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Breach of Tolerance: Primary Biliary Cirrhosis

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Abstract

Keywords

- ▶ apoptosis
- ▶ autoreactive T cells
- ▶ antimitochondrial antibodies
- ▶ liver-tolerance effect
- ▶ pyruvate dehydrogenase complex

In primary biliary cirrhosis (PBC), the breach of tolerance that leads to active disease involves a disruption in several layers of control, including central tolerance, peripheral energy, a “liver tolerance effect,” and the action of T regulatory cells and their related cytokines. Each of these control mechanisms plays a role in preventing an immune response against self, but all of them act in concert to generate effective protection against autoimmunity without compromising the ability of the host immune system to mount an effective response to pathogens. At the same time, genetic susceptibility, environmental factors, including infection agents and xenobiotics, play important roles in breach of tolerance in the development of PBC.

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by immunomediated destruction of the small- and medium-sized intrahepatic bile ducts, mediated by a selective loss of self-tolerance.¹ Epidemiological and genetic studies indicate that PBC is triggered in genetically susceptible individuals following exposure to environmental factors, including microbes and chemical compounds.^{2,3} Loss of tolerance occurs either via molecular mimicry and/or the formation of neoantigens and the development of cross reactivity.⁴ This process results from dysfunction in both innate and adaptive immunity, leading to the expansion of autoreactive T- and B-lymphocyte populations.⁵ In PBC, the multiorchestrated immune effector mechanisms lead to bile duct injury.

Immune Tolerance and the “Liver Tolerance Effect”

In the 1890s, Paul Ehrlich described a phenomenon in which a host immune system turns on itself, attacking and damaging its own organs and tissues. He coined the term “horror autotoxicus” to describe how the phenomenon is teleologi-

cally impossible and that nature would or should not allow this to happen. As a result, despite research supporting the existence of autoimmunity early in the 20th century, little attention was paid to the concept of immune tolerance. The discipline of autoimmunity was eventually recognized by the 1940s, and in the ensuing 20 years, an appreciation of the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus was increasingly attributed to an autoimmune phenomenon. In 1948, Frank Macfarlane Burnet deduced the nature of immunological inertness to self, first naming this as “tolerance” and then proposing it to be a characteristic acquired in developmental life rather than innately as earlier believed.^{6,7} In 1953, Medawar and his colleagues experimentally demonstrated the induction of immune tolerance in inbred mice.⁸ Ultimately, the conception of immune tolerance was defined as an ability of the immune system to prevent itself from targeting self-molecules, cells, or tissues; this has led to seminal research in our understanding of autoimmunity.⁹

The hallmark of the immune system is its ability to maintain this tolerance to self-antigens, and yet still be able to mount effective immune responses against pathogens and

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malignant cells (danger signals). Breaking the balance between tolerance and immunity can lead to disease manifestations, resulting in infections, neoplasia, or autoimmunity. To avoid harmful self-reactivity, self-tolerance within the T- and B-cell repertoire are achieved through central and peripheral tolerance mechanisms.

Central tolerance in the thymus and bone marrow play key roles in shaping immune system homeostasis, especially in early life. In the thymus, with no marked reactivity against self-peptides, developing lymphocytes undergo positive selection in the thymic cortex, then become mature lymphocytes and enter the circulation. Conversely, developing lymphocytes with marked reactivity against self-peptides are negatively selected and deleted in the thymic medulla.^{10–14} After exiting the thymus, mature T cells are subjected to secondary selection (peripheral tolerance) by which the majority of self-reactive T cells are deleted or rendered anergic.^{15,16} Meanwhile, when immature B cells express surface IgM that recognize ubiquitous self-cell-surface antigens, they are eliminated by a process known as clonal deletion or anergy.¹⁷ Autoreactive B cells can escape deletion by a process known as receptor editing.^{18,19} Mature B cells are also under the control of peripheral tolerance.²⁰

Abundant evidence suggests that even under the regulation of central and peripheral tolerance, small numbers of potentially hazardous self-reactive lymphocytes still can “leak out” into the periphery in normal individuals.^{21,22} But these residual self-reactive T cells normally remain in an inactive state due to several factors, including lack of costimulation from antigen-presenting cells, low avidities of their T-cell receptors (TCRs) for self-antigens, or seclusion of self-antigens.²³ At the same time, mature self-reacting B cells may survive intact and rarely be activated because they need costimulatory signals from T cells, as well as the presence of its recognized antigen to proliferate and produce antibodies.

