Chapter 6
Immunopathology of Bile Duct Lesions of Primary Biliary Cirrhosis

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Abstract Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease that predominantly affects women and is characterized by chronic progressive destruction of the small intrahepatic bile ducts with portal inflammation and ultimately fibrosis. The serologic hallmark of PBC is the presence of anti-mitochondrial autoantibodies (AMA). Several mechanisms may now be proposed regarding the immune-mediated bile duct damage in PBC, including the possible roles of T cells, B cells, AMA, and other cell phenotypes. Weakness of biliary epithelial cells in association with apoptosis, senescence, and autophagy has also been noted recently. In PBC, several complex steps and mechanisms may be involved in the induction and progression of cholangitis and biliary degeneration, followed by bile duct loss.

Keywords Anti-mitochondrial autoantibodies (AMA) • Immune-mediated bile duct damage • Biliary epithelial degeneration

6.1 Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by chronic nonsuppurative destructive cholangitis (CNSDC) associated with selective destruction of the intrahepatic small bile ducts (interlobular bile ducts and septal bile ducts) by inflammatory cells, mainly lymphocytes and plasma cells. The

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appearance of anti-mitochondrial autoantibodies (AMA) (90–95%) and a high serum IgM level are characteristics of PBC patients' sera [1, 2]. Progressive biliary damage eventually causes bile duct loss and fibrosis. The mechanisms by which such antibodies produce liver tissue injury are still unknown; however, exposure to infectious microbes and/or xenobiotics is conjectured to initiate an immune reaction stemming from an individual's genetic predisposition and the participation of innate immunity at the primary stage of PBC.

As the mechanism of biliary damage, immunological interaction between biliary epithelial cells and surrounding inflammatory cells is important. It is considered that biliary epithelial cells in PBC express various cytokines and chemokines to generate and continue the specific inflammatory condition around them. Damaged biliary epithelial cells have an antigen-presenting ability, with aberrant expression of HLA class II and other co-stimulatory molecules [3, 4]. Various kinds of migratory inflammatory cells become effector cells, which attack biliary epithelial cells and also produce additional cytokines and chemokines to produce a subsequent inflammatory status and progressive fibrosis. Biliary weakness, showing cellular senescence and disturbance of autophagy, accelerates biliary destruction. This report reviews the immunopathological characteristics of injured bile ducts and infiltrating effector cells, including T cells, natural killer (NK) cells, and B cells, in PBC.

6.2 Immunopathological Characteristics of Damaged Biliary Epithelial Cells and Surrounding Inflammatory Cells in PBC

The small bile ducts, not the large bile ducts, are the specific targets of PBC [5]. Chronic nonsuppurative destructive cholangitis (CNSDC) is one of the typical images of the small portal tracts in PBC patients (Fig. 6.1a). Infiltrating inflammatory cells invade the epithelium and cause epithelial interruption and ductal luminal irregularity. Biliary epithelial cells become fragmented and finally disappear (ductopenia, bile duct loss). Lymphocytes, plasma cells, and often eosinophils infiltrate the areas around the damaged bile ducts. Epithelioid granulomas in various sizes constructed by aggregated macrophages frequently appear in the portal area and in the hepatic parenchyma (Fig. 6.1b).

Biliary epithelial cells of PBC aberrantly express various kinds of co-stimulatory factors and adhesion molecules as well as major histocompatibility complex (MHC) class II molecules and may express the target molecules [3, 4, 6]. Biliary epithelial cells themselves also express various kinds of cytokines/chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated on activation, normal T cell expressed and secreted), fractalkine, as well as generating specific immunological microenvironments around them [7–12]. Biliary epithelial cells also bear several cytokine receptors against IL-4, IL-6, interferon-gamma (IFN-γ), and TNF-α, so these cytokines have autocrine and paracrine effects [13].

