# UPDATE ON PRIMARY BILIARY CIRRHOSIS

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## Abstract

Primary biliary cirrhosis (PBC) is an autoimmune chronic liver disease characterized by progressive bile duct destruction eventually leading to cirrhosis, liver failure, and death. The autoimmune pathogenesis is supported by a plethora of experimental and clinical data, such as the presence of autoreactive T cells and serum autoantibodies. The etiology remains unknown, although evidence suggests a role for both genetic susceptibility and environmental factors that remain to be determined. In fact, a number of chemicals and infectious agents have been proposed to induce the disease in predisposed individuals. The recent availability of several murine models will significantly help in understanding pathphysiology mechanisms. In this review, we critically summarize the most recent data on the etiopathogenesis of PBC, discuss the latest theories and developments, and suggest directions for future research.

## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by an immune-mediated destruction of small and medium-size intra-hepatic bile ducts (1). The serologic hallmark of PBC is the presence of high-titer serum anti-mitochondrial autoantibodies (AMA), together with an increased levels of immunoglobulin M (IgM), and several disease-specific anti-nuclear antibodies (ANA) (1). PBC can be considered a peculiar organ-specific autoimmune disease from both pathogenetic and clinical points of view (1,2). Indeed, PBC mainly affects middle-age women with a female to male ratio of up to 10:1 (3, 4), with only anecdotal cases reported in childhood (5). AMA are found in about 95% of patients with a very high specificity, but no direct correlation with and disease severity (6,7). On the contrary, disease-specific ANA are detected in one third of patients and are associated with a more severe and rapidly progressing disease (8–10).

At presentation, patients with PBC may have symptoms such as pruritus, fatigue, and/or jaundice, but the majority are asymptomatic and diagnosed during clinical workup for other reasons, including the common autoimmune comorbidities (11,12). Currently, a definite

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diagnosis of PBC is made on a combination of abnormal serum enzymes indicating cholestasis (i.e. elevated alkaline phosphatase for at least six months), the presence of serum AMA (titer ≥ 1:40), and characteristic histology with florid bile duct lesions (13). A probable diagnosis is made when two out of these three criteria are present but this definition is not widely accepted. Serum AMA may precede disease onset by several years but individuals found positive for these autoantibodies in the absence of other criteria will eventually develop PBC during follow up (14).

Although several experimental as well as clinical findings support autoimmune mechanisms for biliary damage in PBC (2,15), the underlying cause of the disease remains largely unknown. The current hypothesis on the etiopathogenesis of PBC implies that susceptibility is secondary to genetic predisposition elements that are permissive for host-environmental interactions, similar to other autoimmune diseases (16). However, the past decade has witnessed several key advances in understanding the effector mechanisms of PBC. Several lines of evidence suggest that the primary event in PBC is the loss of tolerance to the E2 subunit of pyruvate dehydrogenase (PDC-E2), the immunodominant AMA autoantigen. They also suggest that the destruction of biliary epithelium is based in part upon its unique apoptotic properties in which the mitochondrial autoantigens remain immunologically intact (17). Furthermore, several animal models with autoimmune cholangitis have now been described, each with unique features that recapitulate the human condition.

This review is timely, since we are witnessing an enormous amount of solid data on the immunomolecular mechanisms underlying the disease onset and perpetuation, which we believe will allow soon to give fundamental answers. To this regard, we will first discuss the role of genetic, epigenetic, and environmental factors in triggering the autoimmune aggression against bile ducts with focus on the recent data from a genome wide association study. We will then discuss the female predominance in autoimmunity focusing on the presence of major sex chromosome defects in women with PBC. We will then illustrate several new lines of research on the target organ and the role of innate immunity, mainly based on animal model studies. Finally, we will discuss the expanding repertoire of immune-serological diagnostic and prognostic markers while newer treatments will not be discussed (18–20).

## GENETICS FACTORS

As for many autoimmune disorders, genetic factors are known to play a decisive role in conferring PBC susceptibility (21) but are not related to a single gene but to a complex multi-genes trait.

### Familial and twin aggregation data

The first insights in a genetic component came from early epidemiological studies showing a higher incidence of disease among first-degree relatives of patients (11). Cumulatively, family aggregation data indicate that up to 6% of PBC patients have at least one family member manifesting the disease. It is to note that a recent study from the US demonstrated that there is an increased incidence of AMA without any sign of disease in first-degree relatives and offspring of patients with PBC, thus indirectly suggesting the existence of a strong genetic predisposition (22).