In addition to these passive mechanisms, evidence indicates that suppressive regulatory mechanisms also exist. CD4 + CD25+ T regulatory cells (Tregs) are immune regulatory cells that play an important role in immune homeostasis. These cells are involved in the maintenance of peripheral self-tolerance and downregulation of immune responses.^{24–26} PDCD5 acetylates FOXP3 and affects Treg cell modulation of immune function, thereby playing a role in the development of autoimmune disease.²⁷

Liver tissue itself also possesses the ability to mediate local and systemic tolerance to self and foreign antigens.²⁸ This is known as the “liver tolerance effect.” The liver is a depot for clearance of toxins and metabolic products that result from physiologic and pathogenic processes. Thus, there is a need to prevent activation of the immune system by exposure to “nonpathogenic” molecules (including food components) and microorganisms (gut microflora), while continuing to mount an effective immune response against pathogens.^{29,30} Non-parenchymal liver cells including the liver sinusoidal endothelial cells, resident DCs, Kupffer cells, and hepatic stellate cells are likely responsible for hepatic tolerance. These cells can generate anti-inflammatory cytokines including IL-10

and TGF β or express the negative costimulators of T-cell activation that mediate immune suppression, such as programmed cell death ligand-1 (PD-L1).^{31,32} In addition, hepatocytes and Treg cells are also involved in mediating T-cell tolerance in the liver.

The normal physiologic mechanisms of immune tolerance should prevent autoimmune diseases. The concept of autoimmunity is often thought to require the presence of autoantibodies (antibodies directed against normal self-tissues or substances).^{33,34} However, autoimmunity may exist in the absence of a known autoantigen. Autoimmunity generally results from a defect in central tolerance mechanisms that allows the generation and proliferation of a limited number of autoreactive cells, which can mature and enter the periphery. However, it has been repeatedly demonstrated in animal and human studies that the presence of autoreactive cells does not universally lead to autoimmune disease. A breach of tolerance that occasionally occurs during the process of mounting an immune response to a pathogen is often, but not always the trigger for the development of autoimmune diseases. Autoimmune diseases are generally classified on the basis of the organ or tissue involved. Autoimmune diseases that are restricted to specific organs of the body are known as *organ-specific* autoimmune diseases, including type 1 diabetes mellitus, multiple sclerosis, PBC, and psoriasis. Conversely, if many tissues of the body are affected, the disease is regarded as a *systemic* autoimmune disease, such as systemic lupus erythematosus and rheumatoid arthritis.

Primary Biliary Cirrhosis and a Breach of Tolerance

In 1851, Addison and Gull first described nonobstructive biliary cirrhosis. The disease was further characterized clinically by Ahrens et al in 1950. After a considerable period of research inactivity, a cDNA encoding the autoantigen targeted by these antimitochondrial antibodies (AMAs) was cloned in 1987 by Gershwin and colleagues.³⁵ Subsequently, the antigen was identified as the E2 subunit (E2) of the pyruvate dehydrogenase complex (PDC).³⁶ More recently, other autoantibodies have been detected in PBC patients, including those directed against the nuclear pore complex (NPC).³⁷ A series of interrelated immunoreactive events have been discovered to occur in primary biliary cirrhosis, which upon characterization will contribute to our understanding of the mechanisms of breach of tolerance (►Fig. 1).

