Published in final edited form as:

Semin Liver Dis. 2014 August ; 34(3): 273–284. doi:10.1055/s-0034-1383727.

Role of Cholangiocytes in Primary Biliary Cirrhosis

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Abstract

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by selective destruction of intrahepatic cholangiocytes. Mechanisms underlying the development and progression of the disease are still controversial and largely undefined. Evidence suggests that PBC results from an articulated immunologic response against an immunodominant mitochondrial autoantigen, the E2 component of the pyruvate dehydrogenase complex (PDC-E2); characteristics of the disease are also the presence of disease-specific antimitochondrial autoantibodies (AMAs) and autoreactive CD4 and CD8 T cells. Recent evidence suggests that cholangiocytes show specific immunobiological features that are responsible for the selective targeting of those cells by the immune system. The immune reaction in PBC selectively targets small sized, intrahepatic bile ducts; although a specific reason for that has not been defined yet, it has been established that the biliary epithelium displays a unique heterogeneity, for which the physiological and pathophysiological features of small and large cholangiocytes significantly differ. In this review article, the authors provide a critical overview of the current evidence on the role of cholangiocytes in the immune-mediated destruction of the biliary tree that characterizes PBC.

Keywords

cholangiocytes; intrahepatic bile ducts; apoptosis; mitochondrial antigens

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by selective destruction of intrahepatic cholangiocytes.¹ Evidence suggests that PBC results from an

articulated immunologic response against an immunodominant mitochondrial autoantigen, the E2 component of the pyruvate dehydrogenase complex (PDC-E2); characteristics of the disease are also the presence of disease specific anti-mitochondrial autoantibodies (AMAs) and autoreactive CD4 and CD8 T cells.^{2,3}

Similar to many autoimmune diseases, the etiology and pathogenesis of PBC remains largely unknown, even though there is increasing evidence for the interplay of genetic and environmental factors in individual host susceptibility.⁴ A major void in the bridge from the loss of tolerance to clinical pathology is the enigmatic observation that while mitochondria are found in all cells, only cholangiocytes are destroyed in PBC. Moreover, PBC does not target homogeneously the biliary tree because it selectively affects small- to medium-sized intrahepatic bile ducts whereas large intra- or extrahepatic bile ducts are not targeted by this pathology.² Such a feature influences the clinical presentation of the disease and its complications.¹ The reasons why PBC selectively targets small bile ducts are still unclear; however, there is evidence to believe that it may depend on the heterogeneous response of cholangiocytes to the immune-mediated injury. Indeed, cholangiocytes are active players in both innate and adaptive immune responses through various immunological pathways, and are actively involved in the first line of defense of the biliary system against foreign substances.⁵ Of relevance in PBC, PDC-E2 remains immunologically intact within human intrahepatic cholangiocytes undergoing apoptosis,⁶ it translocates into apoptotic bodies,⁷ and it is still recognizable within them as such by AMAs.⁷ Further, we have shown the critical requirement of innate immune cells from PBC patients to produce proinflammatory cytokines in response to biliary apoptoses in the presence of AMAs.⁸ Finally, PBC reoccurs after liver transplantation, suggesting that all cholangiocytes, even from unaffected subjects, have unique biological properties that in the right setting can trigger the development of autoimmune cholangitis.⁹ In this review article, we will provide a critical overview of the current evidence on the role of cholangiocytes in the immune-mediated destruction of the biliary tree that characterizes PBC.

Morphological and Functional Heterogeneity of the Biliary Epithelium

The biliary epithelium is a complex interconnected system of tubular conduits, lined by epithelial cells named cholangiocytes, which drains canalicular bile into the duodenum. In human liver, the nomenclature of the different branches of the biliary epithelium refers to the classification originally proposed by Ludwig in 1987.¹⁰ Bile ducts are thus divided according to their diameter: bile ductules (or cholangioles) (< 15 μ m), interlobular ducts (15–100 μ m), septal ducts (100–300 μ m), area (or zonal) ducts (300–400 μ m), segmental ducts (400–800 μ m), and hepatic ducts (> 800 μ m). At the periphery of the biliary epithelium, bile ductules, which are entirely constituted by cholangiocytes, cross the limiting plate and continue with the canal of Hering. The latter is lined by both hepatocytes and cholangiocytes and represents the physical link between the bile canaliculus, formed by the apical membrane of hepatocytes, and the biliary tree.¹¹

Apart from the sake of classification, the distinction of bile ducts according to their diameter allows the identification of substantial differences in the biology of the different cholangiocytes subpopulations that line bile ducts of different sizes.¹²

Morphological Heterogeneity

Numerous studies have shown that cholangiocytes forming the finest branches of the biliary tree (namely the bile ductules in humans and small ducts of diameter < 15 μm in rats)^{10,11} display profound differences with regard to (1) morphological features, (2) secretory function, and (3) proliferative and apoptotic responses to liver injury.¹¹