More recent data further strengthen the relevance of the multifactorial genetic basis in PBC, including a high concordance rate among monozygotic (identical) twins (23), and the observation that lymphocytes from women with PBC preferentially loose one X chromosome (24,25). It is of note that while among autoimmune disorders concordance rate in monozygotic twins have been shown to be on average below 50%, the PBC concordance rate is as high as 63% in 8 monozygotic sets but null among dizygotic twins (23). However, since in some

concordant sets, PBC phenotype varied significantly within one pair, it can be hypothesized that other factors, including epigenetics (4,26–28), exposure to environmental factors, or mere serendipity, may play a role complementary to genetics.

### Case-control association studies

Up until most recently, to identify susceptibility gene(s) that predispose for the development of PBC remained a challenge. The majority of studies on the etiopathogenesis of PBC have focused upon candidate gene based association studies and were limited by sample size and poor selecting and control matching criteria. They mainly focused on immune-related genes with role in maintaining tolerance and belonging to both the HLA loci and non-HLA immune modulators genes (29,30). Unfortunately, the large part of these studies reported weak and often contrasting associations (29,30), with the only exception of the consistent associations found within the HLA region (31–33). Indeed, in contrast to earlier work, we and others recently demonstrated that PBC is not only associated with the HLA DRB1*08 allele but also with two protective alleles, HLA DRB1*11 and DRB1*13 (31,32,34). Furthermore, a most recent Canadian-US study reported the first genome-wide association study and confirmed the key role in PBC susceptibility of common genetic variants in the HLA class II loci (33). Of great interest, this study also demonstrated that genes encoding for interleukin 12 and its receptor are associated with susceptibility to PBC (33). These findings needs to be confirmed in independent cohorts with a larger number of subjects and variants genotyped, but the functional role of these molecules should be carefully evaluated in the near future.

## ENVIRONMENTAL FACTORS

Despite the key role played by genetics in PBC susceptibility, genes are not sufficient to trigger the disease and we submit that exposure to certain environmental factor(s), even not harmful *per se*, may cause the breakdown of immune tolerance and PBC onset. The role of two main environmental factors have been evaluate in PBC, i.e. xenobiotics (i.e. chemical compounds), and infectious agents (viruses and bacteria) (35,36).

### Xenobiotics

Experimental and epidemiology evidence, as well as animal models, support the strong role of environmental factors including xenobiotics in the development of PBC, as illustrated by the discordant monozygotic twin sets previously discussed.

Xenobiotics are compounds foreign to the human system. The possible mechanisms through which xenobiotics may trigger an auto-immune response to self proteins are based on the hypothesis that they may modify their molecular structures or complex to self or non-self proteins to generate neoantigens. Therefore, the altered protein may induce an auto-immune response, as is the case for molecular mimicry. Our group recently suggested a possible pathogenic role of an organic compound in PBC. In particular, a specific halogenated organic compound was able to elicit AMA production by sera from patients with PBC once attached to the major mitochondrial epitope backbone (37). This because antibodies against such modified mitochondrial epitope had a higher affinity than antibodies directed against to the native epitope (37). With a subsequent study based on a multiplex approach, 2-nonynoic acid was found to be recognized by patient serum antibodies which did not cross-react with the PDC-E2 native form. This is very important since 2-nonynoic acid does not occur naturally and is found in several cosmetic products, including nail polish (11).

### Infectious agents

Early epidemiological studies provided the first insight on the role of infectious agents as potential triggers of PBC (38). Indeed, several authors reported that patients with PBC have

urinary tract infections more frequently than controls, *E. coli* being the main etiological agent. More recently, an epidemiological study on 1032 patients with PBC and 1041 controls (11) not only confirmed the association of an enhanced risk of PBC and recurrent urinary tract infections but also increased risk of vaginal infections along with lifestyle factors such as smoking. Interestingly, we also showed that subjects who frequently use nail polish was associated with an enhanced risk of develop PBC.

