

## Role of autoimmunity in primary biliary cirrhosis

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### Abstract

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by the presence of serum autoantibodies and chronic nonsuppurative destructive cholangitis. The pathogenesis of PBC involves environmental factors, genetic predisposition and loss of immune tolerance. In recent years, it has become univocally accepted that an inappropriately activated immune response is one of the most important factors in PBC. In this study, the role of autoimmunity in PBC is summarized and a feasible research orientation is recommended.

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**Key words:** Primary biliary cirrhosis; Autoimmunity; Humoral immunity; Cellular immunity; Nonspecific immunity

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### INTRODUCTION

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease of the liver characterized by the presence of serum antimitochondrial antibodies (AMAs) and the destruction of small and medium-sized bile ducts. The histological manifestations are damaged biliary epithelial cells (BECs) and infiltration of T cells, B cells, macrophages, eosinophils and natural killer (NK) cells in the portal area, which eventually leads to cirrhosis and liver failure<sup>[1]</sup>. PBC affects middle-aged women, and the natural disease history is 10 to 20 year. The annual incidence rates range between 0.7 and 49 cases per million persons, while the prevalence rates range between 6.7 and 402 cases per million persons<sup>[2,3]</sup>. Advanced biochemical assays and improved acquisition of disease will lead to increased detection worldwide. Currently, the only recommended first-line therapy, early treatment with ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/kg per day, can delay progression of histology and ameliorate long-term prognosis<sup>[4]</sup>. However, approximately 25% of patients have no response to UDCA, and in these cases liver transplantation is needed<sup>[5]</sup>. Exploration of pathogenesis is needed to discover novel target treatments. Deficiencies in autoimmune tolerance are critical factors of disease initiation and perpetuation. Therefore, the aim of this study was to demonstrate the function of autoimmunity in PBC.

### HUMORAL IMMUNITY

#### Autoantibody-AMA

High titer of AMAs is the serological hallmark of PBC. It can be detected even before clinical symptoms or bio-

chemical anomalies. The proportion of AMA-negative patients has been minimized due to the development of sensitive detection technology<sup>[6,7]</sup>. The highly disease-specific autoantibody, AMA-M<sub>2</sub>, recognizes components of the oxo-acid dehydrogenase complex (OADC) that are ubiquitously expressed on the inner mitochondrial membrane, including the E<sub>2</sub> subset of the pyruvate dehydrogenase complex (PDC-E<sub>2</sub>). The antigens are released from apoptotic blebs of the BEC, or come from molecular mimicry of infectious agents, or from alteration of xenobiotics<sup>[8]</sup>. However, transgenic mice aberrantly expressing PDC-E<sub>2</sub> components on BECs do not show serological and histological features of PBC<sup>[9]</sup>, indicating that aberrant PDC-E<sub>2</sub> expression is not sufficient for disease development.

Although AMA is found in most PBC patients, it may have no pathogenic role due to the following observations: (1) the titer of AMA is not associated with biochemical or histological manifestations, and there is no indication for a significant change in AMA levels after drug therapy<sup>[10,11]</sup>; (2) approximately 5% of patients are AMA-negative with typical histological injury and exhibit the same treatment response as AMA-positive patients<sup>[12,13]</sup>; and (3) although the antigens are ubiquitous, the disease is organ-specific.

However, some studies have identified novel features of AMAs in recent years. Lleo and colleagues<sup>[14,15]</sup> demonstrated that PDC-E<sub>2</sub> with antigenic reactivity was only detectable in apoptotic blebs of human intra-hepatic BECs; Moreover, in the presence of AMA, macrophages increased the expression of TNF-related apoptosis-inducing ligand (TRAIL) and produced intense inflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin (IL)-6. These results provide a mechanism for the biliary specificity and a physiopathologic role of AMA in PBC. Rigopoulou *et al.*<sup>[16]</sup> found that AMA-IgG<sub>3</sub> was associated with a more severe disease. The presence of IgA-anti-PDC-E<sub>2</sub> in sera or saliva might be associated with the progression of PBC<sup>[17]</sup>. The mechanism responsible is likely that a greater concentration of IgA in bile ducts can make cells more susceptible to apoptosis through constant transcytosis, resulting in subsequent bile duct damage<sup>[18]</sup>. In addition, the production of AMA-IgM from peripheral blood mononuclear cells (PBMCs) from PBC patients was reduced after exposure to UDCA<sup>[19]</sup>.