Previous studies in cholangiocytes isolated from normal rat liver by counterflow elutriation identified two distinct subpopulations of small (~ diameter of 8 μm) and large (~ diameter of 14 μm) cholangiocytes.¹³ Despite that the area of cholangiocytes measured in morphometric studies varies between 3 and 80 μm^2 , the study demonstrated a significant relationship between the diameter of the ducts and the area of the cholangiocytes, demonstrating that small cholangiocytes originate from small bile ducts (< 15 μm in diameter), whereas large cholangiocytes were isolated from large bile ducts (>15 μm in diameter).¹³ The same findings have been confirmed in small and large bile duct units (IBDU) isolated from rat liver.¹² Both small and large cholangiocytes have a multilobulated nucleus, tight junctions, numerous microvilli, and show abundant subapical vesicles.¹⁴ Another study further characterized the morphological heterogeneity of bile ducts showing that the nucleus-to-cytoplasm ratio of cholangiocytes is inversely correlated to the size of the ducts, and that the shape of the cells changes from roughly cubic in the smaller ducts to columnar in the larger,¹⁴ confirming the presence of two distinct subpopulations of cholangiocytes shaping the biliary tree.

Functional and Secretory Heterogeneity

A striking heterogeneity in gene and protein expressions along the biliary epithelium is well described.^{15,16} For example, microarray analysis in small and large cholangiocytes from normal mice identified as much as 230 cDNA (4.73% of the total amount of cDNA screened) to be differentially expressed in the two subpopulations of biliary cells.¹⁷ This diverse protein expression undoubtedly affects the secretory functions of small and large cholangiocytes. To this extent, cholangiocytes actively participate in up to 40% of the bile flow¹⁸ (the so-called bile salt-independent bile flow), for the most part through the activation of a series of coordinated events that induce the active secretion of bicarbonate in bile. Indeed, upon activation of the basolateral secretin receptor (SR),¹⁹ cAMP levels increase in cholangiocytes leading to the PKA-dependent phosphorylation of CFTR, with subsequent extrusion of Cl^- .²⁰ As a consequence of the favorable Cl^- gradient across the plasma membrane, the activation of the $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger 2 (AE2), located at the apical membrane of cholangiocytes,²¹ leads to the secretion of bicarbonate into bile.^{22,23} Interestingly, the expression of the machinery required for bicarbonate secretion is constitutively present mainly in normal large cholangiocytes. Specifically, studies have shown that small cholangiocytes lack the expression of SR, CFTR, and AE2, whereas these cells express the mRNA for both γ -glutamyltranspeptidase (γ -GT) and cytokeratin-19 (CK-19).^{13,24} Moreover, only large IBDU functionally respond to secretin stimulation with increased levels of cAMP, $\text{Cl}^-/\text{HCO}_3^-$, AE2 activity, and ductal secretory activity.¹² Of note, various studies demonstrated the presence of impairments in the expression or regulation of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger in PBC patients,^{25,26} and recent reports suggested that a defective biliary bicarbonate “umbrella” on the outer membrane of cholangiocytes

might be involved in the development of certain vanishing bile duct syndromes such as PBC.^{27,28} Interestingly, it has been recently demonstrated that miR-506 is upregulated in cholangiocytes from PBC patients, binds the 3'UTR region of AE2 mRNA, and prevents protein translation, leading to diminished AE2 activity and impaired biliary secretory functions.²⁹

Several different pathways (differentially expressed along the biliary tree) have been shown to alter secretin-stimulated ductal secretion. In line with the heterogeneous expression of SR, the somatostatin receptor (SSTR₂) is only expressed in large cholangiocytes, where its activation inhibits SR gene expression and secretin-induced cAMP synthesis in vitro,³⁰ and secretin-stimulated choleresis in rats with common bile duct ligation (BDL) in vivo.³¹ Similarly, the endothelin receptors ET_A and ET_B,³² the CCK-B/gastrin receptors,^{33,34} the D2 dopaminergic receptors,³⁵ and the M3 acetylcholine receptors³⁶ are solely expressed by large cholangiocytes. A recent review by Han et al has discussed these studies in detail.³⁷ Recently, adenylyl cyclase (AC) isoforms, the enzymes responsible for the production of intracellular cAMP, have also been shown to be differentially expressed along the biliary tree.³⁸ In particular, the Ca²⁺/calmodulin-stimulated AC8, the Ca²⁺-inhibitable AC9, and the soluble AC mediate cholangiocyte secretion in response to secretin, β -adrenergic agonists, or changes in the intracellular concentration of HCO₃⁻, respectively, whereas small cholangiocytes predominantly express the Ca²⁺-insensitive isoforms AC4 and AC7.³⁸

