What Causes Biliary Atresia? Unique Aspects of the Neonatal Immune System Provide Clues to Disease Pathogenesis

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SUMMARY

Biliary atresia is a devastating cholestatic liver disease of children of unknown etiology. Research pertaining to the immunopathogenesis of biliary atresia should focus on unique aspects of neonatal immunity that promote aggressive and ongoing inflammation and fibrosis early in life.

Biliary atresia (BA) is the most frequent identifiable cause of neonatal cholestasis, and the majority of patients will need liver transplantation for survival. Despite surgical intervention with the Kasai portoenterostomy, significant fibrosis and cirrhosis develop early in life. An increased understanding of what causes this inflammatory fibrosing cholangiopathy will lead to therapies aimed at protecting the intrahepatic biliary system from immune-mediated damage. This review focuses on studies pertaining to the role of the adaptive immune response in bile duct injury in BA, including cellular and humoral immunity. The neonatal presentation of BA prompts the question of what potential modifications of unique aspects of the neonatal immune system set the stage for the progressive biliary disease. This review also discusses the characteristics of neonatal immune response and the theories on how alterations of this response could contribute to the pathogenesis of BA. These include aberrant type 1 helper T-cell (Th1) and Th17 responses, deficiencies in regulatory T cells, activation of humoral immunity, and autoimmunity. To advance our understanding of the etiology of BA, future studies should focus on the unique aspects of the neonatal immune system that have gone awry. (Cell Mol Gastroenterol Hepatol 2015;1:267–274; http://dx.doi.org/10.1016/j.jcmgh.2015.04.001)

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Biliary atresia (BA) is the most frequent identifiable cause of neonatal cholestasis, occurring in approximately 1 out of 12,000 live births in the United States and accounting for an estimated 350 new cases annually. It is most common in Taiwan (~1.5,600 live births) and occurs more frequently in females, Asians, and African Americans. There are three types of BA: isolated BA (84% of cases), BA with at least one malformation but without laterality defects (6%; cardiovascular, gastrointestinal, or genitourinary defects), and BA splenic malformation, a syndrome associated with laterality defects and polysplenia or asplenia (4% to 10%). In isolated BA, meconium and initial stools are normal in color, suggesting early patency of the ducts. However, within the first 3 months of age, the extrahepatic biliary tree becomes obstructed, and the pathology is consistent with an inflammatory fibrosing cholangiopathy. At diagnosis, the extrahepatic biliary remnant is removed, and a Kasai portoenterostomy is performed in an attempt to reestablish bile flow. This results in initial restoration of bile flow in up to two-thirds of patients if performed within 60 days of life.

Even with surgical intervention, significant fibrosis and cirrhosis develops early in life, and the majority of patients will need liver transplant for survival. Analysis of liver tissue from BA patients >4 years old after a Kasai portoenterostomy revealed that, despite resolution of cholestasis in 83% of patients, 100% of patients had fibrosis (Metavir stage >2) or cirrhosis. On average, 20% of children with BA will enter adulthood with their native liver, and the vast majority of those patients will have evidence of chronic liver disease or cirrhosis. An increased understanding of what causes the inflammatory sclerosing cholangiopathy of BA could lead to therapies aimed at protecting the intrahepatic biliary system from inflammatory-mediated damage and fibrosis.

The etiology of BA is unknown, and theories of its pathogenesis include perinatal virus infection targeting cholangiocytes, chronic inflammatory or autoimmune-mediated bile duct injury, and abnormalities in bile duct development. A recent retrospective study of neonatal direct bilirubin levels obtained at 24 to 48 hours of life has shed light on the timing of the initial bile duct injury in BA. In that study, neonatal direct bilirubin levels were obtained for all newborns in a single hospital between 2007 and 2010, and the infants who later developed isolated BA were compared with newborns from the same period who did not have BA. The BA newborns had mean direct bilirubin levels
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Adaptive Cellular Immunity: T-Cell Subsets

