

# The Immunophysiology and Apoptosis of Biliary Epithelial Cells: Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis

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**Abstract** Biliary epithelial cells (BECs) provide the first line of defense against luminal microbes in the biliary system. BECs express a variety of pathogen recognition receptors and can activate several intracellular signaling cascades to initiate antimicrobial defenses, including production of several anti-microbial peptides, cytokines, chemokines, and adhesion molecules. BECs also secrete immunoglobulin A and interact with other cells through expression and release of adhesion molecules and immune mediators. Recently, several reports suggest a correlation between apoptosis and autoimmunity through ineffective clearance of self-antigens. Primary biliary cirrhosis (PBC) is a slowly progressive, autoimmune cholestatic liver disease characterized by highly specific antimitochondrial antibodies (AMAs) and the specific immune-mediated destruction of BECs. We have demonstrated that the AMA self-antigen, namely the E2 subunit of the pyruvate dehydrogenase complex, is detectable in its antigenically reactive form within apoptotic blebs from human intrahepatic biliary

epithelial cells and activates innate immune responses. Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by inflammation and the presence of concentric fibrosis of intrahepatic and/or extrahepatic bile ducts, eventually leading to cirrhosis. However, apoptosis does not appear to play a central role in PSC. Despite both diseases involving immune-mediated injury to bile ducts, apoptosis occurs more commonly overall in PBC where it likely plays a unique role.

**Keywords** Biliary epithelial cells · Apoptosis · Autoimmunity · Primary sclerosing cholangitis · Primary biliary cirrhosis

## Abbreviations

AMAs	Antimitochondrial antibodies
ANA	Anti-nuclear antibody
BCOADC-E2	The E2 subunit of the branched chain 2-oxo acid dehydrogenase complex
BECs	Biliary epithelial cells
BrEPCs	Human bronchial epithelial cells
CX3CR1	CX3C chemokine receptor 1
DC	Dendritic cells
DEC1	2,4-Dienoyl coenzyme A reductase 1
DR	Death receptor
FasL	Fas ligand
gp210	Glycoprotein 210 kD
hBD	Human $\beta$ -defensin
HiBECs	Human intrahepatic biliary epithelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN- $\beta$ 1	Interferon $\beta$ 1
IgA	Immunoglobulin A
IL-12A	Interleukin-12A

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IL-12RB2	Interleukin-12 receptor beta2 subunit
IRAK-1	IL-1 receptor-associated kinase
IRAK-M	Interleukin-1 receptor-associated kinase M
IRF-3	Interferon-regulatory factor-3
LFA-1	Lymphocyte function-associated antigen-1
LFA-3	Lymphocyte function-associated antigen 3
LPS	Lipopolysaccharide
MAdCAM1	Mucosal addressin cell adhesion molecule-1
MaEPCs	Human mammary epithelial cells
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MDA-5	Melanoma differentiation associated gene 5
MPT	Mitochondrial membrane permeability transition
MxA	Myxovirus resistance protein A
MyD88	Myeloid differentiation factor 88
(NF)-kB	Nuclear factor
OGDC-E2	The E2 subunit of the oxo-glutarate dehydrogenase complex
PAMPs	Pathogen-associated molecular patterns
pANCA	Perinuclear anti-neutrophil cytoplasmic antibody
PBC	Primary biliary cirrhosis
PDC-E2	The E2 subunit of the pyruvate dehydrogenase complex
poly I:C	Polyinosinic–polycytidylic acid
PSC	Primary sclerosing cholangitis
Rig-1	Retinoic acid inducible gene-1
STAT4	Signal transducer and activator of transcription 4
SSA/Ro	Sjogren’s syndrome antigen A
TRAIL	TNF-related apoptosis-inducing ligand
TLRs	Toll-like receptors
TNF-R1	TNF- $\alpha$ receptor-1
TUNEL	TdT-mediated deoxyuridine triphosphate nick-end labeling
UCDA	Ursodeoxycholic acid
UQCRC2	Ubiquinol cytochrome c reductase complex core protein II

## Introduction

The biliary system of the liver functions to deliver bile to the intestinal lumen. Biliary epithelial cells (BECs) form a continuous mucosal surface from the smallest biliary unit the bile ductule, through intralobular and septal bile ducts and finally to the large intrahepatic and extrahepatic bile ducts. Due to the exposure of the biliary tract to microbes, BECs

are equipped to respond through several immunological pathways including a variety of pathogen pattern recognition receptors [1–3]. Activation of pathogen pattern recognition receptor-associated intracellular signaling cascades result in the expression of several anti-microbial peptide, cytokines/chemokines, adhesion molecules, and immunoglobulin A (IgA), initiating epithelial immune responses against microbial infection. However, a balance between inflammatory responses and tolerance is a key in mucosal environments.