Small cholangiocytes, however, are far from being an inert compartment of biliary cells that only allow the flowing of bile to progressively larger ducts. The presence of adenosine triphosphate (ATP) in bile,³⁹ and the finding that ATP stimulates Cl⁻ currents in biliary epithelia in a CFTR-independent fashion have recently added an alternative pathway to the secretin-stimulated ductal secretion relying on cAMP-dependent opening of CFTR channels. Interestingly, biliary cells along the bile ducts evenly express a repertoire of purinergic (P2) receptors, the activation of which induces a Ca²⁺-activated Cl⁻ efflux and consequently fluid secretion in both large and small cholangiocytes.⁴⁰ The nature of the Cl⁻ channel responsible for the Ca²⁺-activated Cl⁻ efflux has been identified with TMEM16A, a 114-kDa membrane protein with eight putative transmembrane domains present in both small and large biliary epithelial cells,⁴¹ which has also been involved in mechanosensitive Cl⁻ secretion in the biliary epithelium.⁴² Moreover, because the apical ATP release is twofold greater in small than in large cholangiocytes, a study has proposed the hypothesis of a possible axis along the intrahepatic biliary tree, with the ATP released by small ducts acting as a paracrine signaling molecule for the secretion in larger ducts.⁴⁰

Taken together, the peculiar cellular morphology of small cholangiocytes described before (large nucleus and small cytoplasm)¹⁴ and the lack of the secretin-stimulated ductal secretion may suggest a more undifferentiated status of these cells when compared with large cholangiocytes.⁴³

Heterogeneity of Cholangiocyte Proliferation and Survival

Important differences in the specific proliferative or apoptotic responses to several models of liver injury have also emphasized the distinction between large and small cholangiocytes, and at least in part supported the concept that the latter might represent a stem cell

compartment within the liver. It is well established that in a common model of cholestasis induced by BDL, only large cholangiocytes proliferate in response to damage.^{11,30,44} To this extent, several studies have helped elucidate the mechanisms underlying the proliferative capacity of cholangiocytes after BDL. Thus, among others, the expression level of estrogen receptor (ER)- β increases in biliary epithelial cells of rats after BDL, and treatment with tamoxifen or with the ER antagonist ICI 182,780 markedly decreases cholangiocyte proliferation.⁴⁵ On the same line, ovariectomy in BDL female rats reduces the proliferation of large biliary cells.⁴⁶ Also, gastrin inhibits large cholangiocytes proliferation after BDL in rats via a Ca^{2+} /PKC-dependent inhibition of cAMP levels.³⁴ Similarly, somatostatin has been shown to inhibit DNA synthesis solely in large cholangiocytes.³⁰ Inhibitory autocrine/paracrine effects of serotonin, secreted by proliferating cholangiocytes, have also been identified in vivo; indeed, cholangiocyte proliferation markedly decreases after 1 week of administration to BDL rats of the selective agonists of both serotonin 1A and 1B receptors.⁴⁷ Moreover, vagotomy in BDL rats significantly decreases M3 acetylcholine receptor expression, ductal mass, and biliary secretion.⁴⁸ Recently, the expression levels of both VEGF and VEGF receptors have been found to increase in biliary epithelial cells after BDL, and the neutralization of VEGF in vivo by anti-VEGF-A or anti-VEGF-C antibodies has proven effective in decreasing cholangiocyte proliferation.⁴⁹ Given the potent angiogenic effects of VEGF, it is interesting to note that the proliferative response of the peribiliary plexus to BDL only happens after the adaptive modifications of cholangiocytes,⁵⁰ suggesting a central role of these cells in orchestrating the complex events occurring in the liver in case of injury. In fact, in response to damage, cholangiocytes not only proliferate but acquire a neuroendocrine-like phenotype characterized by (1) the expression of neuroendocrine marker such as chromogranin A, (2) the secretion of a variety of cytokines, growth factors, neuropeptides, and hormones, and (3) an enhanced response to circulating hormones and neuropeptides,⁵¹ as briefly discussed in the present paragraph. Such neuroendocrine-like phenotype allows cholangiocytes to regulate the complex and still not completely defined interactions with all the other cells that populate the liver, such as hepatocytes, endothelial cells, hepatic progenitor cells (HPC), and also with immune cells that are recruited in the liver in case of damage.⁵¹