### **Autoantibody-antinuclear antibodies**

In addition to AMA, serum antinuclear antibodies (ANAs) are positive in approximately 1/3 patients with PBC. These antibodies provide important evidence for AMA-negative PBC. The typical staining pattern and clinical significance<sup>[20,28]</sup> of ANAs are shown in Table 1.

### **B cells and plasma cells**

Plasma cells that originate from B cells are sources of antibodies. As antigen presentation cells, B cells can secrete many kinds of cytokines and present costimulatory signals

to active antigen-specific T lymphocytes. Migita *et al.*<sup>[29]</sup> found that serum B-cell-activating factor (BAFF) levels were significantly higher in PBC patients than in healthy controls and HCV-infected patients, and were positively correlated with aspartate amino-transferase (AST) and total bilirubin levels. In liver, CD5<sup>+</sup> and CD20<sup>+</sup> cells were associated with BEC damage, suggesting that B cells have a role in regulating the portal destruction in PBC<sup>[30]</sup>. Therefore, B cell depletion therapy might be an alternative to UDCA. In murine experiments, I $\mu$ (-/-)NOD.c3c4 mice demonstrated a decreased number of activated NK cells in the liver. The degree of granuloma formation, bile duct destruction, and salivary gland histology were also shown to be significantly attenuated<sup>[31]</sup>. Moreover, anti-CD20 therapy every 2 wk in transforming growth factor-beta receptor II dominant negative (dnTGF- $\beta$ R II) mice at age of 4-6 wk could reduce the number of B cells and CD8<sup>+</sup> T cells in liver<sup>[32]</sup>. In clinical therapy, two doses of 1000 mg rituximab separated by 2 wk were safe and effective in patients with an incomplete UDCA response. After treatment, not only did serum levels of total IgG, IgM, and IgA decrease significantly, but T regulatory (Treg) cells also increased, which was associated with increased mRNA levels of forkhead box 3 (Foxp3) and TGF- $\beta$  in CD4<sup>+</sup> T cells<sup>[33]</sup>.

In contrast, in 2-octynoic acid-bovine serum albumin (2OA-BSA)-induced mice, treatment with anti-CD20 and anti-CD79 antibodies increased the number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrating around damaged bile ducts in portal areas, leading to more severe cholangitis<sup>[34]</sup>. A similar phenomenon occurred in I $\mu$ (-/-)dnTGF- $\beta$ R II mice. Adoptive transfer of CD19<sup>+</sup> cells from dnTGF- $\beta$ R II mice into recombination activating gene-1 (Rag-1)(-/-) mice resulted in decreased liver inflammation and bile duct damage<sup>[35]</sup>. However, anti-CD20 therapy in dnTGF- $\beta$ R II mice at age of 20-22 wk had little effect on liver lesions<sup>[32]</sup>. The efficacies of different B cell depletion approaches in diverse murine models are shown in Table 2. Together, these findings suggest that there is a subclass of B cells that have a regulatory role by producing IL-10<sup>[36]</sup>. However, further exploration of the function of B cells in PBC is needed before B cell depletion therapy can be applied to routine clinical work.