Molecular mimicry is a widely accepted mechanism by which infectious agents may trigger autoimmune aggression in autoimmune diseases, including PBC. Infectious agents may indeed trigger a promiscuous antibody- and cell-mediated immune response, because they share a good degree of amino acid similarity. The highly conserved sequence of mitochondrial enzymes across all species also strengthens this view. Besides *E. coli*, a number of other bacteria have been shown as cross-reactive agents in PBC, including *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Salmonella minnesota*, *Mycobacterium gordonae*, and *Trypanosoma brucei* (38). We recently provided serological and molecular evidence suggesting that *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, is possibly an ideal candidate for the induction of PBC (39) for two reasons: first, it contains two proteins with the highest degree of homology with the major epitope of PDC-E2, and secondly *N. aromaticivorans* can metabolize organic compounds and estrogens. We also reported that the bacterium can elicit a specific antibody reactivity (up to 1000-fold higher than against *E. coli*) in PBC but not in control sera (39). Furthermore, N. aromaticivorans induced serum autoantibodies and PBC-specific liver features in a murine model (40).

It has been previously reported that a novel human beta-retrovirus was found in peri-hepatic lymph nodes and other biological samples from patients with PBC. However, our group could not replicate these findings and neither confirm such hypothesis in an independent study based on a larger series of cases and controls (41). In addition, human beta-retrovirus has been recently found in the liver of patients affected by other liver diseases, including autoimmune hepatitis and viral hepatitis as well as healthy controls (42), thus excluding a specific role of this virus in PBC if not as an epiphenomenon. These data cumulatively discourage the use of antiviral therapies proposed to treat PBC (43).

## FEMALE PREPONDERANCE

Similar to other autoimmune diseases, PBC is characterized by a striking female predominance, with a female to male ratio estimated as 10 to 1 (3,4,26). So far, the reason for this observation remains unknown, but a role of fetal microchimerism, sex hormones, or X chromosome defects has been proposed.

### Fetal microchimerism

One hypothesis on the PBC female predominance was the persistence of fetal cells and genomic materials in women years after pregnancy, a phenomenon coined fetal microchimerism. Fetal cells are semi-allogenic to the maternal immune system and thus might mediate a graft-versus host disease-like reaction in women. Fetal cells were reported in blood and tissues from women with autoimmune diseases, such as scleroderma. Conversely, most of the studies failed to find significant difference in frequency of fetal microchimerism in women with PBC compared to controls (44,45). Based on these data, we are convinced that fetal microchimerism does not play a major role in PBC, although it is possible that it is involved in the pathogenesis of other autoimmune diseases.

### Sex hormones

Although sex hormones were widely investigated in the last five decades and have a number of immunomodulatory functions (46), we rule them out as major responsible for the female predominance in PBC. However, estrogens could have direct effects on cholangiocytes, the specific target organ in PBC, since these cells express estrogen receptors (47). In particular, it has been shown that cholangiocytes from patients with advanced histological stages do not express estrogen receptors, thus suggesting a role of estrogen deficiency in the development of ductopenia in PBC. However, the possible influence of estrogens on PBC onset and perpetuation needs to be confirmed and further investigated (47).

### Sex chromosomes

Few studies have investigated sex chromosomes in autoimmunity. We have recently proposed a novel hypothesis on the female predominance of autoimmunity based on major defects of sex chromosomes (3,4). This theory is based on three observations. First, diseases due to X monosomy or its major abnormalities, such as Turner's syndrome (48) or premature ovarian failure (49), are frequently associated with autoimmune features and in some cases chronic cholestasis. Second, a number of genes that are key factors in the maintenance of immune tolerance, such as FoxP3, map on the X chromosome (21). Third, diseases due to defect in single X-linked genes, such as X-linked immunodeficiency, are characterized by a plethora of autoimmune features (50). The biology of X chromosome is quite complex compared to other chromosomes as women are functional mosaics for X-linked genes, with most genes on one X chromosome being silenced as a result of X-chromosome inactivation (XCI) to achieve equivalent levels of X-linked gene products between sexes. However, more recent data have shown that the picture is even more complex by demonstrating that at least 15% of X-linked genes escape XCI in healthy women and are thus expressed from both X chromosomes (51). A role for X chromosome was first proposed based on experimental evidence that women with PBC have a significantly higher frequency of peripheral blood cells with a single X chromosome (i.e. X monosomy) compared to healthy age-matched women (24). Importantly, this difference was confirmed also in other autoimmune diseases, such as systemic sclerosis and autoimmune thyroid disease (52), but not in women with systemic lupus erythematosus (53,54). We also demonstrated that in PBC (and possibly in other autoimmune disorders) X chromosome loss is preferential and involves more frequently a single parentally inherited X chromosome (25). Other than chromosome loss, it has been also reported that women with autoimmune diseases have a non-random XCI pattern in their circulating blood cells (55,56), thus suggesting a gene dosage effect of X-linked genes but such preferential inactivation was not found in PBC (25) and in other autoimmune disorders (57).