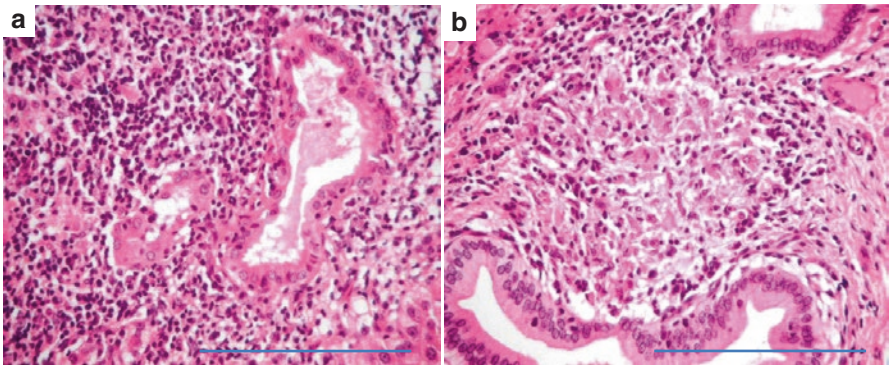

Fig. 6.1 Typical images of chronic nonsuppurative destructive cholangitis (CNSDC) (**a**) and epithelioid granuloma near a damaged bile duct (**b**) in a patient with primary biliary cirrhosis (PBC) (scale bar, 100 μm)

Various studies have described the profiles of the inflammatory cells around the bile ducts in PBC [9, 14–17]. It is considered that the cellular profile may change depending on the process of biliary inflammation. Different immune cell profiles are frequently observed at different portal areas of the same hepatic tissue section. However, it is generally believed that T-cell-related cellular immunity is involved in the pathogenesis of CNSDC. Indeed, it has been demonstrated that CD8+ and CD4+ lymphocytes are the predominant cell types in the inflammatory cells within the portal area in PBC [18, 19]. CD8+ lymphocytes are mostly cytotoxic T cells and affect the targets via the perforin/granzyme exocytosis pathway [20, 21]. CD4+ lymphocytes, especially pathogenic autoreactive T cells, regulate autoimmunity around the bile ducts in PBC. In the early stage, a Th1-predominant cytokine milieu is characteristic in CNSDC [22]; however, as the disease progresses, both IL-12/Th1 cells and IL-23/Th17 cells appear in various degrees. In the late stage of PBC, the ratio of cell formation shifts from Th1 cells to Th17 cells [16]. A decreased number of portal-infiltrating regulatory T cells and an imbalanced ratio with cytotoxic T cells may be associated with disease progression [23, 15].

While T cells comprise 55% of the cellular infiltrate, macrophages make up about 30% [24], and B cells/plasma cells account for about 10% of the population [19, 25]. Eosinophils may make up some proportion of the inflammatory cells [26, 27, 9]. Natural killer (NK) cells and NK T cells account for approximately 5% of the cellular infiltrate and play important roles in initiating the breakdown of tolerance [28]. Interdigitating dendritic cells are found between the biliary epithelial cells, often near breaks in the basement membrane and in the periductal granulomatous response [4, 29, 30].

Lymph follicles are frequently observed in the portal tracts of PBC patients, both in the early stage and the late stage [31] (Fig. 6.2). In immunohistochemical analysis, lymph follicles of PBC are considered as tertiary lymphoid organs (TLOs) (Fig. 6.3). Briefly, high endothelial venules (HEV) showing MECA-79 expression are located in the center of the lymph follicles. A follicular dendritic cell (FDC)

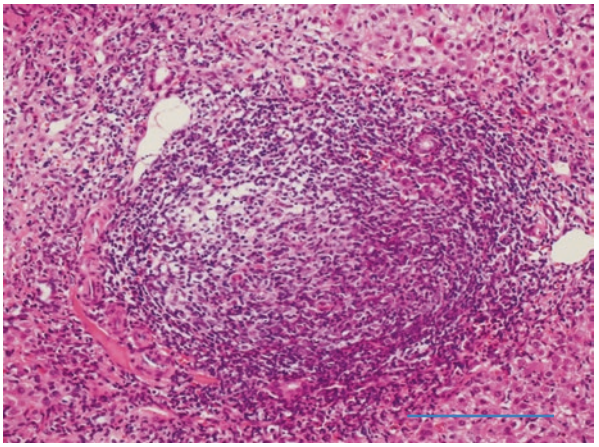

Fig. 6.2 Lymph follicle formation with germinal center in a portal tract of a patient with primary biliary cirrhosis (PBC) (scale bar, 100 μm)