Primary biliary cirrhosis, with the presence of autoantibodies (AMAs), PDC-E2-specific autoreactive CD4, CD8 T cells, autoreactive B lymphocytes in liver tissue, and three separate genetically based mouse models,^{38–40} fulfills the criteria for an autoimmune disease.⁴¹ Autoimmune mechanisms of PBC are multifactorial,⁴² consisting of a breakdown in central or peripheral tolerance, as well as the aforementioned liver tolerance effect.

Specifically, the loss of tolerance in PBC patients may occur as a result of three main conceptual mechanisms: (1) molecular mimicry and the presence of neoantigens, developed through cross reactivity to PDC-E2; (2) a genetic

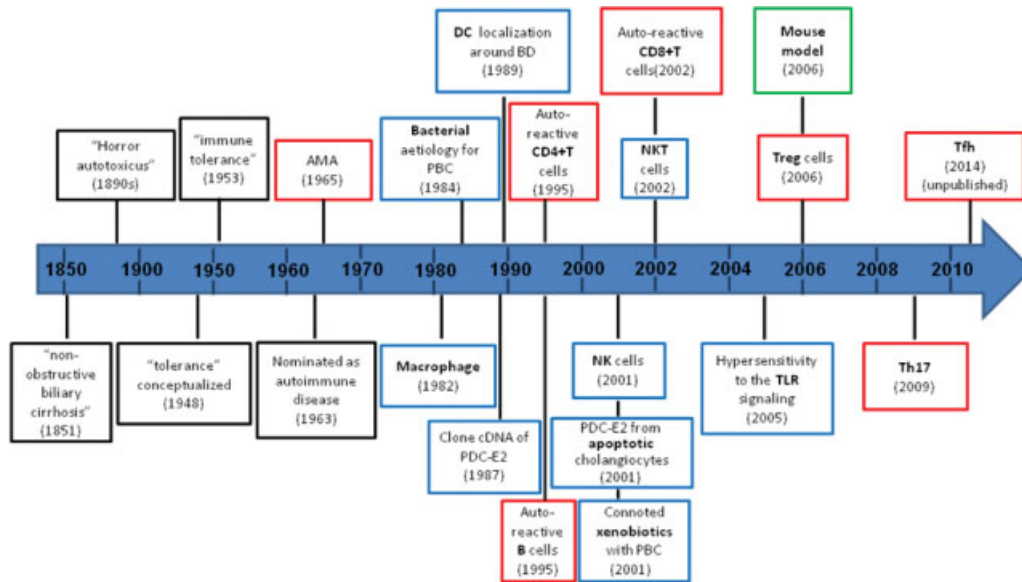


Fig. 1 Timeline of identifying immune parameters in primary biliary cirrhosis (PBC). Black, clinical immunology-related events and conception; blue, innate immunity; red, adaptive immunity; green, PBC mouse models. AMA, antimitochondrial antibody; BD, biliary ducts; DC, dendritic cell; NK, natural killer; NKT, Natural killer T; TLR, Toll-like receptor.

susceptibility, responsible for inheritable abnormalities in the regulation of immune responses; and (3) an imbalance in the immune system, leading to an overactive innate immune system, which inappropriately activates nonspecific immune responses that lead to the expansion of autoreactive populations of T and B lymphocytes (→Fig. 2).

Mitochondrial Autoantigens in Primary Biliary Cirrhosis

The 2-oxoacid dehydrogenase complex (2-OADC) autoantigens are multienzyme complexes essential in the mitochondrial respiratory chain.⁴³ This enzyme family includes three