## ANIMAL MODELS

As in other complex diseases, the development of an animal model is of great importance in dissecting the mechanism underlying the initiation and progression of PBC. In the last few years, several murine models of PBC (2,58) have been proposed and their major features are illustrated in Table 1. Mouse strains have been reported to be spontaneous PBC model animals and two among these animal models, IL-2Rα knockout and dnTGFβRII selective knock-out mice, strongly indicate the possible role of Tregs deficiency in PBC onset. In particular, the mouse deficient for IL2 receptor α (IL-2R α), which is highly expressed on T regs, developed AMA positivity against PDC-E2 in all animals, 80% ANA positivity, and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury (59). The dominant negative form of transforming grown factor β (TGFβ) receptor II, (dnTGFβRII) manifests PBC-like liver disease, such as 100% AMA positivity against PDC-E2 (60). TGFβ receptor II is known to be essential for signal transduction of TGFβ to regulate lymphocytes activation (61). A third animal model is a variant of the non-obese diabetic (NOD) mouse model

(NOD.c3c4). It has been described that NOD.c3c4 mouse develops autoimmune cholestasis and PBC-specific serology, showing AMA positivity in up to 60% of sera and ANA positivity in about 90%. Histologically, there is lymphocyte infiltration around portal tracts with epithelioid granuloma formations and chronic nonsuppurative destructive cholangitis; However, the morphological features of bile duct damage differ from those in human PBC, particularly because of the occurrence of cystic changes (62).

Other useful models for PBC have been subsequently developed by immunization with xenobiotically modified molecular variants of the PDC-E2 epitope (2,58). Firstly, we showed loss of tolerance in rabbits immunized with 6-bromohexonate, a xenobiotically modified hapten mimicking lipoic acid. The immunized rabbits produced very high titer AMA directed at PDC-E2 other than antibodies against the xenobiotic, but did not induce PBC-specific hepatic lesions at least in the short follow-up (63). Finally, induction of specific PBC features was obtained in guinea pigs (64) and in a NOD background (65) exposed to xenobiotic immunization. All these models share some similarities with the human condition (66), yet manifest specific peculiarities.

## CELLULAR IMMUNITY

### Autoreactive T cells

The involvement of cellular immune mechanisms in the biliary damage is clearly suggested by the presence of high number of helper (CD4+) TCR αβ+ and CD8+ T cells in the portal tracts from patients with PBC (67–72). Autoreactive PDC-E2-specific CD4 T cells have been reported both in peripheral blood and liver tissue of patients with PBC but not in healthy and disease controls. In support of their role in the liver damage, a 150-fold increase in number of CD4 T cells specifically targeting PDC-E2 was found in the peri-hepatic lymph nodes and liver compared with blood of patients with PBC. Our group also characterized the antigen specificity of these cells and demonstrated that in HLA DR4*0101 positive patients autoreactive CD4 T cells recognized a single epitope of 163–176 aa sequence which encompass the lipoic acid binding residue of the inner lipoyl domain of PDC-E2 which is shared by serum AMA. Finally, we showed that these cells are of pro-inflammatory nature only in PBC patients but not in controls, based on the production of pro-inflammatory cytokines such as IFN-γ (69), as later confirmed in peripheral blood (73).

Based on a plethora of data, autoreactive CD4+ and CD8+ T cells are believed to be involved in the pathogenesis of PBC and liver infiltration of these cells is one of the major features of the disease (2), including in AMA-negative cases (74). However, findings point to a predominant role for the CD8+ T subpopulation in PBC (75,76). It is of note that the HLA class I restricted epitope for CD8+ T cells, i.e. 159–167 aa sequence, maps closely to the epitopes recognized by serum AMA as well as by CD4 T cells, that is the autoepitope for both CD4 and CD8 T cells overlaps with the B cell (AMA) epitope. As for autoreactive CD4+ T cells, we showed a 10-fold higher frequency of PDC-E2159-167 specific CD8 T cells within the liver compared to blood of PBC patients. Functionally, it has been shown that autoreactive CD8 T cells in this disease have the ability to produce IFN-γ rather than IL-4/IL-10 cytokines (77), but also IL-17 has been recently suggested to be crucial in PBC (76).