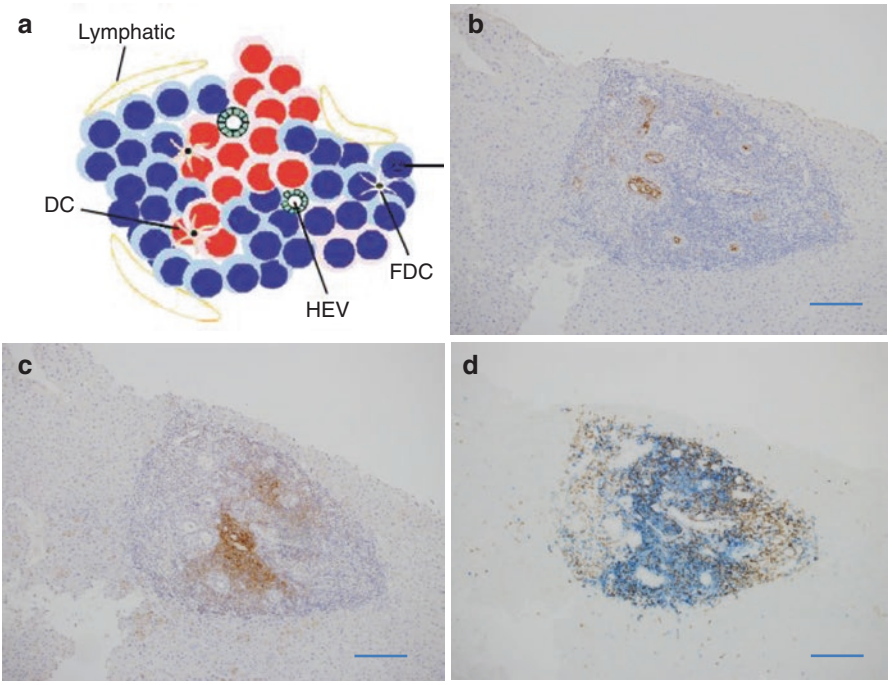

Fig. 6.3 (**a**) A schema of tertiary lymphoid organs (TLOs) in a portal tract of a patient with PBC (*DC* interdigitating dendritic cells, *HEV* high endothelial venules, *FDC* follicular dendritic cells). (**b**) Immunostaining of MECA-79, which is a marker of high endothelial venules (HEV) (*brown color*, positive; counterstaining was performed by hematoxylin). (**c**) Immunostaining of CD21, which is a marker of follicular dendritic cells (*brown color*, positive; counterstaining was performed by hematoxylin). (**d**) Double immunostaining of CD3, which is a marker of T cells and CD20, which is a marker of B cells (*brown color*, CD3; *blue color*, CD20) (scale bar, 100 μm)

network with CD21 expression is observed around the HEV. B cells are seen to be regularly accumulated along the FDC network and located in the center part of the lymph follicles. A layer of T cells is observed in the outer part of the B-cell layer. In our unpublished data, 78% of PBC cases with inflammatory cell infiltration had MECA-79-positive HEV formation. A continuous inflammatory process may induce MECA-79-positive HEV initially and cause subsequent TLO formation in the portal area. These TLOs often include injured bile ducts; however, TLOs formed far away from the injured bile ducts have also been observed. Any direct correlation between TLOs and injured bile ducts is still unknown. Takahashi et al. reported that, rather than follicle-like aggregation of CD20-positive B cells, periductal infiltration of CD38-positive plasma cells is highly associated with bile duct injury [32]. TLOs may correlate indirectly with bile duct injury via maturation of effector cells.

6.3 Mechanism of Bile Duct Injury in PBC

There are several pathogenetic possibilities for the mechanism of bile duct injury in PBC. One hypothesis for the selective destruction of biliary epithelial cells is that the pyruvate dehydrogenase complex (PDC)-E2 subunit, which is normally located in the mitochondrial inner membrane, is aberrantly expressed on the surface of biliary epithelial cells [3]. Not only PDC-E2 itself but also its mutant form and tissue-specific variants may cause the same phenomenon. Lleo et al. reported the existence of intact immunoreactive PDC-E2 within apoptotic blebs of cholangiocytes during the process of apoptosis in PBC [33, 34]. An autoimmune response may accelerate the process against these modified intrinsic PDC-E2 or related molecules.

Another hypothesis is the occurrence of an immune reaction against extrinsic antigen located in the biliary epithelium. The microbial mechanism termed "molecular mimicry" is a strong hypothesis being advanced to account for the breaking of tolerance against mitochondrial antigens.