Apoptosis, the major mechanism of programmed cell death, is essential to regulate and maintain tissue growth and homeostasis. Normally, apoptotic bodies are efficiently cleared by neighboring tissue cells or professional phagocytes without inciting an inflammatory reaction [4, 5]. However, several reports suggest a correlation between apoptosis and autoimmunity through an impairment of apoptosis or an ineffective removal of apoptotic bodies [6–8] leading to the release of intracellular components that are a potential source of autoantigenic stimulation [7, 9]. The presence of intact autoantigens within apoptotic bodies [10] and their ability to activate innate immunity through macrophage cytokine secretion [11] provide the strongest evidence linking apoptosis and autoimmunity [12].

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts with cholestasis, portal inflammation, and fibrosis. PBC may lead to cirrhosis and its complications, and eventually to liver transplantation or death. PBC is characterized by multi-lineage T and B cell responses against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), which is contained in the mitochondria of all nucleated cells [13, 14]. However, a major unanswered question regarding the pathogenesis of PBC is the specific targeting of small BECs despite the presence of mitochondria in all nucleated cells. We reported that PDC-E2 is preserved in apoptotic bodies from human intrahepatic biliary epithelial cells (HiBECs) but not other epithelial cells during apoptosis [15, 16]. Furthermore, the triad of HiBEC apoptotic bodies, macrophages from patients with PBC, and antimitochondrial antibodies (AMAs) produce inflammatory cytokines and TNF-related apoptosis-inducing ligand (TRAIL) [17]. We have proposed that the unique apoptotic features of BECs allow the exposure of a potent intracellular autoantigen to the PBC-associated multi-lineage autoimmune response that leads to the tissue-specific autoimmune injury. Although genetic susceptibility and environmental factors have gained much recent attention, mounting evidence continues to suggest that BEC apoptosis plays an important role in PBC pathogenesis. Primary sclerosing cholangitis (PSC) is another liver disease likely of autoimmune etiology in which small as well as large bile ducts are destroyed and leads to biliary cirrhosis. However, based

upon in situ staining, apoptosis is much more frequent in PBC compared with PSC [18]. This review summarizes the current understanding of the immunophysiology and apoptosis of BECs, as well as how they relate to PBC and PSC.

## Basic Mechanism of Biliary Immunity

### Toll-Like Receptors

Toll-like receptors (TLRs) are key responders to pathogen-associated molecular patterns (PAMPs) and are characterized by a variable leucine-rich extracellular domain and an intracellular toll/interleukin (IL)-1 receptor domain [19, 20]. The former domain responds to PAMPs, and the latter induces the production of antiviral cytokines and inflammatory cytokines/chemokines via intracellular transcription factors such as nuclear factor (NF)- $\kappa$ B, mitogen-activated protein kinases (MAPK), and interferon-regulatory factor-3 (IRF-3) followed by the initiation of inflammatory responses. Epithelial expression of TLRs is highly regulated, reflecting the specific microenvironment and function of each epithelial cell type. BECs express TLRs 1, 2, 3, 4, and 5, and signaling proteins myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK-1), retinoic acid inducible gene-1 (Rig-1), and melanoma differentiation-associated gene 5 [21, 22]. Moreover, immunohistochemistry has confirmed that TLR1-TLR5, MyD88, and IRAK-1 are distributed diffusely in the intrahepatic biliary tree of normal human liver [21]. Upregulation of TLRs in BECs has been demonstrated in patients with PBC, PSC, and hepatolithiasis [23, 24]. In addition, BECs have also demonstrated responsiveness to the corresponding PAMPs. Lipopolysaccharide (LPS), a ligand of TLR4, binds to the surface of cultured BECs and induces the production of TNF- $\alpha$  mRNA in an NF- $\kappa$ B-dependent manner [25]. Stimulation of BEC with polyinosinic-polycytidylic acid (poly I:C), a ligand of TLR9, induces the activation of NF- $\kappa$ B and interferon regulatory factor (IRF3) and the production of interferon  $\beta$ 1 (IFN- $\beta$ 1) and MxA as potent antiviral responses [26]. Therefore, BECs possess functional TLR signaling systems and participate in innate immunity.

Epithelial cells have also developed multiple strategies for negative regulation of TLR signaling that initiate events leading to resolution of the inflammatory responses. These strategies include the release of extracellular soluble decoy TLRs, interference of ligand-receptor interaction through transmembrane negative regulators, and activation of intracellular antagonists to downregulate TLR signaling [27]. Moreover, a function for some of these negative regulators has recently been elucidated in human BECs. Upregulation of interleukin-1 receptor-associated kinase M (IRAK-M), a negative regulator of TLRs signaling, was demonstrated in

freshly isolated human BECs upon stimulation with TLR 2 and TLR 4 [28]. This study demonstrated endotoxin tolerance in the intrahepatic biliary epithelium and suggests that the expression of IRAK-M is crucial for LPS-induced tolerance in BECs. It is speculated that endotoxin tolerance is important in maintaining innate immune biliary homeostasis and that an impaired tolerance to LPS may be associated with the pathogenesis of biliary diseases.