### Regulatory T cells (Tregs)

Despite extensive data on both autoantibody and autoreactive T cells, the mechanisms that lead to loss of tolerance in PBC have proven elusive. From a generic perspective there is considerable discussion that suggests that defects in the regulatory T cell (Treg) compartment are responsible for antigen specific loss of tolerance (78), possibly based on genetic mechanisms (79). However, this has been difficult to prove in vivo and, despite widely

observed, quantitative and/or functional impairments of Tregs in humans and animal models, it has been difficult to link these observations to bile duct specific autoimmunity. PBC is overwhelmingly a syndrome of adults, although interestingly there is a PBC-like disease reported in a child with IL-2 receptor α (CD25) deficiency (80). This observation is particularly intriguing because of data from murine models of autoimmune cholangitis (59,60,81) in which CD8 T cells play a critical role in the loss of Treg function in mice (75). We should also note that quantitative and functional analysis of intrahepatic and circulating Tregs in humans with PBC suggest a loss of T regulatory function (82–84) but these studies have focused entirely on CD4+CD25 Treg cells. Based on the murine data, in the future it would be important to specifically address and analyze the CD8 Treg populations in patients with PBC.

## Innate immunity cells

While adaptive immunity recognizes antigens with high specificity, the innate immunity system, including monocytes, dendrocytes, and natural killer (NK) cells, recognizes distinct evolutionarily conserved structures generally shared by pathogens and known as pathogen-associated molecular patterns (PAMPs), and thus allow a rapid recognition and elimination of infectious agents. PAMPs are known to bind to toll-like receptors (TLRs) which then modulate the function of both adaptive cellular and humoral immunity (85). Of note, the liver is considered both structurally and functionally as a major organ of innate immunity, since it contains the largest resident population of cells of the innate immune system. Growing data indicate that the innate immune system contributes to the triggering and perpetuation of liver damage. In particular, PBC exhibits specific immunological features in support of this view, such as elevated levels of serum IgM in response to bacterial antigens, the presence of epitheliod granulomas, increased levels of cytokines response and enhanced-responsiveness to PAMPs by NK cells and monocytes, as explained in details below. Almost all patients with PBC have elevated IgM levels, independently of their AMA or ANA status (1). A polyclonal hyper-IgM was found to be secondary to a chronic polyclonal innate immune response of memory B cells to bacterial unmethylated CpG motifs (86). Moreover, our group also demonstrated that B cells exposed to CpG motifs express increased amount of CD86 and TRL9 as well as increased production of autoantibodies. These data support a link between bacteria and PBC and strongly suggests a key role for B cells dysregulation in PBC (87).

PBC monocytes have a pro-inflammatory activity which is enhanced in PBC. More specifically, monocytes activated by PAMPs through TLRs release pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, and TNF-α which then amplifies the adaptive T cell mediated immune response against infectious agents. We demonstrated that circulating monocytes from PBC patients challenged with various PAMPs specific for TLR2, TLR3, TLR4, TLR5, and TLR9 lead to high levels of all pro-inflammatory cytokines when compared with cells from controls (88). The mechanisms for such increased sensitivity may well be secondary to the higher frequency of recurrent urinary tract infections reported in PBC. It is also possible that both monocytes and B cells constantly exposed to bacterially derived products (PAMPs) gathered from the portal circulation participate in modulating the adaptive cellular immune response.

In more recent years, NK T cells are attracting growing attention in autoimmunity (89), being innate effector cells regulated by self and non-self glycolipid antigens presented by the antigen-presenting molecule CD1d (90). Such activation leads to a rapid production of cytokines and chemokines by NK T cells, with consequent modulation of both the adaptive and innate immune responses. In an early study, we reported a higher frequency of CD1d-restricted NKT cells in PBC patients compared to controls and that these were more frequent in the liver compared to peripheral blood of patients (72). Subsequently, we confirmed an increased number of CD1d-restrcited NKT cells also in the liver of dnTGFβRII mice, one of the

comprehensive PBC murine models. Finally, we also reported that CD1d-deficient dnTGFβRII mice had a reduced hepatic lymphoid cell infiltrates and milder cholangitis compared to controls (89).