Epidemiological studies have also suggested that infectious agents can trigger or even exacerbate the disease [35]. Both gram-positive and gram-negative bacteria have been suspected, especially *Escherichia coli* and *Novosphingobium aromaticivorans*, which are the most commonly associated agents that have been reported to date [36–38]. Another candidate for the role of extrinsic antigen is xenobiotics (chemicals). Many chemicals, including pharmaceuticals and household detergents, have the potential to form metabolites that show molecular mimicry to PDC-E2 [39, 40]. Amano et al. reported that 2-octynoic acid was unique in both its quantitative structure-activity relationship analysis and reactivity. Sera from PBC patients demonstrate high Ig reactivity against 2-octynoic acid-PDC-E2 peptide. Not only does 2-octynoic acid have the potential to modify PDC-E2 in vivo, but, importantly, it is widely used in the environment including in perfumes, lipstick, and many common food flavorings [41]. Mice immunized with 2-octynoic acid serve as a unique PBC animal model showing autoimmune cholangitis, typical anti-mitochondrial autoantibodies, and increased number of liver lymphoid cells with an increase in the number of CD8(+) cells in the liver [42].

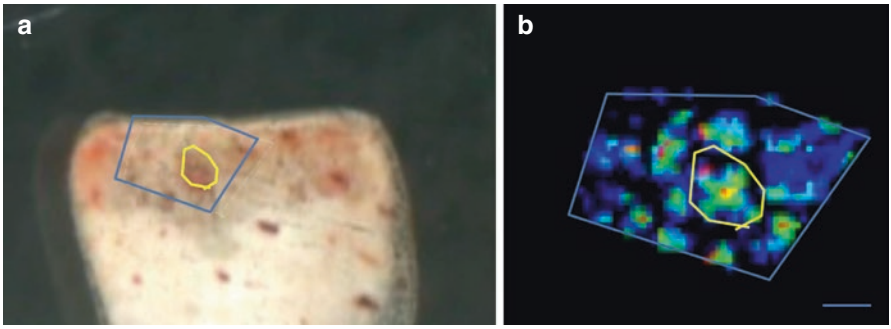

Fig. 6.4 Localization of 2-octynoic acid on the liver of model PBC mice (frequent abdominal injections of 2-octynoid acid). (**a**) Image of a frozen section. *Blue* rounded area was analyzed by imaging mass spectrometry using the nanoparticle-assisted laser desorption/ionization (nano-PALDI) method. The *yellow* rounded area is the portal area. (**b**) Enlarged image of nano-PALDI mass spectrometry of *blue* rounded area. Not only the hepatic parenchyma but also the portal area has positive signals of 2-octynoic acid (scale bar, 100 μm)

However, demonstrating the localization of 2-octynoic acid in the liver has been difficult because of its small molecular size. We have recently developed a new technique to highlight the expression of low-molecular-weight molecules without any labeling on frozen liver using nanoparticle-assisted laser desorption/ionization (nano-PALDI) imaging mass spectrometry (IMS) [43]. We examined the localization of 2-octynoic acid in the liver by using 2-octynoic acid-induced PBC model mice (Fig. 6.4). Interestingly, 2-octynoic acid was not only located in the hepatic parenchyma but also in the portal area. Because 2-octynoic acid was also detected in bile juice, we speculated that 2-octynoic acid may deposit in biliary epithelial cells. These results imply that aberrantly deposited extrinsic xenobiotics (chemicals) or their metabolites can act as pathogens. We are planning to examine frozen liver samples from PBC patients to clarify the pathogenic roles of various xenobiotics.

The activation of the innate immune response seems to be another key event in early PBC that leads to autoimmune injury of the small intrahepatic bile ducts. Biliary epithelial cells possess an innate immune system consisting of the Toll-like receptor (TLR) family, which recognizes pathogen-associated molecular patterns (PAMPs). In PBC, deregulated biliary innate immunity, namely, hyperresponsiveness to PAMPs, is associated with the pathogenesis of cholangiopathy. Moreover, the targeted biliary epithelial cells may play an active role in the perpetuation of autoimmunity by attracting immune cells via chemokine secretion. Biliary innate immune responses induce the production of two chemokines, fractalkine and several Th1 shift chemokines, causing the migration of inflammatory cells including NK cells. TLR4 ligand-stimulated NK cells destroy autologous biliary epithelial cells in the presence of IFN-α synthesized by TLR3 ligand-stimulated monocytes. These findings give new insights into the pathogenesis of PBC [44, 45].