### **Elevated IgM**

In addition to high titers of circulating AMAs, PBC patients have high levels of serum IgM that are not related to titers of AMAs. Compared with AMA-positive PBC, the level of serum IgM was lower in AMA-negative patients<sup>[37,38]</sup>. Plasma cells in the portal tracts of PBC patients are found to be predominantly IgM-positive, while those cells predominantly express IgG in other forms of liver disease, such as autoimmune hepatitis and chronic hepatitis C<sup>[39,40]</sup>. It has been shown that after treatment with UDCA, the level of IgM decreases at both short-term and long-term follow-up<sup>[41]</sup>, which is possibly due to a reduction in naïve B cell and IgM-memory B cell activation through down-regulation of the NF- $\kappa$ B signal-

Table 1 The characteristics of antinuclear antibodies in primary biliary cirrhosis patients

Staining pattern	Autoantigen	Prevalence (%)	Clinical significance
Nuclear dot	SP100 <sup>[20,28]</sup>	25	Highly specific for PBC; Urinary tract infections
	PML <sup>[21,28]</sup>	19	Highly specific for PBC; Coexistence with anti-sp100
	SP140 <sup>[22]</sup>	15	Coexistence with anti-sp100 and anti-PML
Nuclear periphery (Nuclear pore complex)	gp210 <sup>[23]</sup>	25	Association with disease severity and poor prognosis
	p62 <sup>[24]</sup>	30-55	Association with disease severity and poor prognosis
Nuclear periphery (Nuclear envelope)	Lamin <sup>[25]</sup>	6-8	Not highly specific for PBC
	LBR <sup>[24]</sup>	2-6	Highly specific for PBC
Anticentromere	Centromere <sup>[26]</sup>	30	Association with portal hypertension
?	AchR M3 <sup>[27]</sup>	83	Unknown

PBC: Primary biliary cirrhosis; PML: Promyelocytic leukemia; LBR: Lamin B receptor.

Table 2 Differences in B cell depletion therapies in primary biliary cirrhosis murine models

Results	Model	Therapy (time)
Amelioration	NOD.c3c4 mice	Igμ knockout
	dnTGF-βR II mice	Anti-CD20 (4-6 wk)
Exacerbation	2OA-BSA-induced mice	Anti-CD20/Anti-CD79 (6 wk)
	dnTGF-βR II mice	Igμ knockout
No effect	dnTGF-βR II mice	Anti-CD20 (20-22 wk)

ing pathway<sup>[19]</sup>. The mechanism of IgM elevation is still unclear in PBC, but abnormal Ig class switching may be involved. Lleo *et al*<sup>[42]</sup> showed that IgM was inversely associated with levels of CD40L promoter methylation in CD4<sup>+</sup> T cells, suggesting that the CD40-CD40L interaction is involved in the production of high amounts of IgM. Moreover, CD40L is an essential molecule involved in Ig class switching.

## CELLULAR IMMUNITY

It is thought that activated CD4<sup>+</sup> T cells can recognize peptide PDC-E<sub>2</sub>163-176, while activated CD8<sup>+</sup> T cells can recognize peptide PDC-E<sub>2</sub>159-167 and PDC-E<sub>2</sub>165-174 in PBC. Moreover, a large number of autoreactive T lymphocytes infiltrate the portal area.

### CD4<sup>+</sup> T cells

**Effector CD4<sup>+</sup> T cells:** In patients with PBC, it is well accepted that an enhanced ratio of Th1 to Th2 cells is one of the most important factors<sup>[43,44]</sup> in the onset of disease. After treatment with UDCA, serum interferon-gamma (IFN-γ)<sup>[45]</sup> and liver IL-2<sup>[45,46]</sup>, which are Th1 cell-related cytokines, are decreased. However, the level of serum IFN-γ rebounded after 6 mo of therapy<sup>[45]</sup>. Recently, besides Th1 cells, studies have shown that IL-17<sup>+</sup> cells accumulate around the damaged bile ducts<sup>[47,48]</sup>, and BECs produce Th17-inducible cytokines (IL-6, IL-1β and IL-23) when stimulated with pathogen-associated molecular patterns (PAMPs)<sup>[47]</sup>. In addition, the ratio of Th17 to Tregs is enhanced in PBMCs<sup>[49]</sup>.