Although innate immunity hyper-responsiveness is likely not sufficient to cause the loss of immune tolerance, we hypothesize that these alterations might play a role in triggering the autoimmune pathology. In this scenario, it is intriguing that in a murine model of PBC *N. aromaticivorans* was able to induce the production of serum AMA and chronic cellular-mediated autoimmunity against small bile ducts in an NK T cell dependent fashion (40).

# AUTOANTIBODIES

## Anti-mitochondrial antibody (AMA)

Serum AMA are widely accepted as the diagnostic hallmark of PBC and found in nearly 100% of affected individuals when tested using techniques based on recombinant mitochondrial antigens (via immunoblotting or ELISA) (7,91). The extremely high sensitivity and specificity of AMA make them one of the most specific diagnostic tests of human diseases (6,91). AMA specifically recognizes lipoilated domains within components of the 2-oxoacid dehydrogenase (OADC) family of enzymes within the mitochondrial respiratory chain, particularly the dihydrolipoamide acetyltransferase (E2 component) of the pyruvate dehydrogenase complex (PDC). Less frequent autoantigens are the E2 components of 2-oxo glutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes, the E3 binding protein (E3BP) and the E1α subunit of the pyruvate dehydrogenase complex (PDC-E1α) (7,92,93) (Table 2). Indirect immunofluorescence (IIF) using rodent liver, kidney and stomach sections as substrate, is still the most widely used screening assay for AMA in the routine setting (13), although immunoblotting and ELISA have an higher sensitivity, and the use of cloned mitochondrial antigens and bead assay testing system (94) allow to identify AMA in the sera of patients previously defined as AMA negative. Although extremely useful as diagnostic marker, AMA are not clinically helpful during follow-up as several studies demonstrate that they do not correlate with stage (95). It is also to note that AMA are often detectable for several years before the onset of overt clinical disease (14).

## Anti-nuclear antibody (ANA)

Serum ANA are detected in approximately one third of sera from patients with PBC, and reportedly more frequently in AMA-negative cases (8,96). Over the last three decades, several nuclear structures have been identified as specific targets of ANA in PBC (10), with the two most frequent patterns being "multiple nuclear dots" (ND) in which the antigens recognized are the Sp100 and promyelocytic leukemia proteins (PML), and "perinuclear" based on gp210 and nucleoporin p62 antigens localized within the nuclear pore complex (NPC). Both the perinuclear and nuclear dot ANA patterns are very specific for PBC (97) (Table 2), while anti centromere autoantibodies (ACA) are not specific and found in only 10% of PBC patients (98), similar to other autoantibodies (99). Of interest, the ANA specificities have been found more frequently in patients with severe disease in cross-sectional studies (8,96–98,100,101) and, even more interestingly, the presence of anti-NPC is associated with worst prognosis (9, 102–104) in longitudinal observations. These data have obvious relevant implications for the clinical management of PBC since anti-NPC and ACA testing are important for identifying asymptomatic patients with an unfavorable disease outcome and warranting early therapy (7). Unfortunately, the pathogenic role of these antibodies has been poorly investigated and remains unknown.

## BILIARY EPITHELIAL CELLS (BEC)

PBC is characterized by a highly selective destruction of the small and medium size intrahepatic bile ducts, lined by BEC (i.e. cholangiocytes). It has been demonstrated that BEC express cell surface adhesion molecules which permit adhesion and recognition of lymphocytes. In addition, a number of studies have demonstrated that BEC of both healthy and diseased liver have the capacity to increase the expression of adhesion molecules, such as ICAM-1 and others, TNF-α, MHC class I and II, IFN-γ and IL-1 upon stimulation with pro-inflammatory cytokines. Adhesion molecules expressed on BEC, along with the enhanced levels of pro-inflammatory cytokines, allow BEC to modulate the intensity and localization of inflammatory reactions. Moreover BEC have the capacity to act as antigen presenting cells, expressing HLA class II (105,106), and accessory molecules responsible for the co-stimulatory signal to T cells, CD80,86 (B7-1, B7-2). The interactions between BEC and T cells might be responsible for bile duct loss, a key characteristic of progression of disease.