Injured bile ducts and bile ductules of PBC indicate a cellular senescence. Senescent biliary epithelial cells can modulate the microenvironment around bile ducts by expressing senescence-associated secretory phenotypes (SASP) and contribute to maintaining inflammation and fibrosis around bile duct lesions in PBC. Deregulated autophagy followed by cellular senescence in biliary epithelial cells may be closely related to the abnormal expression of mitochondrial antigens and subsequent autoimmune pathogenesis in PBC [46, 47]. Biliary epithelial cells of PBC suffer strong oxidative stress because of a decrease in their antioxidative ability [48]. Oxidative stress may accelerate cellular senescence and disturbance of the autophagy system, causing complex biliary damage.

6.4 Relationship Between the Manifestation of AMA and Bile Duct Injury

Various ideas have been offered regarding the participation of AMA in bile duct injury; the author recently suggested a protective contribution of AMA against biliary damage [31]. The degree of bile duct damage around the portal areas was significantly milder in AMA(+) PBC than that observed in AMA(-) PBC in liver biopsy examination of Chinese PBC patients [31]. Conversely, Lleo et al. suggested that AMA promotes the inflammatory process by demonstrating that there is intense inflammatory cytokine production in the presence of biliary epithelial cell apoptopes, macrophages from patients with PBC, and AMA. The cytokine secretion was inhibited by anti-CD16 and was not due to differences in apoptope uptake. Moreover, mature monocyte-derived macrophages from PBC patients cultured with biliary epithelial cell apoptotic bodies in the presence of AMA markedly increased tumor necrosis factor-related apoptosis-inducing ligand expression [33].

Several reports on AMA in animal models have also been published [23]. A unique murine PBC model expressing a dominant negative form of transforming growth factor beta receptor II (dnTGFbetaRII) under control of the CD4 promoter developed both colitis and autoimmune cholangitis with elevated serum levels of IL-6. Based on this observation, IL-6-deficient mice with a dnTGFbetaRII background (dnTGFbetaRII IL-6(-/-)) were produced and examined for the presence of AMA, cytokine levels, histopathology, and hepatic immunohistochemistry. Serum AMA levels decreased in the dnTGFbetaRII IL-6(-/-) mice; however, autoimmune cholangitis was significantly exacerbated, including elevated levels of inflammatory cytokines, increased number of activated T cells, and worsening hepatic pathology. These results suggest an inflammatory inhibitory action of AMA.

To inhibit AMA secretion, autoreactive B-cell depletion therapy using several PBC model mice was also performed. Moritoki et al. examined the therapeutic

efficacy of B-cell depletion using anti-CD20 [49]. In mice whose treatment was initiated at 4–6 weeks of age (early treatment group), anti-CD20 therapy demonstrated a significantly lower incidence of liver inflammation associated with reduced number of activated hepatic CD8(+) T cells. In contrast, in mice treated at 20–22 weeks of age (late treatment group), anti-CD20 therapy had relatively little effect on the liver. All treated animals had reduced levels of B cells, absence of AMA, and increased levels of inflammatory cytokines such as TNF-α in sera. AMA may play some roles for the induction state of pathogenesis, but not for disease progression in this model [49]. However, B-cell depletion using another murine PBC model (genetic B-cell-deficient Igmu(-/-) NOD.c3c4 mice) demonstrated reduced levels of B cells, absence of AMA, and a decreased number of non-B cells in the liver accompanied by reduced number of activated NK cells. Since liver inflammation was significantly attenuated, B cells and AMA may play important roles in pathogenesis in the model [31]. Given the disparate nature of these results, we consider that the role of B cells and AMA may depend on the disease phase and a variety of other factors.

6.5 Hyper-IgM Production and Immunopathology

Elevated levels of IgM and the presence of AMA are characteristic of the sera of PBC patients. The increase in serum IgM is considered to be the result of chronic B-cell activation induced via the TLR-signaling pathway. Indeed, peripheral blood mononuclear cells (PBMCs) from PBC patients produce significantly higher levels of polyclonal IgM and secretion of AMA than controls after exposure to CpG, which is a natural ligand for TLR9 [50, 51].