Several experiments using murine models have indicated a central role of CD4<sup>+</sup> T cells in the pathogenesis of PBC. NOD.c3c4-SCID mice can develop autoim-

mune cholangitis after adoptive transfer of splenocytes or CD4<sup>+</sup> T cells<sup>[50]</sup>. In IL-2Rα(-/-) mice, marked aggregation of IL-17<sup>+</sup> cells within portal tracts compared to the periphery has been demonstrated. Interestingly, CD4<sup>+</sup> T cells from the livers of normal C57BL/6J mice can secrete higher levels of IL-17 compared to those from spleens, indicating the role of the liver microenvironment in Th17 induction<sup>[48]</sup>.

It is currently unknown whether Th1 or Th17 cells are more important in the pathogenesis of the disease. IL-12p40(-/-)dnTGF-βR II mice have a dramatic reduction in histological autoimmune cholangitis and a significant decrease in the levels of intra-hepatic proinflammatory cytokines<sup>[51]</sup>, while worsening hepatic histology and elevated inflammatory cytokine production have been observed in IL-6(-/-)dnTGF-βR II mice<sup>[52]</sup>. These findings might suggest that IL-12-inducible Th1 cells are more important than IL-6-inducible Th17 cells. However, the definitive conclusions on these mechanisms will require further investigation.

**Treg cells:** CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells play a critical role in self-tolerance and the prevention of autoimmune disease. Patients with PBC display a relative reduction of circulating CD4<sup>+</sup>CD25<sup>high</sup> Tregs compared to controls<sup>[53,54]</sup>. The frequency of circulating Tregs can increase after 1 year of treatment with UDCA<sup>[13]</sup>. However, the number of Foxp3<sup>+</sup> cells is higher in the liver in PBC<sup>[53]</sup>, which is most likely due to the localization of CD8<sup>+</sup> T cell blasts in the liver portal area<sup>[55]</sup>.

In addition to CD4<sup>+</sup> Tregs, Bernuzzi *et al*<sup>[56]</sup> found that the CD8<sup>+</sup> Treg (CD8<sup>+</sup>CD28<sup>-</sup>) population has striking phenotypic alterations, including decreased CD39 and increased CD127. Although CD8<sup>+</sup> Tregs were not significantly different quantitatively between patients and healthy subjects, the *in vitro* induction of CD8<sup>+</sup> Tregs by incubation with IL-10 was significantly reduced in PBC patients.

Murine models constructed by altering the signaling pathway of Treg cells can imitate PBC-like manifestations, which underscores the vital function of Tregs in disease. Zhang *et al*<sup>[57]</sup> demonstrated that Scurfy mice, which have complete ablation of Foxp3<sup>+</sup> Tregs, exhibited a high titer of AMAs and elevated serum cytokines

Table 3 The characteristics of primary biliary cirrhosis murine models and primary biliary cirrhosis patients

	NOD.c3c4 <sup>[50]</sup>	dnTGF-βR II <sup>[59]</sup>	IL-2Rα(-/-) <sup>[58,85]</sup>	Ae2a,b(-/-) <sup>[86]</sup>	2OA-BSA induced <sup>[87]</sup>	Human
Disease onset	8-20 wk	4 wk	4 wk	6 mo?	4-12 wk	40-60 yr
AMA	56%	100%	100%	40-80%	100%	95%
ANA	80%-90%	(-)	80%	(-)	?	30%
Cytokines	Th1 ↑	Th1 ↑	Th1, Th17 ↑	Th1 ↑	Th1 ↑	Th1, Th17 ↑
Treg cells	↓	↓	↓	↓	↓	↓
Ig	IgG, IgM ↑	IgA ↑	IgG, IgA ↑	IgG, IgM ↑	?	IgM ↑
Liver inflammation						
CD8+/CD4+T	↑	↑	↑	↑	↑	↑
Treg	?	↓	↓	↓	?	↑
Fibrosis	?	Slight	?	Slight	Slight	Evident in late stage
Complication	Bile cyst and Extrahepatic damage	Colitis	Colitis Anemia	?	?	Thyroid disorder Sjogren syndrome

ANA: Antinuclear antibodies; AMA: Antimitochondrial antibodies.