### Anti-microbial Peptide

Defensins and cathelicidin are anti-microbial peptides belonging to the innate immune system [29]. They protect the mucosal barrier against bacteria, mycobacteria, fungi, and viruses via mechanisms such as the disruption of microbial membranes. However, they also participate in adaptive immunity through the recruitment of CD4<sup>+</sup> T cells and immature dendritic cells [30]. Granulocytes express the highest density of defensins, however, cholangiocytes also produce these peptides in basal and diseased states [1, 29]. Defensins are divided into two major groups,  $\alpha$ -defensins and  $\beta$ -defensins, based on their structure [31].  $\alpha$ -Defensins are particularly abundant in neutrophils and Paneth cells of the small intestine [31]. Of the six human  $\beta$ -defensins (hBD-1 to hBD-6) that have been identified, human  $\beta$ -defensin 1 (hBD-1) is diffusely expressed in the cytoplasm of normal BECs [31]. Moreover, because hBD-1 is constantly detectable in bile samples, hBD-1 is believed to play a role in the constitutive antimicrobial defense of the hepatobiliary system [1]. hBD-2 is undetected in normal BECs. However, hBD2 is induced by bacterial and viral infections and cytokines in the liver. Therefore, hBD-1 plays a constitutive role in biliary antimicrobial defense, while the expression of hBD-2 is induced in response to local infections and may play a role in additional antimicrobial defenses.

Cathelicidin is expressed in normal human BECs, as well as hepatocytes. Moreover, bile acids, including chenodeoxycholic acid and UCDA, enhance cathelicidin expression through the farnesoid X receptor and the vitamin D receptor [32].

### Cytokines and Chemokines

HiBECs constitutively express IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) [2, 33], which are important chemotactic agents for neutrophils, monocytes, and T cells. Treatment of HiBECs with PAMPs induces the production of IL-6 and MCP-1 via activation of transcription factors NF- $\kappa$ B and MAPK [2]. In addition, LPS induces expression and secretion of IL-12 and TNF- $\alpha$  in immortalized mouse BECs through activation of TLR4 [25]. Fractalkine is a chemokine with both chemoattractant and cell adhesive functions, and it is involved with its receptor CX3C

chemokine receptor 1 (CX3CR1) in the chemoattraction and recruitment of intraepithelial lymphocytes. LPS induces the upregulation of fractalkine expression in cultured HiBECs, followed by the chemoattraction of CX3CR1-expressing mononuclear cells [34]. Shimoda et al. [35] demonstrated the significance of fractalkine and the precise mechanism of its production using populations of multiple intrahepatic cell types, including endothelial cells, liver sinusoidal endothelial cells, and BECs, to directly study the interaction of fractalkine producing cells with liver-infiltrating mononuclear cells. Endothelial cells produced large amounts of fractalkine upon stimulation by PAMPs. In contrast, liver sinusoidal endothelial cells produced no fractalkine. Moreover, TLR3-stimulated BECs produced fractalkine after direct contact with TLR4-stimulated autologous monocytes. These findings suggest that cytokines and chemokines secreted by BECs recruit and activate immune cells such as T cells, macrophages, and natural killer cells to protect against biliary infection [36].

#### Adhesion Molecules

Normal BECs express low levels of lymphocyte adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3). However, previous study reports a lack of specific ICAM-1 immunohistochemical staining in normal livers [37]. ICAM-1 is critical for leukocyte migration to inflammatory sites. It binds to lymphocyte function-associated antigen-1 (LFA-1) and macrophage antigen-1 expressed on leukocytes such as neutrophils, macrophages, and lymphocytes [38]. BECs from patients with PBC exhibit increased expressions of ICAM-1, with a corresponding expression of LFA-1 on infiltrating lymphocytes [37]. The presence of the adhesion molecule LFA-3 on the surface of BECs allows their interaction with CD2 molecules expressed on cytotoxic T lymphocytes and natural killer cells [39]. T cells are also activated by CD40, a member of the TNF receptor superfamily, which is expressed on BECs [40]. The CD40–CD40L and CD2/CD58 complexes induce production of IL-12, which plays an important role in the cytotoxic response of BECs. CD40 and CD40L expression in BECs are increased, respectively, by stimulation with IFN- $\gamma$  and activation of CD2 on T cells [40]. The increase in leukocyte adhesion molecules facilitates tissue-specific migration by the slowing down of leukocyte circulation near the damaged epithelium, encouraging trafficking to the target site.

#### Secretory IgA

BECs also transport protective immunoglobulins from the circulation into bile. The predominant immunoglobulin in bile is IgA, although IgM and IgG are present but at much

lower levels. IgA could be protective for bile ducts by preventing bacterial infection or inhibiting bacterial toxins from interacting with BECs [41]. IgA is synthesized by plasma cells around bile ducts and secreted into bile after binding to the polymeric Ig receptor located on the basolateral membranes of BECs. Aagaard and colleagues demonstrated that natural IgA antibodies against a variety of intestinal bacteria are present in bile, and after inoculation of various antigens into the intestinal lumen or intestinal lymphoid tissues, a consequent secretion of specific IgA antibodies that protect from biliary tract infections is evident in the bile of experimental animals [42]. Additionally, IgA has been demonstrated to neutralize intracellular microbes and their products during its transit through mucosal epithelium. Immune complexes of IgA and foreign antigen in the lamina propria may also be transported to the lumen via secretory components, excreting pathogens to a proteolytic mucosal environment [43].