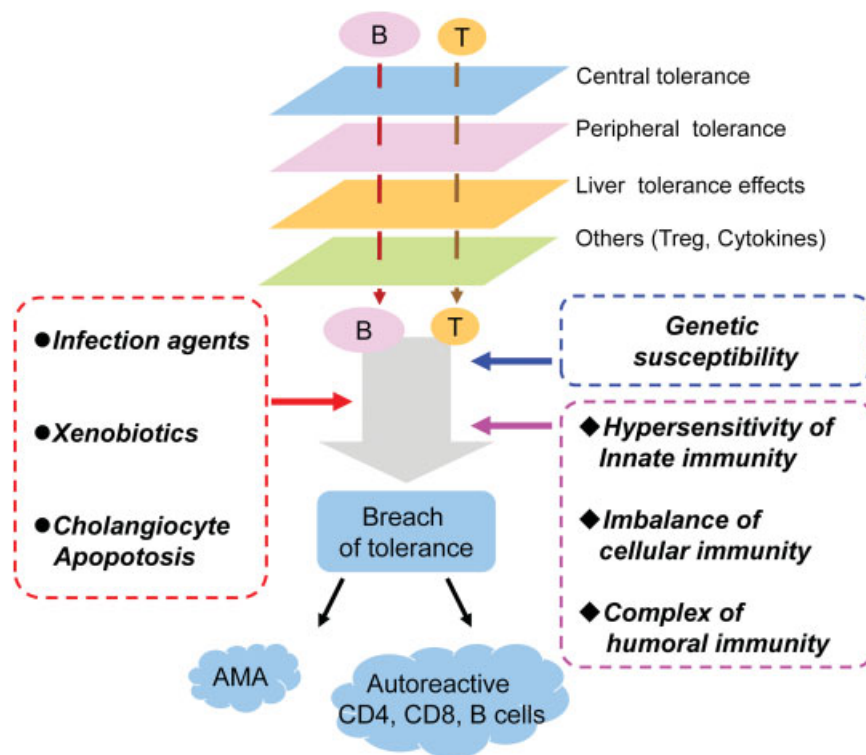


Fig. 2 Mechanisms that are involved in breaching immune tolerance in primary biliary cirrhosis. The breach of tolerance that leads to active disease involves a disruption in several layers of control, including central tolerance, peripheral energy, a “liver tolerance effect,” and the action of T regulatory cells and their related cytokines. At the same time, genetic susceptibility, environmental factors, including infection agents and xenobiotics, also play important roles in breach tolerance.

complexes: PDC, the 2-oxo glutarate dehydrogenase complex (OGDC), and the branched chain 2-oxoacid dehydrogenase complex (BCOADC). Each of the three complexes consists of three subunits: E1, E2, and E3. Antimitochondrial antibodies specifically recognize the lipoylated domains of the 2-OADC family of enzymes, and all immunodominant epitopes contain an ExDKA (glutamic acid -E-, x, aspartic acid, -D-, lysine -k-, and alanine -A-) motif, with lipoic acid attached to K at position 173. This configuration is necessary and/or sufficient for antigen recognition. Interestingly, there are only five proteins in mammals that contain lipoic acid, and four of the five are autoantigens in PBC. Among the 2-OADC constituents, the major autoantigen is the PDC-E2, which was the antigen first cloned in 1987³⁵; less frequent autoantigens include OADC-E2, BCOADC-E2, and E3BP.⁴⁴

In autoimmunity, the paradox is that autoantigens are unable to elicit a primary immune response themselves, but can be recognized as targets for effector T cells stimulated by a pathogenic cross-reactive epitope. To break self-tolerance to the autoantigen, the epitope mimic or mimotope needs to induce activation and proliferation rather than anergy of autoreactive T cells. Subsequently, the autoantigen presented by the host cells of a certain tissue must be recognized by reactive epitope-specific T cells. In the case of PBC, either a mimotope carried by a microbe or a neoantigen generated by xenobiotic-modified self-antigen can mimic mitochondrial proteins, and subsequently may activate autoreactive lymphocytes that have leaked out into the peripheral repertoire. The process may become self-perpetuating because of the cross-reactive unmodified self-antigens (→ Fig. 3).⁴⁵