Small cholangiocytes, which do not proliferate after BDL, are however activated in other models of damage. Interestingly, carbon tetrachloride (CCl_4) administration to BDL or normal rats selectively damages large cholangiocytes by inducing an apoptotic response.^{52,53} Nonetheless, small cholangiocytes, which are probably protected from CCl_4 toxicity because of a lack of the cytochrome P450E1 (the enzyme that initiates CCl_4 -induced hepatobiliary damage), concomitantly proliferate to replenish the loss of larger biliary cells and de novo express the SR gene and show secretin-stimulated cAMP production.⁵³ Chronic bile acid feeding is also known to induce cholangiocyte proliferation.⁵⁴ Following administration of taurocholate or tauroolithocholate to rats for one week, not only large and small cholangiocytes proliferate, but also the latter de novo express ASBT in a Ca^{2+} /PKC-dependent fashion and exhibit bile acid transport activity.⁵⁵ Small cholangiocytes have been shown to proliferate also in a model of intrahepatic cholestasis, in which proliferation and apoptosis of small and large cholangiocytes coexist, induced by the administration of α -naphthylisothiocyanate (ANIT).⁵⁶ Interestingly, though the biology of

large cholangiocytes seems to rely mainly on cAMP-dependent signaling pathways,^{13,57} IP₃/Ca²⁺-dependent pathways are prevalent in small cholangiocytes.^{40,55,58} On this line, histamine has been recently shown to stimulate the proliferation of small bile ducts by the activation of the histamine receptor H1 via an IP₃/Ca²⁺-dependent fashion.⁵⁹ Activation of Ca²⁺-related pathways seems important also in inducing small biliary cells to differentiate into large biliary cells.⁶⁰ Indeed, after selective damage of large cholangiocytes by GABA administration to BDL rat, small cholangiocytes proliferate in vivo and acquire in vitro ultrastructural and phenotypical characteristic that are typical of large bile ducts and that can be inhibited by treatment with Ca²⁺ chelators.⁶⁰ Taken together, in addition to unveiling important aspects of intracellular pathways, the aforementioned studies strongly suggest the possibility that small cholangiocytes lining in the canal of Hering are the precursor of larger cholangiocytes and that they may be important in the replenishment of damaged or senescent biliary cells.

Highlights from Animal Models

Animal models represent an important tool to study the pathophysiology of chronic liver diseases. These models have been predominantly developed in rodents and can be roughly divided in two major categories: (1) general models of liver injury, in which hepatocytes and/or cholangiocytes are damaged by specific interventions that cause the final development of liver fibrosis; and (2) specific models that aim to resemble the pathophysiological alterations of a particular chronic liver disease, such as PBC.⁶¹

General Models of Cholestatic Liver Diseases

Among the first group, the ligation of the common bile duct, which involves a ventral laparotomy, the isolation of the common bile duct, and its resection between two ligatures, has been extensively used in rodents.⁶² After BDL in rodents, large cholangiocytes respond to the injury by increasing their proliferative activity and the induction of portal inflammation and fibrosis ultimately leads to the development of cirrhosis in 2 to 6 weeks.⁶¹ Interestingly, in contrast to other models of damage, BDL in rats appear not to be associated with apoptosis in cholangiocytes,⁴⁸ and some authors have speculated that the absence of apoptosis in the BDL model may be at least in part responsible for the lack of proliferation of small rat cholangiocytes.¹⁶ Bile duct ligation has also been successfully used in mice, despite a less reproducible response and higher mortality rates. The causes of such problems have been, at least in part, attributed to a differential dilatation of the gallbladder, which is absent in rats, and its possible rupture with bile leakage into the peritoneum, respectively.⁶³

Liver damage via a model of intrahepatic cholestasis can also be achieved by the administration of ANIT.⁶⁴ The exact mechanism of action of this toxin is still debated, but an important role has been attributed to the formation of radical species and lipid peroxidation,⁶⁵ and infiltration of neutrophils via a CD18-dependent mechanism.⁶⁶ Administration of α -naphthylisothiocyanate causes liver fibrosis in mice after 4 to 8 weeks, with a slow progression to cirrhosis.⁶⁷ Interestingly, ANIT has been also shown to cause proliferation and apoptosis of large and small cholangiocytes via a mechanism possibly involving the generation of reactive oxygen species (ROS) induced by ANIT itself.⁵⁶

Despite involving mainly large bile ducts and more closely resembling sclerosing cholangitis, the feeding of 0.1% DDC-supplemented diet in mice for up to 8 weeks induces a biliary-type liver fibrosis with the formation of portal-portal septa.⁶⁸ The damage induced by DCC has been related to an increased porphyrin secretion with formation of porphyrin-containing pigment plugs, some degree of cholestasis, and impaired glutathione excretion due to downregulation of Mrp2. However, no difference in the bile flow and bile salt excretion in bile could be detected in treated mice.⁶⁸

The effect of bile acids in the pathophysiology of hepatobiliary system has been long studied.⁶⁹ Previous reports have suggested that the cholestatic effect of lithocholic acid (LCA), a hepatotoxic bile acid, is in part caused by alteration of the canalicular membrane of hepatocytes,⁶⁹ and by an impaired hepatobiliary trafficking of export pumps.⁷⁰ In a recent work, however, feeding of 1% LCA-supplemented diet has proven effective in directly damaging cholangiocytes by formations of crystal precipitates that obstruct the biliary tree and presumably favor the intrinsic toxic effect of lithocholate.⁷¹ As a consequence of the LCA-supplemented diet, mice develop bile infarcts, destructive cholangitis, and periductal fibrosis, supporting the concept that a “toxic bile” may be an important element in the pathophysiology of cholestatic liver diseases.⁷²

Specific Models of Primary Biliary Cirrhosis

Specific models of PBC, aimed to recapitulate the main pathophysiological characteristics of the disease, have been developed greatly in the last decade. Despite the fact that none of the proposed model can perfectly resemble the complex scenario of the human disease, murine experimental models remain a valuable tool to help unveiling the etiopathogenetic aspects of PBC and defining possible new therapeutic targeted strategies.