Antigenicity of BEC self-molecules, or highly homologous epitopes, could also be related to their role in mucosal immunity. As other epithelial cells, BEC actively transfer IgA-AMA specific for PDC-E2. Interestingly, these specific IgA-type AMA can be detected in all body fluids of patients with PBC, including saliva, bile, and urine (107,108). Matsumura and colleagues provided evidence for direct toxic effects of AMA-IgA by exposing canine kidney cells transfected with the human polymeric Ig receptor to highly purified AMA-IgA (109). Overall, the immunogenic characteristics of BEC in PBC are summarized in Table 3.

Apoptosis of BECs in PBC may prove crucial for immune tolerance loss (17,110), as in other conditions (110). Odin and colleagues reported that the glutathiolation of the lysine-lipoic acid moiety of PDC-E2 was reduced by serum AMA (111). Most recently our group demonstrated that BEC expose intact immunoreactive PDC-E2 within apoptotic blebs from cells undergoing apoptosis (17), thus suggesting that the unique characteristics of BECs during apoptosis may explain the tissue specificity of the autoimmune injury in PBC (112) although experimental data suggest that BEC may in fact be innocent victims of the autoimmune injury (68).

## HYPOTHESIS ON PBC ETIOPATHOGENESIS

Following this discussion of available data, it is possible to propose a unifying view. Three major events are crucial to the proposed mechanism leading to the breakdown of tolerance and the resulting PBC onset and perpetuation, i.e. BEC apoptosis, female predominance, and genetic susceptibility. A microorganism (possibly the ubiquitous *N. aromaticivorans*) with highly similar proteins to human PDC-E2 enters the human system through the digestive mucosa and its mimicking proteins are modified within the liver by xenobiotics to form immunoreactive antigens. These modifications could be then sufficient to trigger the innate immune system to initiate a cascade of local inflammatory events resulting in local dendritic cell activation and antigen processing. Mucosal antigen-presenting cells in turn could activate autoreactive T and B cells (16) that are directed to the liver through the portal system. T cells, therefore, could participate directly not only to the autoimmune injury, but also to its amplification and perpetuation (68). B cells, on the other hand, could secrete AMA, particularly of the IgA type. AMA-IgA could be then transported to the vascular side of biliary epithelial cells where they could recognize PDC-E2-like molecules located on the luminal surface cell membrane. AMA-IgA/PDC-E2-like molecules engagement could initiate apoptotic signaling cascade. Ultimately, the immune complexes of post-apoptotic PDC-E2 and IgG-AMA and the direct cytopathic effects of autoreactive T cells (and possibly AMA) lead to the selective BEC targeting and autoimmune cholangitis development.

## CONCLUSIONS AND FUTURE PERSPECTIVES

There have been substantial advances in the understanding of PBC pathogenesis since the molecular identification of PDC-E2 in 1987 as the major autoantigen of AMA (92). A number of questions on the etiology and pathogenesis of PBC still need to find an answer but we believe that soon we will be able to solve the puzzle. We are also convinced that to achieve this goal, our efforts should be mainly dedicated to overcoming some logistic difficulties. Firstly, we encourage the collection of very large series of patients and controls, possibly by mean of multicentric studies, and the use of genome-wide analysis on thousands of genetic and epigenetic variants. This will allow defining the individual bases of PBC. Secondly, based on the most recent evidence, the role of innate immunity in the onset and perpetuation of PBC should be further studied. Third, it is time to prove the AMA pathogenic role in PBC. Fourth, it will be important to develop additional animal models to better dissect the molecular mechanisms underlying the disease. Finally, we are convinced that the growing evidence on the key role of apoptosis in PBC will provide some intriguing data in the near future. Ultimately, we believe that while new frontiers are being proposed (113,114) we will be able to understand the etiopathogenesis of PBC only through a multidisciplinary approach uniting clinicians, basic immunologists, geneticists, chemists, and microbiologists, possibly through the proposed role of an autoimmunologist (115).