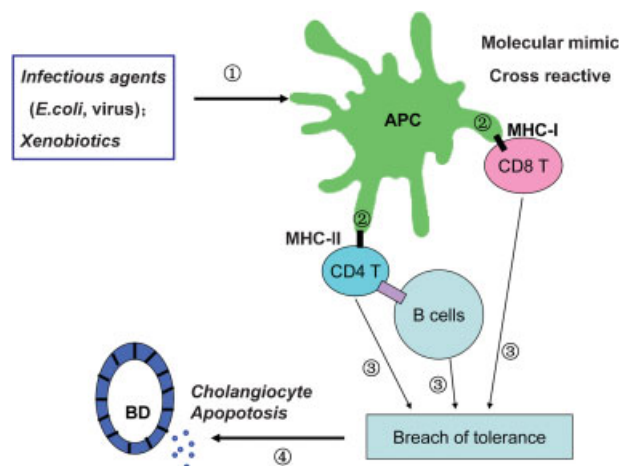


Fig. 3 Molecular mimicry of PDC-E2 helps breach tolerance in primary biliary cirrhosis (PBC) patients. (1) Infectious agents (*E. coli*, viral pathogens) and xenobiotics can be recognized and phagocytosed by antigen-presenting cells (APCs); (2) APCs process and present molecular mimicry of PDC-E2 to CD4 or CD8 T cells by MHC-II or MHC-I; (3) autoreactive CD4 T, CD8 T cells are activated, and CD4 T cells provide help to autoreactive B cells to produce antimitochondrial antibodies (AMA), which lead to the breach of tolerance; (4) intact PDC-E2 released from biliary epithelial cells recognized by reactive epitope-specific T cells lead to PBC.

Molecular Mimicry of PDC-E2 and Cross Reactivity

Two triggers, infections and xenobiotics, have been extensively investigated with regard to their role in molecular mimicry. Central to molecular mimicry is the ability of microorganisms that contain immunogenic epitopes (similar to self-antigens) to trigger a cross-species immune response.⁴⁶ Xenobiotics are chemicals that can generate a loss of tolerance to self-proteins, usually as a result of changed immunogenicity through alteration of or complexing to either self-proteins or nonself-proteins.^{47,48}

Molecular Mimicry

In 1964, the term “molecular mimicry” was coined by Damian, who suggested that select antigenic determinants of microorganisms may resemble host epitopes.⁴⁹ Numerous specific infectious agents, mainly bacteria (both gram negative and positive), and viruses (herpes simplex viral,⁵⁰ mouse mammary tumor virus,^{51,52} and Epstein-Barr virus⁵³), parasites (trypanosomes and *Ascaridiagalli*⁵⁴), and fungi (*Saccharomyces cerevisiae*⁵⁵), have been implicated in PBC.⁵⁶ The classic example is *Escherichia coli* and it has been reported that urinary tract infections (UTIs) are frequently observed in PBC.⁵⁷ Sera from PBC patients react with both *E. coli* and human PDC-E2. We also note that *Novosphingobium aromaticivorans*, a gram-negative bacterium, contains two proteins that share highly homologous amino-acid sequences with the immunodominant epitope of PDC-E2. Specific antibody reactions to this organism have also been detected in PBC patients, and in fact exhibit up to a 1000-fold stronger response than those against *E. coli*.⁵⁸ In addition, a peptide derived from *Pseudomonas aeruginosa* has been found to be partially homologous to PDC-E2_{159–167}, which is the HLA-A*0201-restricted epitope of PDC-E2 recognized by autoreactive cytotoxic T lymphocytes in PBC (→ Table 1).⁵⁹

Autoimmune cholangiopathy, which possesses some similarity to human PBC, has occurred after exposure to bacterial components. Lipopolysaccharide (LPS), a specific component of gram-negative bacterial cell walls, injected into mice either alone or in combination with PDC-E2, induces the appearance of portal lymphocytic infiltration and cholangiocyte degeneration such as that seen in human PBC liver.⁶⁰ Furthermore, lipoteichoic acid (LTA), a gram-positive cell wall component, is also involved in systemic multifocal epithelial inflammation in chronic colitis-harboring TCRα(-/-)xAIM(-/-) mice.⁶¹ Serum levels of LTA-specific IgA are also significantly higher in PBC than in normal controls.⁶² Unmethylated CpG motifs from bacterial DNA triggers a PDC-specific Th1 response in peripheral blood mononuclear cells (PBMCs) from mice immunized with PDC.⁶³