Adaptive cellular immunity involves the interaction of antigen-presenting cells with T cells, resulting in activation of T cells with the production of cytokines. Adaptive immune responses are triggered by repeat exposure to both pathogen and non-microbial antigens, resulting in highly specific memory T-cell activation. Aspects of adaptive cellular immunity that characterize the neonate include decreased frequencies and function of dendritic cells (antigen-presenting cells) compared with adults and T-cell responses that are skewed to a type 2 helper T cell (Th2) profile, with the production of interleukin 4 (IL-4), IL-5, and IL-13. It is becoming clear that neonates are also capable of generating adult-like Th1 responses (IL-2, interferon-γ [IFN-γ]) when the conditions for antigenic priming are optimized. Over 30 years ago, Hoffman et al described T-cell responses in neonatal mice and found that with a high dose of a murine leukemic virus (>1,000 plaque-forming units) led to nonprotective Th2 responses and disease. In stark contrast, a low exposure of virus (0.3 plaque-forming units) to the neonate induced a virus-specific Th1 response with clearance of virus. These studies have led to the hypothesis that BA pathogenesis could be related to low-dose neonatal virus infection with proinflammatory Th1 immune responses. Multiple studies have since shown that neonates are able to mount fully mature Th1 responses under certain circumstances, which increases costimulatory signals on antigen-presenting cells. It can therefore be theorized that an abnormal skewing of the T-cell response in the neonate from the default Th2 response to the inflammatory Th1 response could be an early event that promotes ongoing T-cell-mediated bile duct injury in BA.

The predominant cellular immune response in BA at diagnosis encompasses activated CD4+ and CD8+ T cells within portal tracts that produce Th1 cytokines (IL-2, IFN-γ, tumor necrosis factor α [TNF-α]) and macrophages secreting TNF-α, IFN-γ, and IFN-γ. These lymphocytes have been found invading between bile duct epithelia, resulting in degeneration of intrahepatic bile ducts. With the aim of understanding whether the inflammation is nonspecific (bystander activation) versus antigen specific with expansion of clones of T cells, T-cell receptor characterization was performed. Analysis of the T-cell receptor variable region of the β-chain (Vβ) within BA liver and extrahepatic bile duct remnants revealed that the T cells were indeed oligoclonal in nature with a limited T-cell receptor Vβ repertoire, suggesting antigen-specific activation. The exact antigen(s) stimulating the clonal expansions remains a mystery that if solved will provide a wealth of information on the processes of T-cell-mediated bile duct injury in BA.

Th1 Cellular Immunity

To perform mechanistic studies of immune-mediated hypotheses, the Rhesus group A rotavirus (RRV)-induced mouse model of BA (murine BA) has been employed by many investigators. This model mimics many aspects of the human disease, including bile duct epithelial apoptosis, portal inflammation, intrahepatic bile ductule proliferation, and extrahepatic biliary obstruction. The main limitation of the mouse model is that the extrahepatic biliary fibrosis is minimal compared with humans and the biliary obstruction is mainly due to inflammation and edema.

Many investigators view the findings in the mouse model as representative of the early events in human BA. In murine BA the virus is cleared within the first 2 weeks of life, a time point when extrahepatic biliary obstruction is complete. The CD4+ Th1 cellular inflammatory environment found in murine BA recapitulates the human disease, and the progressive inflammatory destruction and obliteration of the bile ducts leads to death by 3 weeks of age. In support of a Th1 cytokine environment in BA mice, many investigators have described increased levels of chemokines that promote Th1 cellular differentiation [chemokine (C-C motif) ligand 2, chemokine (C-C motif) ligand 5, C-X-C motif chemokine 10]. IFN-γ is a necessary cytokine in the pathogenesis of murine BA, as RRV-infected IFN-γ knockout mice are protected from developing biliary obstruction and have a dramatic increase in survival.
Depletion of the CD8\(^+\) T-cell subset was also associated with increased survival, and the CD8\(^+\) T cells from BA mice were found to be directly cytotoxic to cholangiocytes in vitro.\(^{25}\) A recent study by Zheng et al.\(^{26}\) further analyzed the CD8\(^+\) T-cell response in murine BA. Multiple RRV nonstructural protein 4 (NSP4) constructs were created to assess which viral epitope was responsible for CD8\(^+\) T-cell activation. A computer-based program was utilized that predicted which NSP4 viral epitopes would most likely interact with CD8\(^+\) T cells. A fusion protein composed of glutathione S-transferase and NSP4 (GST-NSP4), as well as NSP4\(_{144-152}\) and NSP157-170 epitopes, were all recognized by CD8\(^+\) T cells and induced CD8\(^+\) T-cell IFN-\(\gamma\) production, similar to that found with RRV stimulation. Injection of neonatal mice with GST-NSP4, and not other viral constructs, led to biliary obstruction similar to RRV-infected mouse pups. Liver CD8\(^+\) T cells from NSP4\(_{144-152}\), NSP157-170, and GST-NSP4 injected mice that were cultured with bile duct epithelial cells led to direct epithelial cytotoxicity. The fact that the viral epitope-specific liver CD8\(^+\) T cells also recognized proteins within bile duct epithelia led to direct epithelial cytotoxicity. The fact that the viral epitope-specific liver CD8\(^+\) T cells also recognized proteins within bile duct epithelia and elicited cellular damage suggests molecular mimicry as a potential mechanism of autoimmune activation. The researchers concluded that NSP4 is a pathogenic immunogen that initiates the inflammatory response, resulting in bile duct epithelial injury in murine BA. Collectively, these studies suggest that CD4\(^+\) T\(_{H1}\) cells may activate CD8\(^+\) cytotoxic T cells and that both subsets contribute to the biliary injury and obstruction in BA.