#### Biliary Immunity and Apoptosis

##### Apoptosis of Biliary Epithelial Cells

The decision of a cell to undergo apoptosis can be influenced by a wide variety of regulatory stimuli acting either as inhibitors or inducers of apoptosis. Apoptosis contributes to duct loss and is induced by signals such as activation of death receptors, immune-mediated injury, oxidative stress, infections, and toxins. Two main pathways of apoptosis regulation have been identified: termed the intrinsic and extrinsic pathways. The intrinsic pathway is characterized by mitochondria dysfunction or possibly by endoplasmic reticulum stress [44]. The extrinsic pathway is mediated either by cell surface receptors, including Fas and TNF- $\alpha$  receptor-1 (TNF-R1), or by perforin and granzyme B released from activated cytotoxic lymphocytes. Upon activation by Fas ligand (FasL) and TNF- $\alpha$ , both Fas, TNF-R1 recruit adaptor proteins, and aggregation of this complex initiates cleavage of procaspase 8 into its active form. TRAIL binds two death receptors (TRAIL-R1/death receptor 4 (DR4) and TRAIL-R2/death receptor 5 (DR5)) and has been demonstrated as a critical molecule in the extrinsic pathways [45, 46]. Cytotoxic lymphocytes express FasL and release granules containing granzyme B and perforin, which allows granzyme B to enter target cells. Granzyme B then directly cleaves critical cellular proteins and activates procaspases [47]. At the earliest stages of apoptosis, the phagocytes and the dying cells have to be in proximity, which is achieved through “find-me” signals; the phagocytes then specifically engage the dying cells, achieved via “eat-me” signals on dying cells and receptors for the “eat-me” signals on phagocytes; the phagocytes then physically

engulf the dying cells through signaling induced by engulfment receptors and cytoskeletal reorganization. After internalization, actin is displaced from the phagosome, which matures by a series of fusion and fission events with components of the endocytic pathway [48, 49].

There is controversial evidence concerning the expression of programmed-death (PD) ligands in BECs of biliary diseases. PD molecules are expressed on leukocytes, induce apoptosis in the leukocyte, and may be another method of limiting the immune response [50, 51]. Death receptor 5 (DR5) is constitutively expressed by BECs in normal conditions and is significantly upregulated in BECs of some biliary diseases, which may result in their apoptosis via ligation by TRAIL expressed by activated cytotoxic lymphocytes [46]. Stimulation of BEC with poly I:C induced the activation of NF- $\kappa$ B and IRF-3, followed by the production of antiviral IFN- $\beta$ 1 and also enhanced apoptosis via production of TRAIL [26]. The expression of TRAIL by diseased BECs in PBC and PSC may be an attempt by BECs to control the inflammatory responses in these diseases by targeting DR5-expressing leukocytes [46]. The induction of apoptosis in BECs by leukocytes is also important for the clearance of pathogens and may play a role in the induction of BECs damage in biliary diseases. CD40 binds to the CD40L found on leukocytes such as T cells, B cells, and macrophages. In the case of PBC, infiltrating macrophages and T cells express CD40L, with a more pronounced expression on macrophages [52]. In the same study, cultured cholangiocytes demonstrated constitutive expression of CD40, Fas, and FasL. Ligation of CD40 increased FasL expression and led to a three- to fourfold increase in Fas-dependent apoptosis of cultured BECs, suggesting that the binding of CD40 on BECs by inflammatory leukocytes causes apoptosis and perhaps, the clearance of infectious agents. Thus, apoptosis is an important mechanism in the control of immune responses and may contribute to biliary diseases.

#### Apoptosis and Autoimmunity

The clearance of apoptotic cells is a highly regulated process, essential to avoid the outflow of intracellular contents and to limit the immunological response against generated antigens. Indeed, the most significant difference between the phagocytosis of pathogens and the uptake of apoptotic cells has traditionally been considered the immune response: A pro-inflammatory reaction is often induced after the phagocytosis of necrotic cells, whereas the secretion of anti-inflammatory cytokines follows the engulfment of apoptotic cells [53–55]. The formation of apoptotic bodies and fragments is essential during apoptosis to limit the escape of intracellular content and to preclude any ensuing immunological responses against intracellular autoantigens with

inflammatory reactions [54, 56]. Nevertheless, apoptotic bodies and fragments can, under some circumstances, constitute a major source of immunogens in autoimmune diseases that involve the targeting of ubiquitous autoantigens [57]. Although autoantigens have no common cellular distribution or function that distinguishes them from other molecules, a high percentage of autoantigens are specifically cleaved by caspases [58, 59] and become concentrated in cytoplasmic surface blebs or apoptotic bodies during apoptosis [10, 57]. Apoptotic cells engulfed by antigen-presenting dendritic cells (DC) are suggested to serve as a source of self-antigens for the induction of peripheral self-tolerance in normal conditions [60, 61]. Moreover, apoptotic cells are able to promote maturation of DCs by upregulating costimulatory molecules and inducing proinflammatory cytokine release, while functioning as endogenous adjuvants for the induction of specific T cell responses [62]. Further evidence supporting the presentation of neo-antigens derived from apoptotic cells by DCs has been reported [63–65].