Most studies supporting the role of infectious agents in the pathogenesis of PBC are based on linear or conformational mimicry between microbial proteins and human mitochondrial antigens. Shared sequences between human and microbial proteins can disrupt immune tolerance by inducing cross-reactive antibodies or effector T cells and/or by promoting epitope spreading.⁶⁴ However, controversy surrounding the concept of infectious agents and their components in the etiology of PBC still exists.^{65,66}

Table 1 Molecular mimicry and neoantigens to PDC-E2

		Epitope mimic or mimotope	Cross reactivity	References
Molecular mimicry				
	<i>Escherichia coli</i>	PDC-E2 ₂₁₂₋₂₂₆ , PDC-E2 ₁₆₃₋₁₇₆	Ab and CD4	207,245
	<i>Novosphingobium aromaticivorans</i>	PDC-E2 ₂₀₈₋₂₃₇	Ab	84,246
	<i>Helicobacter pylori</i>	PDC-E2 ₂₁₂₋₂₂₆	Only in mouse models	247,248
	<i>Pseudomonas aeruginosa</i>	PDC-E2 ₁₅₉₋₁₆₇	CD8 TCR	59
	<i>Haemophilus influenzae</i>	PDC-E2 ₂₁₂₋₂₂₆	Ab	245
	<i>Lactobacillus delbrueckii</i>	PDC-E2 ₂₁₂₋₂₂₆	Ab	249
	<i>Mycoplasma pneumoniae</i>	PDC	Ab	250
	<i>Mycobacterium gordonae</i>	PDC-E2 ₂₁₂₋₂₂₆	Ab	251
	<i>Borrelia burgdorferi</i>	PDC-E2 ₂₀₈₋₂₃₅	Ab	252
Xenobiotics				
	Lipoic acid moiety replacement	Lipoic acid mimics	AMA	47
	6-bromohexanoate	12-aa replacement within lipoic acid moiety	AMA	68
	2-octynoic acid	PDC-E2	AMA	71
	Structure-activity relationship (QSAR) analysis	Lipoic acid mimics	AMA	70

Xenobiotics

Self- or nonself-proteins can be modified by chemical compounds (i.e., xenobiotics) causing a change in molecular structure that enhances immunogenicity. Xenobiotics are important in PBC because many environmental chemicals are metabolized primarily in the liver and during metabolism, may form reactive metabolites that can modify cellular proteins to form neoantigens. Sufficient data exist supporting the hypothesis that xenobiotic-induced and/or oxidative modification of mitochondrial autoantigens is a critical step leading to loss of tolerance for PBC patients.⁶⁷ As previously noted, lipoic acid is a critical component of the PDC-E2 epitope. Moreover, lipoic acid, at the exterior of the PDC-E2 protein complex, is accessible to chemical modification.

In 2001, Gershwin and his colleagues replaced the lipoic acid moiety with synthetic structures designed to mimic a xenobiotically modified lipoyl hapten and subsequently quantified the reactivity of these structures with sera from PBC patients. The data demonstrate that AMAs from seropositive patients with PBC, but not controls, reacted against three of 18 organically modified autoepitopes significantly better than to the native domain,⁴⁷ suggesting an organic compound may modify a self-protein and serve as a mimotope.