The NOD.c3c4 mouse was the first spontaneous model of PBC described.⁷³ NOD.c3c4 congenic mice were derived in studies with nonobese diabetic (NOD) mice by introgression of genetic regions present on chromosome 3 and 4 of parental mice strains that carry insulin-dependent diabetes resistance alleles.⁷³ Interestingly, while the c3/c4 loci can effectively prevent diabetes development, they result in the development of an autoimmune biliary disease that models human PBC.⁷⁴ Indeed, NOD.c3c4 mice show peribiliary CD3⁺, CD4⁺, and CD8⁺ cells and eosinophil infiltration, and develop auto-antibodies to PDC-E2 specific for the inner lipoyl domain, progressive biliary obstruction, and granuloma formation. The central pathogenetic role of T cells was confirmed by disease development in NOD.c3c4-scid mice after transfer of splenocytes or CD4⁺ T cells from diseased NOD.c3c4 mice, whereas depletion of T cells effectively protects NOD.c3c4 mice from biliary disease. Although many of the features of PBC can be successfully reproduced in this model, it is important to note that NOD.c3c4 mice also develop biliary cysts and large bile duct inflammation, which are not characteristic of the disease.⁷⁵

The dominant-negative TGF- β receptor II (dnTGF- β RII) mice have been described as an additional mouse model of PBC.⁷⁶ TGF- β is a potent cytokine with important pleiotropic functions in the regulation of immune responses, mainly involved in the maintenance of immunological tolerance toward self or nonharmful antigens.⁷⁷ dnTGF- β RII mice overexpress a mutated form of the TGF- β RII (under the control of the CD4⁺ promoter and

with no CD8⁺ silencer, allowing the expression of the transgene in both CD4⁺ and CD8⁺ T cells) that is unable of signal transduction.⁷⁸ Gershwin et al showed that dnTGF-βRII mice develop several features of the human PBC, including spontaneous production of antimitochondrial antibodies to PDC-E2, E2 subunit of the oxo-glutarate dehydrogenase complex (OGDC-E2), and E2 subunit of the branched chain 2-oxo acid dehydrogenase complex (BCOADC-E2); CD4⁺ and CD8⁺ T cells infiltration within the portal tract of 6- to 7-month-old mice; and increased levels of interferon- (IFN-) γ, tumor necrosis factor- (TNF-) α, interleukin- (IL-) 6, and IL-12p40.^{76,79} It is important to note that the biliary involvement is present in the context of a more generalized dysregulation of the immune homeostasis, and that dnTGF-βRII mice also show marked-to-severe inflammatory bowel disease, with distortion of crypt architecture, crypt abscesses and hyperplasia, and mild inflammatory infiltrations in the lungs, stomach, duodenum, pancreas, and kidney.⁷⁸ Interestingly, when unfractionated splenocytes of dnTGF-βRII mice are transferred to Rag1^{-/-} mice, features of liver disease similar to PBC arise, suggesting that there is no specific abnormality in the biliary tree that drives the immune reaction and that loss of self-tolerance in the spleen is sufficient to cause the disease.⁸⁰ Moreover, CD8⁺ T cells appear essential in determining the cholangitis, while CD4⁺ T cells seem more important in the development of the colitis.⁸⁰ The loss of self-tolerance in dnTGF-βRII mice has been associated to a defective suppressor activity of CD4⁺CD25⁺ regulatory T (Treg) cells, possibly due to a lower FoxP3 expression in these cells found in dnTGF-βRII mice.⁸¹

The importance of Treg cell functions in the development of PBC has been further stressed in an additional rodent model, the IL-2Rα^{-/-} mouse. Among other stimulatory functions on effector T cells, IL-2 also promotes the suppression of immune responses through the activation of IL-2R and expansion of Treg cells.⁸² Interestingly, PBC patients show a reduction of Treg cells in peripheral blood compared with control and lower levels of FoxP3-expressing Tregs in affected portal tracts.⁸³ Accordingly, IL-2Rα^{-/-} mice develop portal tract inflammation and biliary damage, with CD4⁺ and CD8⁺ infiltrates. Moreover, mice show increased serum levels of IFN-γ, TNF-α, IL-2, and IL-12p40, and develop AMAs with specificity for PDC-E2.⁸⁴ However, IL-2Rα^{-/-} mice are also prone to develop other autoimmune disorders, such as hemolytic anemia and inflammatory bowel disease, especially after the first 2 months of age.⁸⁵ A later study has clarified that different pathogenic mechanisms are involved in the formation of biliary or colonic lesions, with CD4⁺ cells being responsible mostly for the colon damage and CD8⁺ for the biliary injury, in a similar fashion as for the dnTGF-βRII mouse model.⁸⁶