#### Association with Immunophysiology and Apoptosis in Biliary Disease

##### Primary Biliary Cirrhosis

PBC is a female-predominant, organ-specific autoimmune disease characterized by destruction of intrahepatic small bile duct BECs [66]. The serological hallmark of PBC is the presence of AMAs directed against PDC-E2 located in the inner membrane of mitochondria [14, 67, 68]. In addition, it has been reported that 23% and 57% patients with PBC also produce autoantibodies against two other 2-oxo acid dehydrogenase enzymes, the E2 subunit of the oxoglutarate dehydrogenase complex (OGDC-E2) and the E2 subunit of the branched chain 2-oxo acid dehydrogenase complex (BCOADC-E2), respectively [13, 69–71]. Furthermore, patients with PBC who are negative for AMAs do not have autoantibodies against PDC-E2, OGDC-E2, or BCOADC-E2 but often have autoantibodies to nuclear autoantigens [70, 72–75].

Epidemiologic studies have shown that having a first-degree relative with PBC, a history of urinary tract infections, a history of smoking, or use of hormone replacement therapies increased the risk of PBC [76, 77]. More recently, genome-wide case-control association studies in PBC have identified a significant association with interleukin-12A (IL-12A), interleukin-12 receptor, beta2 subunit (IL-12RB2), and signal transducer and activator of transcription 4 (STAT4) polymorphisms [78, 79]. The IL-12A polymorphism is also associated with celiac disease [80] and multiple sclerosis [81], and the STAT4 polymorphism is also

found in patients with systemic lupus erythematosus and rheumatoid arthritis [82]. The association of these pleiotropic immune function-related genes in PBC and other autoimmune diseases illustrates that multiple genes are shared between clinically distinct but immune-related diseases, and that the immune-mediated pathogenesis of PBC may be secondary to the breaking of tolerance to the PDC-E2 epitope by environmental factors such as xenobiotics [83].

#### *Immune Response in PBC*

CD4<sup>+</sup> and CD8<sup>+</sup> T cells and innate responses have all been demonstrated in PBC patients and proposed as possible etiologies for the biliary injury of PBC [84]. PDC-E2-specific autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been identified in peripheral blood from patients of PBC. Furthermore, PDC-E2-specific autoreactive CD4<sup>+</sup> T cells are 100- to 150-fold higher and CD8<sup>+</sup> T cells are ten- to 15-fold higher in the liver compared with peripheral blood in PBC patients [85–89]. Notably, the frequency of CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells are higher in PBC patients compared with normal controls [37, 38], and T cell clones with PDC-E2 specificity derived from PBC patients were all CD4<sup>+</sup>CD45RO<sup>+</sup> T cell [39]. In addition to the memory CD4<sup>+</sup> T cells, memory CD8<sup>+</sup> T cells are increased in a mouse model of PBC [40]. Although both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are present within portal tract infiltrates, there is a growing body of data that suggest a more direct role of cytotoxic CD8<sup>+</sup> T cells in the biliary destruction pathway [90–92]. Compared with healthy controls, PBC patients had an increased frequency of CD8<sup>+</sup> effector memory T cells (CD8<sup>high</sup>T<sub>EM</sub> cells), characterized as CD45RO<sup>high</sup>CD57<sup>+</sup>CD8<sup>high</sup> T cells and respond specifically to the major histocompatibility class I epitope of PDC-E2. Histologically, CD8<sup>+</sup>CD57<sup>+</sup> T cells accumulate around the portal area supporting their role in the progressive destruction of BECs [93].