The murine BA model has been used to understand the role of cellular autoimmunity in bile duct injury. Periduct inflammation involves an influx of bile duct epithelial-specific IFN-\(\gamma\)-producing T cells.\(^{27}\) This was determined based on liver memory T-cell activation when cells were cultured with a bile duct epithelial homogenate protein source. In vitro analysis revealed that inhibition of CD4\(^+\) T cells, but not CD8\(^+\) T cells, was associated with loss of IFN-\(\gamma\) production, identifying the CD4\(^+\) T cell as the key cell type associated with bile duct-specific autoimmune activation. Adoptive transfer of the liver T cells from BA mice into immunodeficient recipient mice resulted in bile duct-targeted inflammation.\(^{25,27}\) Similar to human BA, the exact identity of the bile duct antigens that are being targeted has not been elucidated.

**T\(_{H17}\) Cellular Immunity**

IL-17 has been implicated as a major pathogenic cytokine contributing to autoimmune-mediated diseases. Neohates have an enhanced ability to mount proinflammatory T\(_{H17}\) responses, based on research showing that TLR-stimulated cord blood cells produce high levels of IL-6 and IL-23, necessary cytokines for T\(_{H17}\) differentiation.\(^{29}\) In addition, cultured cord blood CD4\(^+\) T cells can generate significant amounts of IL-17.\(^{30}\) In the neonatal setting of a fully mature T\(_{H17}\) pathway, is it possible that an aggressive, persistent T\(_{H17}\) response plays a role in bile duct damage in BA? A recent study in BA found that serum IL-17a and IL-23 levels were increased in BA patients compared with healthy age-matched controls.\(^{31}\) In addition, the ratio of T\(_{H17}\) cells/ regulatory T cells (Tregs) was significantly higher in the peripheral blood of BA patients. BA liver tissue had increased mRNA expression of ROR-\(\gamma\) (IL-17 transcription factor), IL-17a, IL-17b, IL-6, and transforming growth factor \(\beta1\), an increased number of IL-17a infiltrating cells, and a decreased ratio of Treg/CD4\(^+\) T cells. This study implies that T\(_{H17}\) inflammatory pathways dominate and overcome the regulatory T-cell response, contributing to biliary injury in BA. T\(_{H17}\) cell-mediated immunity requires further investigation to determine the significance of IL-17 to BA pathogenesis.

**Regulatory T Cells**

The Treg subset of CD4\(^+\) T cells is responsible for controlling immune responses to prevent “bystander damage” of healthy tissue and to prevent activation of autoreactive T cells. The Treg subset expresses the cell surface marker CD25\(_{\text{high}}\) and the transcription factor forkhead box P3 (Foxp3).\(^{32}\) Tregs inhibit cells involved in adaptive immunity (T- and B-cell responses) and innate immunity (macrophages, dendritic cells, and natural killer cells).\(^{33,34}\) In human neonates, the percentage of Tregs in peripheral blood increases significantly in the first 5 days of life, reaching adult levels at that time.\(^{35}\) Recent studies suggest that there is a significantly greater number of Tregs in cord blood compared with adult Tregs.\(^{36}\) Furthermore, cord blood Tregs are highly functional and can suppress T-cell proliferation and T\(_{H17}\) IFN-\(\gamma\) production, similar to adult Treg function.\(^{37}\)

