours later T cell proliferation was determined by measuring incorporated radioactivity on a microplate scintillation counter.

Adoptive transfer of Tregs into BA mice

BALB/c mice were inoculated with RRV as above. Adult spleen cells used for adoptive transfer were purified from BALB/c mice transgenic for green fluorescent protein (GFP) under the control of the Foxp3 promoter (Foxp3-GFP). CD4⁺ cells that were either GFP⁺ (Treg) or GFP⁻ (Teffector- Teff) were purified with MoFlo cell sorter. 3×10^5 cells (either Treg or Teff) were injected IP into 5 day old BA mice.

Histopathology/Immunohistochemistry

Formalin-fixed, paraffin embedded liver and extrahepatic bile ducts were stained with H&E. Additional sections of liver were snap frozen and sections were stained with FITC-conjugated antibodies to CD4, CD8a, or CD11b (eBiosciences, San Diego, CA) or Alexafluor 555-conjugated cytokeratin 19 to identify bile ducts [17] using standard protocols.

Flow cytometry

Fluorochrome-conjugated antibodies to CD3, CD4, CD8a, CD11b, CD45, CD25, CD44, GITR, CD62L, and isotype matched controls were purchased from eBiosciences. Cells were blocked with anti-CD16/32 and stained with PE-conjugated CD45 and other cell-surface antibodies. Leukocyte populations were gated for study based on their CD45-staining. For analysis of Treg marker co-expression, liver leukocytes from Foxp3-GFP mice were purified by Percoll gradient prior to staining, thus eliminating the need for CD45 staining in these particular assays. Expression of cell surface markers was determined on a FACS Caliber flow cytometer (Becton-Dickinson, Mountain View, CA) using CELL Quest software for analysis.

Ex vivo intracellular cytokine staining

Liver cells were cultured in the presence or absence of PMA and ionomycin followed by permeabilization and intracellular staining for fluorochrome-labelled antibodies to IL2, IFN- γ , or TNF- α (eBiosciences). Flow cytometry was performed as above.

In vivo depletion of Tregs

Four day old BALB/c mice were given the CD25-depleting antibody PC61 (400 μ g) (gift from Ron Gill, PhD, UC Denver) or control rat IgG by IP injection. On day 7 of life, mice were administered 2×10^6 pfu RRV. Mice were followed for disease for 2 weeks (day 21), at which point serum and livers were harvested for analysis as above. Confirmation of CD25 cell depletion was determined by flow cytometry of spleens from treated animals on day 21.

Results

BA mice had reduced numbers and diminished function of regulatory T cells

In order to determine if Tregs have a role in biliary destruction, the number of Tregs present in the livers of BA mice was measured. At 2 weeks of age, BA mice had a significant decrease in the percent of CD4⁺ cells that were Foxp3⁺ (BA: $6.3 \pm 0.7\%$, BSS control: $9.1 \pm 0.5\%$, $p=0.006$) (Figure 1A). This difference in percent also translated into a large difference in absolute number of liver Tregs (BA: $18,200 \pm 4,715$ /liver, BSS: $50,510 \pm 9,420$, $p=0.04$) (Figure 1B). This decrease was in contrast to the lack of change in Treg percentages in the spleen of BA and BSS control mice (BA: $12.6\% \pm 3.3\%$, BSS: $12.8\% \pm 0.4\%$, data not shown). This suggests a defect in Treg trafficking to the liver in BA mice.

In addition to diminished Treg liver numbers, we questioned the ability of BA Tregs to suppress effector T cell function. To answer this question, CD4⁺Foxp3⁺ cells were purified

from mice that have the Foxp3⁺ Tregs tagged with GFP. Splenic Tregs were used in these experiments as sufficient numbers of cells could be isolated from this organ and the spleen is a natural reservoir of Tregs. These cells were used in a suppression assay along with T cell responders from normal adult BALB/c mice. As shown in Figure 1C, Tregs from BSS control mice were effective at inhibiting anti-CD3 stimulated proliferation. BSS control Tregs at a 1:4 ratio of Tregs:T responder cells suppressed 79.1±1.5% of maximal stimulation, whereas BA Tregs were less effective as a 1:4 ratio yielded 50±3.8% of maximal stimulation. This deficiency in suppressive ability followed throughout the range of cell ratios tested and was statistically significant (p=0.009).

In order to explore the reason for the defective suppression in BA mice, analysis of markers of activation and homing on Tregs was performed. Expression of the activation marker CD25 on Foxp3⁺ Tregs was significantly reduced in BA mice spleens (p=0.002) and livers (p=0.01) compared to controls. Additionally, the adhesion molecules CD44 and CD62L were diminished on Tregs from BA spleens (p=0.02 and 0.01, respectively) (Figures 1D and 1E). Expression of another regulatory marker, GITR, was unchanged in all populations. These studies revealed deficits in both number and function of Tregs in RRV-infected BA mice.