#### *Apoptosis in PBC*

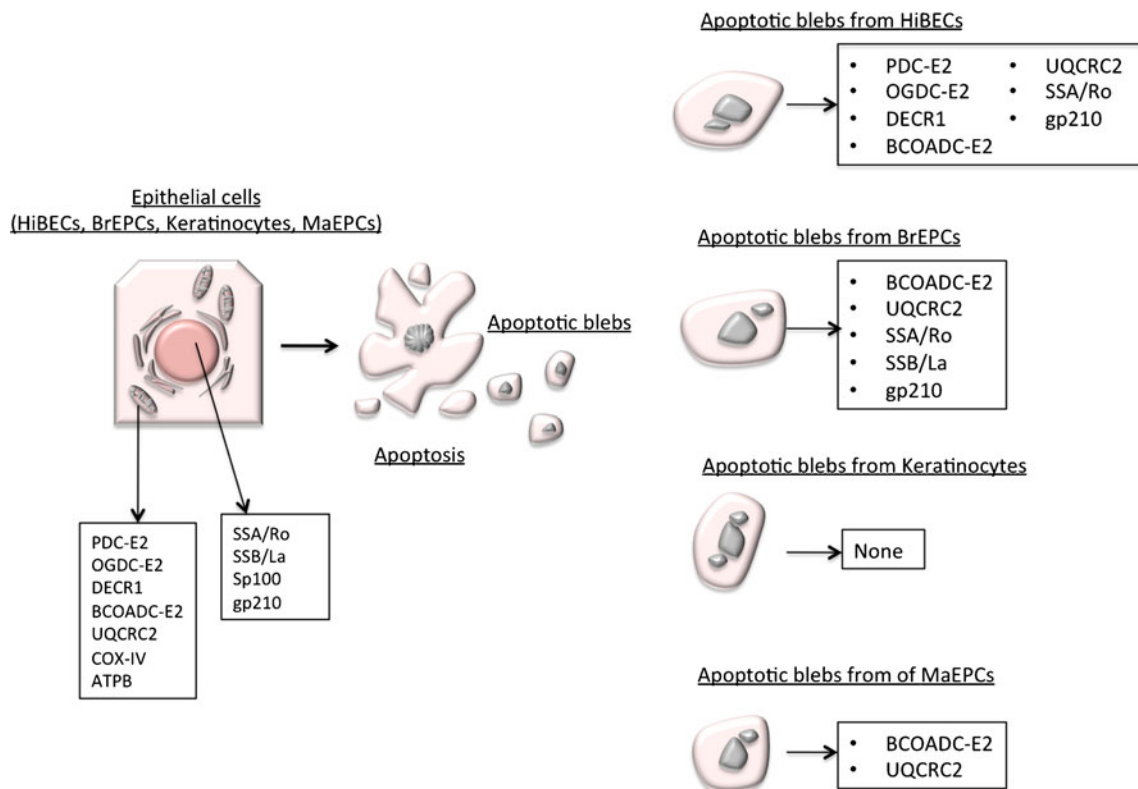
It is clear that mitochondrial proteins are present in all nucleated cells, yet the autoimmune attack is directed with high specificity to BECs. Notably, there are qualitative differences between the metabolic processing of PDC-E2 during apoptosis of BECs compared with other epithelial cells. We confirmed by immunoblotting that seven mitochondrial and four nuclear proteins were present in naive, untreated cultures of HiBECs, human bronchial epithelial cells (BrEPCs), human mammary epithelial cells (MaEPCs), and keratinocytes [16]. Of interest, PDC-E2 was detectable in its antigenically reactive form within apoptotic blebs from HiBECs, but it was not detected in apoptotic blebs from the three other epithelial cell lines [15, 16]. It is reasoned that absence of glutathiolation may contribute to this unique

feature of the BEC [94]. Furthermore, we demonstrated that other mitochondrial inner membrane proteins including OGDC-E2, BCOADC-E2, 2,4-dienoyl coenzyme A reductase 1 (DECR1), and ubiquinol cytochrome c reductase complex core protein II (UQCR2) remain intact in apoptotic blebs from HiBECs. In addition to mitochondrial inner membrane enzymes, Sjogren's syndrome antigen A (SSA/Ro) and glycoprotein 210 kD (gp210), which are nuclear antigens, are detectable in apoptotic blebs from HiBECs (Fig. 1) [16]. Furthermore, we tested the ability of apoptotic blebs from HiBECs to induce cytokine production from mature monocyte-derived macrophage from either patients with PBC or controls in the presence or absence of AMA. We demonstrated that only the unique triad of HiBEC apoptotic blebs, macrophage from PBC patients, and AMA produces inflammatory cytokines and TRAIL [17]. Our observations may help close several remaining gaps in the understanding of PBC including the mechanisms that lead to the selective destruction of small intrahepatic bile ducts. We posit that PDC-E2 in the apoptotic blebs is recognized by circulating AMAs, and the resulting immune complexes stimulate macrophages from subjects with a susceptible genetic background. This event leads to a localized burst of inflammatory cytokines, and the ensuing inflammation is associated with increased apoptosis of surrounding cells, including BECs, and perpetuation of local inflammation with chronic damage to the biliary tract (Fig. 2). Although much work remains to be done in this area, recent findings suggest that BEC apoptosis may be of considerable importance for understanding PBC and that the BEC is more than simply an innocent victim of an immune attack. Rather, it attracts an immune attack by virtue of the unique biochemical mechanisms by which it handles PDC-E2.

Several studies have investigated apoptosis of BECs specifically in PBC. There is increased DNA fragmentation, implying increased apoptosis, in the BEC of patients with PBC when compared with normal controls [95–97]. Fas, FasL, perforin, granzyme B, and TRAIL expressed significantly greater levels on BECs of patients with PBC [46, 96, 98]. In addition, the upregulation of WAF1 and p53 related to biliary apoptosis is found in BECs of PBC [99]. TdT-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining has also shown significantly greater apoptosis of BECs in PBC compared with PSC even when controlled for similar degrees of inflammation [18, 46, 96, 99] (Table 1). These findings suggest that intrinsic pathways induce apoptosis of BECs in PBC and that apoptosis of BECs occurs more commonly in PBC than PSC.