## Acknowledgments

## Abbreviations

| | |
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| PBC | primary biliary cirrhosis |
| AMA | anti-mitochondrial antibodies |
| IgM | immunoglobulin M |
| ANA | anti-nuclear antibody |
| PDC-E2 | E2 subunit of pyruvate dehydrogenase |
| HLA | human leukocyte antigen |
| XCI | X-chromosome inactivation |
| dnTGFβRII | dominant negative form of transforming grown factor β receptor II |
| NOD | non-obese diabetic |
| Treg | regulatory T cell |
| NK | natural killer |
| PAMPs | pathogen-associated molecular patterns |
| TLRs | toll-like receptors |
| OADC | 2-oxoacid dehydrogenase |
| OADC-E2 | E2 components of 2-oxo glutarate dehydrogenase |
| BCOADC-E2 | E2 components of branched-chain 2-oxo acid dehydrogenase |
| E3BP | E3 binding protein |
| PDC-E1α | E1α subunit of the pyruvate dehydrogenase complex |
| IIF | Indirect immunofluorescence |

| ND  | nuclear dots                  |
|-----|-------------------------------|
| PML | promyelocytic leukemia proteins |
| NPC | nuclear pore complex          |
| ACA | anti-centromere antibodies    |
| BEC | biliary epithelial cells      |

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**Table 1**

Similarities of the acquired and innate immunity compartments between the murine models and human PBC.

| Model | Adaptive immunity | Innate immunity | Ref. |
|---|---|---|---|
| **Ae2(a.b)-deficient** | - AMA | -- | (116) |
| | - Lymphocytic CD8+ infiltrates | | |
| | - Decreased T regulatory cells | | |
| | - PBC-like liver lesions | | |
| **Xenobiotic on** | - AMA | -- | (65) |
| **C57BL/6** | - Lymphocytic CD8+ infiltrate | | |
| | - PBC-like liver lesions | | |
| **NOD.c3c4** | - AMA, ANA | -- | (62) |
| | - lymphocytic infiltrate | | |
| **IL2Ra$^{-/-}$** | - AMA | -- | (59) |
| | - portal tract CD4+ and CD8+ cells | | |
| **dnTGFbRII** | - AMA | NKT cells worsen liver injury | (60) |
| | - Deficient T reg function | | |
| *N. aromaticivorans* **on** | - AMA | NKT cells are required | (40) |
| **NOD 1101** | - PBC-like liver lesions | | |
| | - Disease transfer by T cells | | |

**Table 2**

Major autoantigens in PBC.

| **Mitochondrial proteins** | |
|---|---|
| E2 subunits of 2-OADC | PDC-E2 [*] |
| | OGDC-E2 [*] |
| | BCOADC-E2 [*] |
| Pyruvate dehydrogenase complex | E3BP [*] |
| | PDC E1α |
| **Nuclear proteins** | |
| Multiple nuclear dots | Sp100 |
| | PML |
| Nuclear pore complex | gp210 [*] |
| | nucleoporin p62 [*] |
| Centromeres | CENP A, B and C |

[*] Specific molecules detectable by immunoblot or ELISA

Abbreviations: 2-OADC: 2-oxo-acid dehydrogenase complex; PDC: pyruvate dehydrogenase complex; OGDC: oxoglutarate dehydrogenase complex; BCOADC: branched chain 2-oxo-acid dehydrogenase complex; E3BP: dihydrolipoamide dehydrogenase (E3) – binding protein

**Table 3**

Imunopathologic characteristics of cholangiocytes in PBC.

| | PBC | Normal |
|---|---|---|
| PDC-E2 expression | + + + | + |
| Adhesion molecules | | |
| - ICAM-1 | + + | + |
| - VCAM-1 | + | −/+ |
| - LFA-1 | + | −/+ |
| - E-selectins | + + | |
| Biliary intra-epithelial lymphocytes | Small bile ducts, increased CD4$^+$CD28$^-$ | Large bile ducts, few CD4$^+$ |
| Cytokines | | |
| - INF-γ | + + | − |
| - IL-2 | + + | − |
| - IL-6 | + + | − |
| - IL-6 receptor | −/+ | − |
| - TNF-α | + + | −/+ |
| - TNF receptor | + + | −/+ |
| BEC phagocytosis of apoptotic BECs | + + | − |
| Apoptosis-related molecules | | |
| - Fas (CD95) | + | − |
| - granzyme B | −/+ | − |
| - perforin | −/+ | − |
| - bcl-2 | − | + + |