The primary site of IgM production in PBC patients is still unclear. Takahashi et al. reported that CD38-positive plasma cells accumulated around the bile duct in PBC patients [32]. These periductal plasma cells produced IgM and IgG, not IgA, so they may be candidates for the source of serum IgM. Kikuchi et al. focused on the spleens of PBC patients, because B-cell maturation and differentiation occur in the splenic white pulp and produce IgM in response to an innate immunity stimulus of capsular polysaccharide of a pneumococcus. In immunohistochemical analysis using surgically resected spleens and autopsied spleens, IgM-producing plasma cells aggregated near the CD21-positive FDC network into the germinal center of the spleens of PBC patients (Fig. 6.5). A chemokine, CXCL13, which has a chemotactic function for B cells, localized near IgM into the lymph follicles of PBC spleens. Not only portal-infiltrated B cells in the liver but also splenic B cells produced IgM in the PBC patients. In PBC patients, IgM production may be regulated systemically, rather than being a local event in the liver.

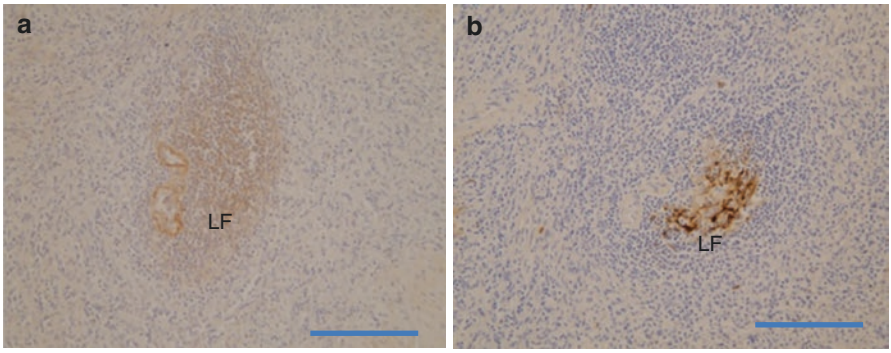

Fig. 6.5 Immunostaining of the lymph follicle of the spleen in patients with primary biliary cirrhosis (PBC). (**a**) CD21 immunostaining showing the follicular dendritic cell (FDC) network. (**b**) IgM immunostaining showing aggregation into the germinal center (*LF* lymph follicle; scale bar, 100 μm)

6.6 Establishment of a New Therapeutic Approach Against B Cells in PBC

A new therapeutic approach targeting B cells in PBC patients has recently been clinically performed. Tsuda et al. reported the safe and potential efficacy of B-cell depletion with the anti-CD20 monoclonal antibody rituximab in patients with PBC who had experienced incomplete response to ursodeoxycholic acid (UDCA) [52]. After treatment, serum levels of total IgG, IgM, and IgA as well as AMA-IgA and AMA-IgM decreased significantly from baseline by 16 weeks and returned to baseline levels by 36 weeks. Transient decreases in memory B cells and T cells and an increase in CD25(high) CD4(+) T cells were observed after treatment. These changes were associated with significant increases in mRNA levels of FoxP3 and TGF-β and a decrease in TNF-α in CD4(+) T cells. Notably, serum alkaline phosphatase levels were significantly reduced up to 36 weeks following rituximab treatment. From the above results, Tsuda et al. concluded that depletion of B cells influences the induction, maintenance, and activation of both B cells and T cells and provides a potential mechanism for treatment of patients with PBC who experience an incomplete response to UDCA. However, in a few examples of PBC patients administered rituximab, the potential for herpes zoster reactivation and upper respiratory infection were acknowledged in this trial. This is regarded as an important problem of infection control in the performance of B-cell removal therapy. We have also experienced a case of PBC that rapidly progressed to liver cirrhosis after treatment including rituximab [53]. A 66-year-old Japanese female patient with PBC, who presented with a gastric lymphoma, had been treated with a regimen containing rituximab for incidental malignant lymphoma. She showed biochemical and

immunological improvements, and her liver histology before and after rituximab treatment confirmed a decrease in liver inflammation. However, she developed liver cirrhosis a short time after rituximab treatment without biochemical or immunological worsening.

In conclusion, autoimmune B-cell removal therapy has the potential to become a new treatment for PBC, but caution should be exercised, and careful patient observation is required.

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