AE2^{-/-} mice were developed by Salas et al based upon the clinical evidence that AE2 gene expression is reduced in liver biopsy specimens and blood mononuclear cells from patients with PBC.^{25,87,88} As mentioned above, AE2 plays an important role in Cl⁻/HCO₃⁻ exchange resulting in bicarbonate secretion by cholangiocytes into bile. AE2^{-/-} mice recapitulate many of the biochemical, histological, and immunological features of PBC irrespective of gender.⁸⁸ As these mice age, there is an increase of hALP and ALT levels along with increased portal inflammation with damaged intralobular bile ducts surrounded by inflammatory infiltrate (T cells, macrophages, and eosinophils).⁸⁸ These changes are accompanied by induction of AMAs (IgG class) against PDC-E2 inner lipoyl domain as observed in PBC.^{88,89} Recent studies have contributed to the development of the hypothesis

that AE2 deficiency or functional impairment may play a key role in the pathogenesis of PBC through defective biliary HCO_3^- secretion leading to a reduction of the protective biliary HCO_3^- umbrella.^{18,27,28}

In addition to genetic models of PBC, two models of immunity induced by xenobiotics in mice have been shown to induce pathologies very similar to PBC. In the first model, guinea pigs were immunized with 6-bromohexanote, which has structural similarities with PDC-E2.⁹⁰ In this model, there are increased serum levels of anti-PDC-E2 antibody as well as anti-BCOADC-E2 and anti-OGDC-E2 antibodies.⁹⁰ However, light changes in liver pathology were observed, which is a limitation of this model.⁹¹ In the second model, mice are immunized with 2-octynoic acid, a xenobiotic used in cosmetics and as a food additive that when coupled to BSA has a structure similar to the inner lipoyl domain of PDC-E2.⁹² Administration of 2-OA to B6 or NOD.1101 mice resulted in high levels of serum AMAs, portal inflammation, and auto-immune cholangitis similar to human PBC.⁹³

The Immunobiology of Cholangiocytes

Bile ducts were once thought to simply be a conduit system for bile transport, and cholangiocytes just the lining of that system. The involvement of cholangiocytes in the immune response was assessed a few decades ago, although it was initially thought to be limited to the secretion of immunoglobulin A in the bile.⁹⁴ Cholangiocytes are in fact the first line of defense of the biliary system against foreign substances, and due to the exposure of the biliary epithelium to foreign antigens, are equipped to participate through various immunological pathways to both innate and adaptive immune responses (Table 1). Indeed, cholangiocytes express Toll-like receptors (TLRs) and antimicrobial peptides⁹⁵; act as antigen-presenting cells (APCs) by expressing human leukocyte antigen molecules and costimulatory molecules⁹⁶⁻⁹⁸; recruit leukocytes to the target site by expressing adhesion molecules,^{99,100} cytokines, and chemokines^{101,102}; and induce apoptosis of leukocytes to limit the immune responses.¹⁰³ As discussed before, in response to injury cholangiocytes proliferate, change their phenotype, and become active players in the immune response.⁵

Toll-like receptors are important mediators of the innate immune response, which upon recognition of structurally conserved molecules derived from microbes, activate specific intracellular pathways that lead to the secretion of proinflammatory cytokines and chemokines. Even though bile is sterile under physiological conditions, cholangiocytes from intrahepatic large bile ducts, septal and interlobular bile ducts, and bile ductules have been shown to express TLRs 2, 3, 4, and 5 in the course of infectious diseases.¹⁰⁴ Moreover, TLR3 has been demonstrated to be upregulated at sites of ductular reaction in PBC, autoimmune hepatitis (AIH), and chronic viral hepatitis.¹⁰⁵ Despite the fact that the role of TLR pathways in noninfectious cholangiopathies is poorly understood, the inflammatory cytokines and other antimicrobial molecules secreted by cholangiocytes subsequent to TLR stimulation mediate various immune responses thought to be important to maintain mucosal homeostasis. Evidence for the maintenance of tolerance by cholangiocytes has been demonstrated by the upregulation of interleukin-1 receptor-associated kinase M,¹⁰⁶ a negative regulator of TLR signaling, in freshly isolated human intrahepatic biliary epithelial cells upon stimulation with TLR-2 and TLR-4.¹⁰⁷