Adoptive transfer of regulatory T cells into BA mice ameliorated disease progression

In many autoimmune mouse models of human disease, replenishment of Tregs into diseased mice is an effective treatment (18–20). In order to determine if Tregs were capable of altering disease progression in the BA mouse model, normal adult BALB/c splenic Tregs or control effector T cells (Teff) were purified from Foxp3-GFP mice and transferred into RRV-inoculated 5 day old mice. (Figure 2A). At 2 weeks of life, RRV-infected BA mice had ~44% survival, RRV-infected BA mice that received control Teff cells had ~16% survival and RRV-infected mice that received Tregs had ~87% survival (Figure 2B: p=0.018 BA+Treg vs BA; p<0.001 BA+Treg vs BA+Teff). Biliary injury was shown to be less severe in the RRV-infected mice that received Tregs based on the significantly reduced direct serum bilirubin (0.25 mg/dL) compared to either BA mice (7.1mg/dL, p<0.001) or BA +Teff mice (4.8 mg/dL, p=0.001) (Figure 2C). Histology findings revealed that transfer of Tregs to RRV-infected mice prevented extrahepatic bile duct obstruction, while biliary obstruction was observed in the BA mice and the RRV-infected mice that received Teff cells (Figure 2D). Immunohistochemistry showed that the inflammatory portal tract infiltrate comprised of CD4⁺ and CD8⁺ T cells and CD11b⁺ macrophages that had been previously described in BA mice [9] was also present in mice that received Teff cells but absent in mice that received Tregs (Figure 2E).

Flow cytometry analysis of 14 day old mouse livers demonstrated decreased amounts of CD8⁺ T cells and CD11b⁺ macrophages in BA+Treg mice compared to BA and BA+Teff mice (Figure 3A). *Ex vivo* stimulation and intracellular cytokine staining of liver leukocytes showed that BA mice that received Tregs had a profile similar to healthy BSS control mice (Figure 3B). Both BA and BA+Teff mice had increased numbers of T cells producing IFN-γ and TNF-α, whereas BSS control and BA+Treg mice showed minimal cytokine production (Figure 3C). These results revealed that adoptive transfer of normal Tregs into RRV-infected mice prevented biliary inflammation and obstruction, suggesting an important role for dysfunctional Tregs in the pathogenesis of disease.

Depletion of regulatory T cells in BA-resistant older mice lead to increased susceptibility of RRV-induced biliary disease

Since it had been determined that transfer of supplemental Tregs into BA mice abrogated disease, the next experiment was to determine if depletion of Tregs from BA-resistant older

mice could lead to bile duct injury. To that end, 4 day old BALB/c pups were given CD25-depleting antibody PC-61 or control IgG. Three days later, mice were inoculated with RRV and followed for disease incidence for 2 weeks. CD25⁺ cell depletion was confirmed at the final time point by flow cytometry of splenic T-cells (Figure 4A). Biliary disease occurred in 67% of Treg-depleted mice as compared to 28% of control mice. All mice that died as a course of this experiment initially presented with biliary disease, as evidenced by either jaundice or acholic stools. Serum direct bilirubin was measured at 2 weeks post-RRV inoculation with Treg-depleted mice having significantly elevated levels (12.3 vs. 3.7 mg/dL in controls, $p=0.005$) (Figure 4B). In addition, bile duct occlusion occurred in Treg depleted mice (Figure 4C) and not in IgG controls. Immunohistochemistry of Treg-depleted mouse livers revealed an inflammatory infiltrate comprised of CD4⁺ and CD8⁺ T cells and CD11b⁺ macrophages (Figure 4D) that was not present in IgG treated livers.

Discussion

The RRV-induced mouse model of biliary atresia is characterized by a bile duct-specific inflammatory infiltrate that contains autoreactive CD4⁺ T cells. This study revealed the significance of Treg deficiencies to disease pathology in murine BA. RRV-infected BA mice had deficits in Treg number and suppressive function. In addition, enrichment of Tregs into RRV-infected mice prevented BA and depletion of Tregs from “BA-resistant” older mice promoted biliary injury. Previous studies on autoimmune models, such as the NOD model of type 1 diabetes, BWF1 lupus mice, and experimental autoimmune encephalomyelitis mice, have demonstrated alterations in either Treg number or function [18–22]. In these and other models, adoptive transfer of functional Tregs have suppressed the autoimmune response and progression of disease [21,23–26]. The impact that an intact Treg population has upon maintaining self-tolerance is also witnessed by the fact that loss of this population results in onset of organ-specific autoimmune inflammation [15, 27–29].