In neonatal mice, Tregs exit the thymus and travel to the spleen and lymph nodes on day 3 of life.\(^{32,33,38}\) Thymectomy in 3-day-old neonatal mice results in a spectrum of organ-specific autoimmune that can be prevented by reconstitution of the thymectomized animals early in life with normal adult Tregs.\(^{38-40}\) Autoimmune disease may also develop when exogenous insults, such as virus infection, disrupt the maturation or functioning of Tregs. Morse et al.\(^{41}\) showed that murine T lymphotropic virus infection on day 1 of life (but not on day 7 or 28) led to decreased release of Tregs from the thymus and the development of autoimmune gastritis. Kobayashi et al.\(^{42}\) found that the administration of a poly I:C virus mimic into neonatal thymectomized mice resulted in worsening incidence and severity of autoimmune gastritis and was associated with a significant...
decrease in the number of splenic Tregs. These studies reveal that neonatal viral infection can induce or exacerbate the propensity for autoimmunity due to Treg deficiencies, which sets the stage to study this mechanism of autoimmunity in the pathogenesis of BA.

In murine BA, RRV infection must take place in the first 48 hours of life to induce biliary disease, and the incidence of disease is highest when virus is administered in the first 24 hours of life. The necessity of early age at the time of viral infection to generate disease leads to the question of whether this neonatal virus infection could alter the release of Tregs from the thymus or decrease their suppressive capacity in the periphery, thus allowing for pathogenic autoreactive T cells and inflammation to flourish, stimulating effector cell functions (Figure 1). Miethke et al. characterized the frequency of Tregs in neonatal mice and found that the liver and spleen of 3-day-old mice had significantly fewer Tregs compared with 7-day-old mice, similar to previous studies showing that Tregs begin migration to the periphery on day 3 of life. The Treg deficit in week 1 was associated with enhanced dendritic cell activation of natural killer (NK) cells, resulting in biliary injury and obstruction. Lages et al. showed that adoptive transfer of total CD4$^+$ T cells, but not Treg-depleted CD4$^+$ T cells, into RRV-infected mice was associated with increased survival and diminished CD8$^+$ T-cell cytotoxicity. Tucker et al. reported significant deficits in liver Treg frequencies as well as Treg suppressive function in BA mice. In addition, adoptive transfer of highly purified adult Tregs into RRV-infected BA mice prevented the development of biliary obstruction, dramatically increased survival and inhibited Th1 cell-mediated biliary injury. These complimentary studies demonstrate that Treg frequency and function are diminished in BA; further analysis of the mechanisms

![Figure 1. Hypothetical model of the role of Treg deficits in BA pathogenesis.](image)

Neonatal virus infection in the genetically predisposed individual may (1) alter the release of Tregs from the thymus or (2) decrease their regulatory capacity in the periphery, allowing for autoreactive CD4$^+$ T-effector cells (Teffs) to flourish and (3) activate macrophages, cytotoxic CD8$^+$ T cells (CTLs), and autoantibody-producing B cells, leading to progressive bile duct epithelial injury.
contributing to the Treg deficiencies is warranted. Future therapies aimed at enhancing Treg numbers and suppressive capabilities in BA may lead to protection of the intrahepatic biliary tree from ongoing damage.

Adaptive Humoral Immunity and B Cells

Humoral immune responses are initiated by interaction of antigen with the B-cell receptor (BCR) and direct cell contact with CD4+ T cells and/or Toll-like receptor ligands. The BCR is composed of a membrane-bound form of IgM (binds antigen [Ag]) and the signal transduction moiety Ig-α/Ig-β that is necessary for activation. The engagement of BCR by Ag leads to activation and proliferation of Ag-specific B-cell clones that differentiate into either plasmablasts or germinal center B cells, which then give rise to plasma cells or memory B cells. In neonatal immunity, murine studies demonstrate that predominantly IgM antibodies respond to T-cell–independent antigens such as plant lectins, polysaccharides, and polymerized proteins, as well as self-bile duct epithelia in the extrahepatic biliary remnant of BA mice and discovered autoantibodies reactive to cytotoxic proteins within bile duct epithelia. One such protein was identified as α-enolase, and significant elevations of α-enolase autoantibodies were uniquely present in BA mouse sera. One possible theory to explain the increase in production of autoantibodies in the setting of previous virus infection is that there is molecular mimicry between virus and self proteins. In this study, a high degree of sequence homology between enolase and rotavirus proteins VP4 and VP8 were identified, and the anti-enolase antibodies bound to both enolase and rotavirus, suggesting molecular mimicry as a mechanism of the autoimmune response. In addition, high levels of IgM and IgG α-enolase autoantibodies were detected in ~40% of infants and children with BA. Interestingly, anti-enolase antibodies have been found in other autoimmune diseases including autoimmune liver diseases, suggesting that this antibody may be a nonspecific marker of autoimmunity.60,61 Future research centered on identifying potentially pathogenic, bile-duct specific autoantibodies should be pursued.