IgA is the major immunoglobulin isotype involved in mucosal immunity, finalized to local pathogen clearance.¹⁰⁸ One of the features of biliary epithelia is the expression of polymeric immunoglobulin receptors (pIgR) involved in the transcytosis of IgA across epithelium. The process of transcytosis, which is used by the body to deliver protective immunoglobulins to mucosal surfaces, involves the movement of polymeric IgA through the cytoplasm from the basolateral to the apical domain. Importantly, in the human liver, the only cells expressing the pIgR are cholangiocytes.¹⁰⁹ IgA transcytosis has been demonstrated to be involved in PBC pathogenesis.¹¹⁰ Defensins and cathelicidins are antimicrobial peptides belonging to the innate immune system, and cholangiocytes have been shown to produce these peptides in basal and diseased states. Specifically, human β -defensin 1 (hBD-1) is diffusely expressed in the cytoplasm of normal intrahepatic bile duct epithelium.¹¹¹ The ability of cholangiocytes to interact with the immune system is crucial for the maintenance of biliary mucosal integrity; moreover, the specific response of cholangiocytes to inflammatory signals (e.g., cytokines) reflects the significance of a concerted immune response.

One of the main roles of cholangiocytes in the immune response is their ability to act as APCs. Cholangiocytes of both normal and diseased human livers express ICAM-1, LFA-3, HLA-I, and HLA-II,^{112–114} as well as EGF, Fas/Apo-1 (CD95), CD40, and CD44,¹¹⁵ suggesting an important involvement of cholangiocytes in antigen presentation and in the recruitment and activation of circulating leukocytes that can migrate across the portal endothelium.^{100,116,117} A combination of inflammatory cytokines such as TNF α , IL-6, and IL-1 inhibits cAMP-dependent fluid secretion in cholangiocytes and impairs the barrier functions of biliary epithelia.¹¹⁸ Moreover, human cholangiocytes secrete IL-8 and MCP-1,¹¹⁹ which act as chemotactic agents for neutrophils, monocytes, and T cells,¹⁰⁰ but also IL-6, that stimulates terminal differentiation of B cells and immunoglobulin secretion, and TNF- α , which increases cytotoxic activities of T cells and induces the expression of adhesion molecules and HLA antigens on cholangiocytes.^{101,102} Cholangiocyte MHC II overexpression has been observed in injured bile ducts from livers with allograft rejection, graft versus host disease, PBC, and PSC.^{101,115} Moreover, TLR signaling has been shown to induce the production of different cytokines, such as IL-6 and IL-1 β , which are necessary for Th17 differentiation, and IL-23, required for phenotypic maintenance in the cultured human cholangiocyte cell line BEC1–3.¹²⁰ In biopsy specimens from patients with PBC, Th17 cells have been found to be distributed around inflamed portal tracts and damaged interlobular bile ducts.¹²¹

Apoptosis of Cholangiocytes in the Pathogenesis of Primary Biliary Cirrhosis

Apoptosis is essential in maintaining immune cell populations. Dying cells undergo morphological modifications including chromatin condensation, nuclear fragmentation, and generation of apoptotic bodies. The clearance of apoptotic cells is a highly regulated process, essential to avoid the outflow of intracellular content and limit the immunological response against generated antigens.^{122,123} The formation of apoptotic bodies and fragments limits the escape of intracellular content and preclude any ensuing immunological response against intracellular autoantigens with inflammatory reactions.^{124,125} However, under some

circumstances, apoptotic bodies and fragments can constitute a major source of immunogens in autoimmune diseases that involve the targeting of ubiquitous autoantigens.^{126,127}

Apoptosis of cholangiocytes in PBC has been investigated in several studies; it has been demonstrated that cholangiocytes from PBC patients, when compared with normal controls, show increased DNA fragmentation, implying increased apoptosis.^{128,129} Moreover, significantly greater levels of Fas, FasL, perforin, granzyme B, and TRAIL are expressed on cholangiocytes from subjects with PBC.^{103,129,130} In addition, the upregulation of WAF1 and p53 related to biliary apoptosis is found in cholangiocytes of PBC,¹³¹ and significantly greater apoptosis has been demonstrated in cholangiocytes of PBC patients than of other chronic cholestatic diseases, even when controlling for similar degrees of inflammation.^{103,129,131,132}