The heightened potential pathogenicity of newborn mouse T cells is demonstrated in several ways. In neonatal mice, thymectomy on day 3 of life can result in spontaneous autoimmune disease, due primarily to reduction of peripheral Tregs prior to emigration from the thymus [12]. In addition, neonatal exposure to self antigens can elicit pathogenic autoimmune responses while adult exposure does not [30, 31]. These facts lend credence to the hypothesis that the neonatal BALB/c environment is unique in its susceptibility to a bile duct-tropic virus-induced autoimmune disease that is affected by regulatory T cell function. It was indeed ascertained that RRV-infected BA mice have both a quantitative and qualitative deficiency in Tregs early in disease.

In order to determine the reason for reduced ability of BA mouse Tregs to suppress anti-CD3 mediated proliferation, we sought to find any differences in effector markers associated with Treg function, namely CD25, CD44, and CD62L. It has previously been demonstrated that Treg cells that are deficient in CD25 expression can lead to increased susceptibility to autoimmune disease [32, 33]. In addition, the adhesion marker CD44 is directly associated with suppressor activity in Foxp3⁺ Tregs and that costimulation via this molecule leads to production of regulatory cytokines [34, 35]. Lastly, Treg function, in the form of proper migration to secondary lymphoid tissue and to delay autoimmune disease progression, is modulated by the adhesion marker CD62L (L-selectin) [36, 37]. As all three of these markers are decreased in BA mice, Tregs in BA mice are likely to have reduced overall ability to suppress effector T cell function and are less able to migrate to inflamed tissue. Of note is the finding that Foxp3⁺ cells in BA mice do not have reduced expression of the glucocorticoid-induced TNFR family-related protein (GITR). This is not surprising due to the fact that the co-expression of Foxp3 and GITR is one of the best markers of T cells which have regulatory potential [38].

While this study does demonstrate the association that Treg function has in murine BA, several questions remain. First, it is unknown what is the cause of the diminished liver Treg number in RRV-inoculated mice. Does presence of rotavirus hinder release of Tregs from the thymus, or are there changes present in the chemokine receptors on BA mouse Tregs which render them less able to home to the site of inflammation, or are Tregs more susceptible to apoptosis following RRV infection? [39] Analysis of splenic Tregs compared to liver Tregs might help address this. The fact that replenishment of BA mice with functional Tregs ameliorates disease raises additional inquiries. As regulatory T cells affect both infectious disease immune responses and autoimmune inflammation [40], the timing of Treg transfer into RRV-inoculated mice may have differential effects. Theoretically, Treg transfer earlier than day 5, when the primary immune response is anti-viral, may have a deleterious effect in reducing the clearance of RRV. Conversely, delayed reconstitution of Tregs beyond day 5 may not overcome a rampant inflammatory response, as suggested in another mouse model of autoimmune disease [41].

One intriguing result of the adoptive transfer study is the fact that mice receiving Teff cells have accelerated disease compared to untreated BA controls. Several other mouse models of autoimmune disease have demonstrated the effect that polyclonal naïve T cells have on disease progression [44, 45]. This may be due to a change in the balance between numerous T cell subsets (i.e. Th1, Th17, Th2) following transfer. Also, potentially autoreactive cells present in the transferred population could be subjected to the inflammatory milieu present in BA mice and undergo stimulation and expansion. In addition, it is likely that polyclonal stimulation of Teffs can increase IL-17, IFN γ , or TNF α , resulting in increased liver-targeted macrophage responses. [42]

A potential limitation of the study presented here is the use of the PC-61 anti-CD25 antibody as a method for eliminating Tregs. Multiple studies have demonstrated that the CD25 marker is insufficient in identifying all functional Tregs in mice. Ono et al. determined that a population of cells that were CD25 negative, yet positive for Foxp3 and GITR, were capable of suppressing autoimmune myocarditis [38]. Other studies have shown that Foxp3⁺ cells remain, albeit at reduced numbers, in mice who have been treated with PC61 [43] [44]. Even so, use of PC61 to deplete Tregs has been demonstrated in the current and other studies [14] [28] [45] to have a significant impact on disease progression. Use of other methods to remove Tregs *in vivo* in BA-resistant mice, such as Foxp3-DTR mice [29] or use of anti-GITR antibodies [38], may result in more severe reconstitution of disease.

In summary, this study conclusively shows that the RRV-induced murine model of BA is associated with defects in the production and function of Tregs, resulting in inflammatory-mediated bile duct destruction. Recently, our laboratory reported that human BA also is associated with a significant decrease in peripheral blood Treg numbers, and that this decrease is even more striking in BA patients that have T cell reactivity to CMV [46]. One can, therefore, postulate that dysfunctional Tregs play an important role in the pathogenesis of human BA. Future studies aimed at expanding Tregs *in vivo* may eventually translate to clinical therapies for the treatment of this destructive pediatric biliary disease. These new treatments that target Treg function have the goal of protecting the intrahepatic bile ducts from inflammatory injury, increasing patient survival with their native liver, and delaying or negating the need for transplant.