#### *Therapy for PBC*

UDCA is well known to be a beneficial agent for the treatment of PBC. Especially in early-stage PBC, a



**Fig. 1** Presence of mitochondrial inner membrane enzymes and nuclear antigens in apoptotic bodies of various cell types undergoing apoptosis. Mitochondrial inner membrane enzymes; PDC-E2, OGDC-E2, DECR1, BCOADC-E2, UQCRC2, COX-IV, and ATPB.

Nuclear antigens; SSA/Ro, SSB/La, Sp100, and gp210. All 11 autoantigens are present in naive, unmanipulated cultures of human primary epithelial cells. PDC-E2, OGDC-E2, and DECR1 are detected in apoptotic bleb from HiBECs only

substantial proportion of patients have a favorable outcome with UDCA treatment [100]. Long-term treatment with UDCA in PBC patients improves biochemical liver tests, delays histologic progression, and prolongs survival without liver transplantation [101, 102]. However, the mechanisms of UDCA action have not been fully clarified. It has been postulated that the inherently greater hydrophilic nature of UDCA prevents hepatocytes and BEC damage by replacing more hepatotoxic hydrophobic bile acids [103]. Several studies demonstrated that, both in vivo and in vitro, UDCA can reduce apoptosis in BEC, hepatocytes, and non-liver tissues [95, 97, 104]. UDCA reduces serum levels of both solubilized FasL and TNF- $\alpha$  in patients with PBC. Also, UDCA reduces not only the mitochondrial MPT in isolated mitochondria but also interferes with alternate and upstream molecular targets such as the E2F-1/Mdm-2/p53 pathway [104, 105]. Reduction of apoptosis in BEC may play a role in the therapeutic response to UDCA in PBC.

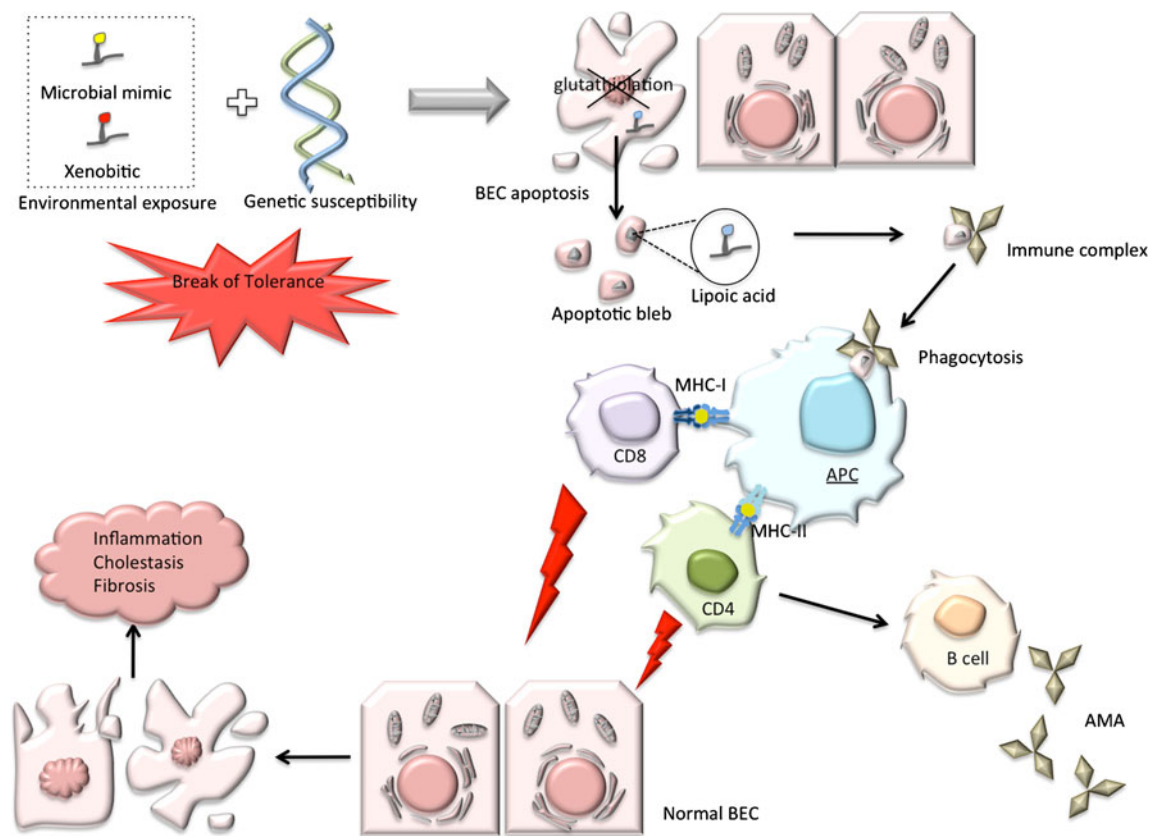
#### Primary Sclerosing Cholangitis

PSC is a chronic inflammatory large duct cholangiopathy that induces fibrosis and dilatations of the intrahepatic and