Recent findings suggest that intrinsic pathways induce apoptosis of cholangiocytes in PBC; indeed, cholangiocytes seem to be more than simply an innocent victim of an immune attack, rather the unique biochemical mechanisms by which they handle PDC-E2 seems to attract the immune response.¹³³ Cholangiocyte apoptosis may be of considerable importance for understanding PBC; however, much work remains to be done in this area. It is clear that mitochondrial proteins are present in all nucleated cells, yet in PBC the autoimmune attack is directed with high specificity to cholangiocytes. Importantly, there are significant differences between the metabolic processing of PDC-E2 during apoptosis of cholangiocytes compared with other epithelial cells. The unique process of PDC-E2, during apoptosis of cholangiocytes, was initially described by Odin et al.⁶ They demonstrated that immunologically active PDC-E2 was recognized in cholangiocytes after apoptosis, whereas in other cell types autoantibody recognition of PDC-E2 was not identified after apoptosis. Further, PDC-E2 was not cleaved by caspases. The covalent modification of a PDC-E2 sulfhydryl group by glutathione was hypothesized to be the cause for the loss of recognition of this antigenic epitope. In fact, both overexpression of Bcl-2 and depletion of glutathione before inducing apoptosis prevented loss of autoantibody recognition, suggesting that glutathiolation, rather than degradation or loss of PDC-E2 was responsible for it. Recently, the same group suggested that estrogens may help preserve mitochondrial function during apoptosis.¹³⁴ Successively Allina et al hypothesized that the absence of lipoylysine oxidation in PDC-E2 may yield unique self-peptides following phagocytosis of the apoptotic cholangiocytes.¹³⁵

We have recently demonstrated that PDC-E2 is not only immunologically intact during apoptosis in cholangiocytes, but it localizes in the apoptotic bodies of cholangiocytes where it is accessible to AMA recognition.⁷ However, the mechanism by which PDC-E2 translocates to the cell membrane is not known. We confirmed by immunoblotting analysis that PDC-E2 was detectable in its antigenically reactive form within apoptotic blebs from human intrahepatic cholangiocytes, but it was not detected in apoptotic blebs from three other epithelial cell lines,^{7,136} whereas seven mitochondrial and four nuclear proteins were present in naive, untreated cultures of cholangiocytes and epithelial controls.¹³⁶

Moreover, recent data show that macrophages from PBC patients cultured with biliary apoptotic bodies in the presence of AMA determine an intense inflammatory cytokine

production.⁸ The cytokine secretion is inhibited by anti-CD16; it is not due to differences in apoptotic uptake. Furthermore, the unique triad of cholangiocyte apoptoses, macrophages from PBC, and AMAs markedly increased TNF-related apoptosis-inducing ligand expression, which induces apoptosis in cholangiocytes.¹⁰³

Conclusions

Cholangiocytes have always been known to be the target of the immune attack in PBC; however, just recently they have been recognized to play an active role in the pathogenesis of PBC. There are no specific predisposing biliary cell phenotypes because PBC may relapse after liver transplantation. Rather, the involvement in PBC of cholangiocytes may depend on their particular biological characteristics. Such properties may include unique processes of apoptosis, the presence of poly Ig receptors and especially in the case of cholangiocytes, their immunobiology. Cholangiocytes are active participants rather than innocent victims in the autoimmune pathology of PBC. These observations may help to understand many open questions in the pathogenesis of PBC (Fig. 1), including the mechanisms that lead to the selective destruction of small intrahepatic bile ducts. Ultimately, these findings will lead to the development of effective therapeutic approaches.

Acknowledgments

Financial Support

Portions of these studies were supported by the Dr. Nicholas C. Hightower Centennial Chair of Gastroenterology from Scott & White, a VA Research Career Scientist Award, and a VA Merit Award to Dr. Alpini, a VA CD2 and Merit Grant Award to Dr. Glaser, and NIH grant DK58411 and DK07698 to Drs. Alpini and Glaser.

The views presented are those of the authors and do not necessarily represent the views of the Department of Veterans Affairs.

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Fig. 1. Scheme of the pathogenic model in primary biliary cirrhosis. During the apoptosis of cholangiocytes, PDC-E2 remains intact because of its lack of glutathiolation in apoptotic bodies. The PDC-E2 present in the apoptotic bodies would be recognized by circulating antimitochondrial autoantibodies (AMAs), and the immune complex would then stimulate the innate immune system in a subject with a susceptible genetic background.

Table 1

Summary of immunobiological characteristics of cholangiocytes

Cholangiocyte expression	Function	Mechanism
TLRs 2, 3, 4, and 5	Recognize pathogens	
IRAK-M	Maintain tolerance	Negative regulator of TLR signaling
PD ligands, TRAIL	Limit immune response	Induce apoptosis of leukocytes
IL-6, IL-8, and MCP-1	Chemotactic	Recruitment of immune cells to protect against infection
Defensins, cathelicidin	Antimicrobial, chemotactic	Disrupt microbial membranes; recruit CD4+ T cells and immature dendritic cells
ICAM-1, LFA-3, VCAM-1	Cholangiocyte–leukocyte interaction	Leukocyte migration to inflammatory sites
HLA class II molecules	Antigen presentation	
CD80, CD86, CD40	Costimulation of T cells	

Abbreviations: HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule 1; IRAK-M, interleukin-1 receptor-associated kinase M; LFA-3, lymphocyte-associated antigen 3; MCP-1, monocyte chemotactic protein-1; PD, programmed death; TLRs, Toll-like receptors; TRAIL, TNF-related apoptosis-inducing ligand; VCAM-1, vascular cell adhesion molecule-1.