Acknowledgments

Grant support: NIDDK 5R01DK78195 (PI: Mack, C), University of Colorado School of Medicine Dean's Academic Fund

Abbreviations

BA	Biliary Atresia
RRV	Rhesus rotavirus
Tregs	regulatory T cells
APCs	antigen presenting cells

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Figure 1. Regulatory T cells are reduced in both number and functional ability in BA mice and have reduced expression of activation and adhesion molecules

A. Determination of Treg frequency as a percent of liver CD4⁺ cells. 2 week old BA or control mouse livers were pooled, leukocytes were purified by Percoll gradient, and cells were stained for CD4 and Foxp3. *p=0.006

B. Determination of absolute numbers of Tregs per individual liver was performed following flow cytometry. *p=0.04

C. Suppression assay using Tregs isolated from either BA or BSS control mouse spleens. CD4⁺Foxp3⁺ cells were purified from Foxp3-GFP mouse spleens at 2 weeks of age and added in increasing amounts to adult mouse naïve CD4⁺FoxP3⁻ responder (Tresp) cells. Proliferation was determined by H³ thymidine incorporation. Percent suppression was calculated as the ratio of proliferation as compared to maximal proliferation (Tresp alone). p=0.009. Standard error ranged from 0–2.1% for control samples and 0–10.6% for BA samples.

D. Summary of flow cytometry of CD4⁺Foxp3⁺ Tregs from either spleens or livers of 2 week old BA and control mice co-stained for additional cell surface markers. *p<0.05, **p<0.005

E. Representative flow cytometry diagrams of BA and control mouse splenocytes. Percent of CD4⁺ cells positive for indicated marker is noted.

Figure 2. Adoptive transfer of Tregs into RRV-infected BA mice on day 5 of life ameliorates disease

A. Purification of cells for adoptive transfer. Treg ($CD4^{+}Foxp3^{+}$) and Teff ($CD4^{+}Foxp3^{-}$) cells were purified from naïve $Foxp3-GFP$ adult mouse splenocytes to >95% purity.

B. Kaplan-Meier survival analysis of mice. 16–19 mice for each group were followed for survival post-transfer. * $p=0.02$ for BA vs. BA+Treg, ** $p<0.001$ for BA+Teff vs. BA+Treg.

C. Direct bilirubin was measured from pools of 2 week old mouse serum. * $p=0.0001$

D. Histological analysis of cellular infiltrate in extrahepatic and intrahepatic bile ducts. H&E staining of extrahepatic (EHD) and intrahepatic (IHD) bile ducts from 2 week old RRV-infected mice that received either Tregs or Teffs. Arrows highlight bile duct epithelia. Magnification 400x. Representative of 3 experiments.

E. Immunohistochemical staining of liver sections from 2 week old RRV-infected mice that received either Tregs or Teffs. Immune cells ($CD4^{+}$, $CD8^{+}$ or $CD11b^{+}$ cells) are stained green, bile duct epithelial cells (cytokeratin 19) are stained red and nuclei are stained blue. Magnification 200x.

Figure 3. Adoptive transfer of Tregs into RRV-infected mice resulted in a non-diseased status profile of infiltrating cells and cytokine production

A. Cellular infiltrate analysis of liver leukocytes in 2 week old mice. Pools of livers (n=2–4 livers/pool) were stained for CD45 to gate for leukocytes and co-stained for other cell surface markers. *p<0.05, **p<0.01.

B. Summary of *ex vivo* intracellular cytokine staining of liver leukocytes from 2 week old mice. Pools of livers were stimulated with PMA and ionomycin for 3 hours. Cells were stained for surface markers, then permeabilized and stained for presence of cytokines. *p<0.05, **p<0.01.

C. Representative flow cytometry diagrams of *ex vivo* intracellular cytokine staining. Percent of CD4⁺ cells positive for indicated cytokine is noted.

Figure 4. Depletion of Tregs in BA-resistant mice lead to onset of biliary inflammation and obstruction

A. Administration of PC61 anti-CD25 antibody effectively depletes Tregs. Flow cytometry of splenocytes from treated animals 2 weeks post-treatment.

B. Measurement of direct bilirubin in pooled serum of PC-61 or control antibody treated mice. * $p=0.005$

C. H&E staining of extrahepatic (EHD) and intrahepatic (IHD) bile ducts from Treg-depleted or control mice 2 weeks following RRV infection. Magnification 400x. Representative of 3 experiments.

D. Immunohistochemical staining of liver sections from Treg-depleted mice or control IgG mice. Immune cells ($CD4^+$, $CD8^+$ or $CD11b^+$ cells) were stained green, bile duct epithelial cells (cytokeratin 19 $^+$) were stained red and nuclei were stained blue. Magnification 200x.