extrahepatic bile ducts, leading to the formation of multifocal bile duct strictures [106]. PSC is a male-predominant disease and is strongly associated with inflammatory bowel disease [106, 107]. Additionally, there is an increased risk of cholangiocarcinoma and gallbladder carcinoma. Histological changes show concentric periductal fibrosis (onion skinning) with progression to stricturing of large bile ducts and obliteration of small bile ducts. Although it has been reported that 50% to 80% and 7% to 77% patients with PSC produce autoantibodies against pANCA and ANA, respectively, they have no role in the routine diagnosis of PSC [108].

#### Immune Response in PSC

CD4+ T cells predominate in the portal infiltrate, and CD8+ T cells predominate in the lobular infiltrate in the liver of patients with PSC [109]. There is a greater percentage of  $\gamma\delta$ + T cells in peripheral blood of patients with PSC compared with healthy controls. The infiltrating T cells express the IL-2 receptor and CD45RO, suggesting that they have an activated memory phenotype [110]. Chemokines and their receptors have specific roles in PSC. Hepatic inflammation upregulates mucosal addressin cell adhesion molecule-1



**Fig. 2** Schema of pathogenic model in PBC. During the apoptosis of BECs, PDC-E2 remains intact because of its lack of glutathiolation in apoptotic blebs. The PDC-E2 present in the apoptotic blebs would be

recognized by circulating AMA, and the immune complex would then stimulate the innate immune system in a subject with a susceptible genetic background

(MAdCAM1) and CCL25, the CCR9 ligand, and increases recruitment of mucosal T cells. CCR9+ liver-infiltrating lymphocytes appear to be specifically increased in PSC compared with PBC. Although the frequency of CCR9+ lymphocytes is not increased in peripheral blood, approximately 20% of lymphocytes from PSC livers express CCR9 compared with <2% in normal livers or PBC [111].  $\alpha 4\beta 7$  and CCR9 expression is functionally relevant because MAdCAM1 and CCL25, which are absent from normal liver, are present on hepatic endothelium in liver diseases associated with colitis and  $\alpha 4\beta 7$ +CCR9+

lymphocytes from livers of patients with PSC bind MAdCAM1 and respond to CCL25 in adhesion and migration assays [78].

*Apoptosis of Biliary Epithelial Cells in PSC*

Histologically, TRAIL expression in BECs has been noted to be significantly greater in both PSC and PBC patients compared with healthy controls and patients with biliary stones, although DR5 expression was not significantly different

**Table 1** Comparison of apoptosis between PBC, PSC, and normal control

	PBC	PSC	Control	Reference
DR5	+	+	+	[46]
TRAIL	++	++	-	[46]
Fas	++	±	-	[96, 112]
WAF1	+++	+	ND	[99]
p53	+++	+~++	ND	[99]
Perforin	++	ND	+	[96, 98]
Granzyme B	+	ND	-	[96, 114]
TUNEL staining				
Overall	+++	+	- ~+	[18, 46, 96, 99]
Duct with inflammation	+++	+	- ~+	[18, 98]
Duct without inflammation	++	+	- ~+	

ND not done



between PSC, PBC, or controls [46]. However, Fas has been shown to be lower in the BEC of PSC when compared with PBC [112]. TUNEL staining score was significantly higher in BECs from PBC than PSC and control group. Furthermore, it was significantly higher in BECs from PBC than PSC in the presence of similar degrees of inflammation [18]. TUNEL staining may identified not only the apoptotic nuclei but also the nuclei in the necrotic tissues; the staining of necrotic cells could be explained either by the activation of an apoptotic pathway in damaged cells or by the degradation of DNA by lysosomal enzymes [113]. Though TUNEL staining may not indicate the exact frequency of apoptotic cells, it is a reliable parameter of comparison between PBC, PSC, and normal control. This difference of apoptosis suggests that BEC apoptosis may be of considerable importance for understanding PBC and suggests different pathogenic mechanisms for bile duct injury in PSC.

## Conclusion

Biliary immunity is speculated to be associated with the pathogenesis of biliary diseases as well as the defense against microbial infection. The clearance of apoptotic cells is considered as a multipurpose process that is critical to the maintenance of tolerance. The presence of intact autoantigens within apoptotic bodies and the activation of innate immunity through macrophage cytokine secretion provide a link between apoptosis and autoimmunity. The roles of genetic susceptibility and environmental factors that modify the autoantigen motif and contribute to the breakdown of tolerance may lead to further understanding of the unique immunophysiology of BEC during apoptosis and the pathogenesis of autoimmune biliary diseases.

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