B cells are not only responsible for production of antibodies, but also play a key role as professional antigen-presenting cells (APCs), with subsequent T-cell activation. Naïve neonatal APCs have limited ability to activate T cells due to low levels of major histocompatibility complex class II and costimulatory molecules.62 However, neonatal mice exposed to low levels of replicating virus display increased antigen-presentation capabilities, with subsequent Tg11 cell activation and cytotoxic T-cell function.63 Perhaps a similar virus-induced APC activation is occurring in the neonate with BA. To assess the importance of B cells in BA pathogenesis, Feldman et al64 used mice deficient in Ig-α (Ig-α−/−) that have loss of BCR expression and function, resulting in defective B-cell antigen presentation and immunoglobulin production. RRV-infected Ig-α−/− mice had dramatically increased survival and lack of bile duct obstruction. Significantly decreased numbers of liver CD4+ T cells, NK T cells, and NK cells and macrophages were observed in RRV-infected Ig-α−/− mice compared with wild-type mice. Similar to other B-cell depletion studies,65-68 the RRV-infected Ig-α−/− mice had increased levels of Tregs, suggesting a link between B-cell activation and Treg inhibition. In addition, lack of T-cell activation in RRV-infected Ig-α−/− mice was demonstrated based on markedly decreased production of IFN-γ and TNF-α from CD4+ T cells and IFN-γ from CD8+ T cells. This implies that without B-cell antigen presentation, the T cells are not activated, which suggests a possible mechanism of protection from disease. B cells appear to play a critical role in the RRV-induced mouse model of BA, and future studies aimed at deciphering the specific role of antigen presentation and production of pathogenic autoantibodies are necessary to understand the impact of B cells in disease pathogenesis.

Summary

Biliary atresia is a devastating disease wherein the vast majority of patients require liver transplantation for

Liu et al57 performed immunoblot analysis of sera from BA mice and discovered autoantibodies reactive to cytotoxic proteins within bile duct epithelia. One such protein was identified as α-enolase, and significant elevations of α-enolase autoantibodies were uniquely present in BA mice. One possible theory to explain the increase in production of autoantibodies in the setting of previous virus infection is that there is molecular mimicry between virus and self proteins. In this study, a high degree of sequence homology between enolase and rotavirus proteins VP4 and VP8 were identified, and the anti-enolase antibodies bound to both enolase and rotavirus, suggesting molecular mimicry as a mechanism of the autoimmune response. In addition, high levels of IgM and IgG α-enolase autoantibodies were detected in ~40% of infants and children with BA. Interestingly, anti-enolase antibodies have been found in other autoimmune diseases including autoimmune liver diseases, suggesting that this antibody may be a nonspecific marker of autoimmunity.60,61 Future research centered on identifying potentially pathogenic, bile-duct specific autoantibodies should be pursued.

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Summary

Biliary atresia is a devastating disease wherein the vast majority of patients require liver transplantation for
survival. It is critical to grasp the immunopathogenesis of BA in order to provide future therapies that control the intrahepatic biliary inflammation and prevent subsequent fibrosis. Evidence exists for a key role of both arms of the adaptive immune response in bile duct injury. The neonatal presentation of BA provides a clue to disease pathogenesis. Early events that impact the neonatal immune system (ie, perinatal virus infection) may alter the immune response and promote a progressive inflammatory or biliary autoimmune disease. To advance our understanding of the etiology of BA, future studies should focus on those unique aspects of the neonatal immune system that have gone awry, as detailed throughout this review.

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The authors disclose no conflicts.

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