(TNF-α, IFN-γ, IL-6, IL-12p40, and IL-23). In addition, these mice also had a substantial number of lymphocytes infiltrated around portal areas with bile duct damage. Both IL-2Rα(-/-)<sup>[58]</sup> and dnTGF-βR II murine models<sup>[59]</sup> show PBC-like characteristics.

### CD8<sup>+</sup> T cells

Compared to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells play a more significant role in mediating the destruction of the bile duct. In PBC patients, CD45RO<sup>high</sup>CD57<sup>+</sup>CD8<sup>high</sup> T cells expressing elevated α4β7 and IL-5 accumulate around the portal area<sup>[60]</sup>. Moreover, the ratio of CD8<sup>+</sup> T cells to FoxP3<sup>+</sup> cells is significantly higher in the liver at late stages of the disease<sup>[53]</sup>. Biliary ductule damage by CD8<sup>+</sup> T cells is also observed in non-obese diabetic autoimmune biliary disease (NOD.ABD) mice<sup>[61]</sup>. CD8<sup>+</sup> T cells from NOD.ABD mice can transfer liver inflammation into NOD.c3c4-SCID recipients. The liver of dnTGF-βR II mice has an increased number of CD8<sup>+</sup> T cells and a higher ratio of CD8 to CD3 or CD4 cells<sup>[59]</sup>. Furthermore, adoptive transfer of CD8<sup>+</sup> T cells from dnTGF-βR II mice to Rag-1(-/-) recipients results in PBC-like liver lesions and AMA-positive sera, while CD4<sup>+</sup> T cells predominantly cause bowel inflammation<sup>[62]</sup>. Relative to IL-2Rα(-/-) mice, IL-2Rα(-/-) CD4(-/-) mice have increased biliary ductular damage. These findings also suggest that CD8<sup>+</sup> T cells may mediate bile ductular injury under the background of Treg function loss<sup>[63]</sup>.

## INNATE IMMUNITY

The innate immune system is the first line of defense against infection. Emerging evidence suggests that the liver is one component of the body's immune system<sup>[64]</sup>. Traditional immunosuppressive drugs are not effective for PBC compared to other autoimmune diseases. Thus, the role of the innate immune system in PBC has garnered a lot of focus in recent years.

### Toll-like receptor

The liver can encounter a number of pathogenic microorganisms and their by-products from the gut by acting

as a traffic hub. The expression of toll-like receptor (TLR) is normally regulated by a negative signaling pathway in the liver, which prevents inappropriate activation of inflammation.

After *in vitro* challenge with TLR ligands, circulating monocytes in PBC patients produce higher pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α<sup>[65]</sup>. The expressions of TLR3<sup>[66]</sup> and TLR4<sup>[67,68]</sup> are increased in BECs. Transfection of poly(I:C) into BECs induces a marked increase in mRNAs encoding a variety of chemokines/cytokines<sup>[66]</sup>. In addition, the expressions of TLR4 in BECs<sup>[67]</sup> and circulating monocytes<sup>[69]</sup> both increase significantly after stimulation with lipopolysaccharide (LPS). Furthermore, the level of RP105, which is involved in the negative regulation of TLR4 signaling, is decreased in PBC monocytes<sup>[69]</sup>. The interplay between LPS and TLR4 results in myeloid differentiation factor 88 (MyD88) recruitment, NF-κB activation and coding gene expression<sup>[67,69]</sup>. After exposure to CpG, PBMCs from PBC patients produce higher levels of IgM and AMAs<sup>[70,71]</sup>. Importantly, the effect can be inhibited by K<sup>+</sup> channel blockers<sup>[70]</sup> and UDCA<sup>[19]</sup>.

These results indicate that patients with PBC exhibit hypersensitivity of TLR signaling, which leads to a breakdown of self-tolerance. However, activation of the TLR signal pathway alone may not explain the entire course of the disease<sup>[72]</sup>. Mice immunized with 2OA-BSA coupled with poly(I:C) showed alterations in the disease process and acceleration of fibrosis of liver.

### NKT cells

NKT cells are a subset of lymphocytes possessing both T cell receptors (TCRs) and NK-specific receptors, and they play an important part in the modulation of the innate immune response and cytotoxicity.

Alpha-galactosylceramide (α-GalCer) is an activator of NKT cells, and the frequency of CD1d-α-GalCer-restricted NKT cells is similar between the peripheral blood and liver of healthy people. In contrast, the frequency of these cells in liver is significantly higher than in peripheral blood in PBC patients, and also higher than in healthy individuals<sup>[73]</sup>.



To define the function of CD1d-restricted NKT cells in the pathogenesis of PBC, Chuang *et al*<sup>[74]</sup> generated CD1d(-/-)dnTGF- $\beta$ R II mice and found that they developed decreased hepatic lymphoid cell infiltration and mild cholangitis. After immunization of 2-OA-BSA-induced PBC mice with  $\alpha$ -GalCer, Wu *et al*<sup>[75]</sup> found that the disease was exacerbated, including signs of portal inflammation, bile duct destruction and liver fibrosis. However, *in vivo* depletion of NK and NKT cells in the same murine model only suppressed AMAs and cytokine production, but did not change the portal cholangitis<sup>[76]</sup>. Therefore, these data support the role of NK and NKT cells in the loss of autoimmune tolerance in PBC; however, the development of PBC also requires other pathological factors.

### NK cells

NK cells are another component of the innate immune system, and function by secreting cytokines and lysing target cells. NK cells account for 30% of the total resident lymphocytes in the liver. Chuang *et al*<sup>[77]</sup> reported an obvious higher frequency and absolute number of NK cells in both the blood and liver of PBC patients. Moreover, the cytotoxic activity and perforin expression of isolated NK cells were increased. Recently, Shimoda *et al*<sup>[78]</sup> demonstrated that TLR4 ligand-stimulated NK cells destroyed autologous BECs in the presence of IFN- $\alpha$  synthesized by TLR 3 ligand-stimulated monocytes. In addition, there was an increased number of CD56<sup>+</sup> cells scattered around the destroyed small bile ducts.

### CHEMOKINES

Chemokines, which are 8-12 kDa heparin-binding cytokines, direct lymphocyte trafficking and positioning in tissues and play roles in modulating immune responses and shaping the severity of disease<sup>[79]</sup>. In PBC, infiltrating memory T cells are culprits regarding the destruction of bile ducts.

Chemokines, interferon-gamma-inducible protein-10 (IP-10) and monokine-induced by gamma interferon (MIG), are increased in plasma and the portal area in PBC patients compared to controls. Moreover, the frequency of CXCR3<sup>+</sup> cells is higher in both PBMCs and injured bile ducts. Intriguingly, daughters and sisters of PBC patients demonstrate increased plasma levels of IP-10 and MIG, but the frequency of circulating CXCR3<sup>+</sup> cells is normal<sup>[80]</sup>. Knockout of CXCR3 in Poly(I:C)-induced mice displayed a delayed and milder progression of cellular inflammation<sup>[81]</sup>.

Other chemokines may also be involved in recruiting inflammatory cells into the liver in PBC. BECs are a source of chemokine production that attracts monocytes into the liver<sup>[82,83]</sup>. Stimulated by poly(I:C), BECs produce augmented CX3CL1 in the presence of monocytes pretreated with LPS through CD40-CD154 contact<sup>[83]</sup>. In addition, BECs pretreated with LPS also secrete elevated CX3CL1<sup>[83,84]</sup>.

### CONCLUSION

Substantial amounts of data to date have illustrated that autoimmunity plays a critical role in the pathogenesis of PBC. The adaptive immune response, the innate immune system and their interplay participate in the development of disease. However, there are clearly limitations and unanswered questions that still remain: (1) although some murine models have been established, they cannot imitate PBC in humans completely (Table 3); (2) *in vitro* experiments based on human samples may not completely reflect the endosomatic problems; and (3) the data to date are mainly based on PBMCs, which lack reliability without assessment in the local liver microenvironment. In order to address these problems, better murine models are needed. In the future, researchers must identify new mechanisms through the analysis of both *in vivo* and *in vitro* approaches for the development of effective treatment strategies for PBC.

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