In 2003, the Gershwin laboratory replaced the lipoic acid moiety of PDC-E2 with a collection of synthetic structures designed to mimic a xenobiotically modified lipoyl hapten on a 12-aa peptide, leading to significantly higher reactivity with AMA. Based on these data, they immunized rabbits with the xenobiotic, 6-bromohexanoate, bovine serum albumin conjugate, and induced AMA production without requirement for the peptide backbone of PDC-E2.⁶⁸ These autoantibodies disappeared when the stimulus was discontinued. In 2007,

the same group reported that oxidative stress-induced liver damage leads to a transiently higher frequency of AMA induction, especially in subjects with acetaminophen (APAP) poisoning.⁶⁹

In 2011, quantitative structure-activity relationship (QSAR) analysis was performed on a focused panel of lipoic acid mimics in which the lipoyl disulfide bond was modified.⁷⁰ 6,8-bis(acetylthio)octanoic acid (SAC), 8-(acetylthio) octanoic acid (OASAc), and 6,8-bis(propionylthio)octanoic acid (SCOEt) were highly reactive to sera from AMA-positive PBC patients, at levels even higher than the reactivity against lipoic-acid-conjugated PDC-E2 peptide. Furthermore, two of these compounds (6-bromohexanoate and 2-octynoic acid⁷¹) were capable of inducing AMA and PBC-like liver lesions in guinea pigs⁷² and NOD.1101⁷³ or C57BL/6⁷⁴ mice, respectively. These models illustrate breakdown of tolerance in the absence of exposure to PDC-E2.

Interestingly, 2-octynoic acid is found in several cosmetic products including nail polish; their frequent use among women may contribute to the female predominance of PBC.^{75,76}

Apoptosis

The clearance of apoptotic cells is normally associated with an anti-inflammatory response; this process plays an important role in tissue homeostasis and immune tolerance.^{77,78} Moreover, increasing evidence suggests that failure in clearance of apoptotic cell debris is linked to the breakdown of tolerance and the development of autoimmunity.⁷⁹⁻⁸¹ Apoptosis of biliary epithelial cells (BECs) has been proposed as a potential source of neoantigens that are responsible for activating autoreactive lymphocytes.⁸² Autophagy, the catabolic process

that leads to cell destruction and the clearance of the resultant cellular debris may also play a significant role in autoimmune diseases.⁸³

Mitochondrial antigens are ubiquitously expressed in all nucleated cells, and are phylogenetically highly conserved.⁸⁴ During spontaneous or induced apoptosis, almost all cell types express mitochondrial antigens on the intact plasma membrane and within apoptotic blebs. The apoptotic cells acquire the ability to initiate an autoimmune response by presentation of 2-OADC-derived autoantigens.⁸⁵ In most cell types, the release of lysine-lipoylated sequences from mitochondria during apoptosis leads to oxidation by glutathiones.⁸⁶ The oxidated forms are not immunogenic and are not recognized by serum AMA because glutathionylation masks autoantibody recognition.⁸⁷ Cholangiocytes fail to covalently link glutathione to lysine-lipoyl groups during apoptosis and immunogenicity is retained. The intact PDC-E2 in apoptotic fragments can then be taken up by local antigen-presenting cells and transferred to regional lymph nodes for priming of cognate T cells. Lleo and colleagues first reported the presence of PDC-E2 in the blebs of human intrahepatic bile duct cells that were undergoing apoptosis.⁷⁷ Autoantigens found in apoptotic blebs (apoptopes) were subsequently taken up by macrophages.⁸⁸ Further study showed that addition of serum AMA to a coculture of macrophages and apoptopes led to a significant increase in proinflammatory cytokine secretion. However, the blocking of CD16 mediated complement receptor 3 (CR3) signaling, possibly resulting from the serum anti-CD16 IgM autoantibodies found in PBC, may be related to the delayed clearance of apoptotic BECs by macrophages.⁸⁹

The unique characteristics of BECs during apoptosis might constitute the pathogenic link between the ubiquitous distribution and high degree of conservation across species of the AMA autoantigen and the organ specificity of PBC pathology.⁸⁵

Genetics and Autoimmunity in PBC

There is a broadly accepted paradigm that genetic susceptibility results in a breakdown in immunological tolerance that may enhance the effect of autoantigens and the degree of an abnormal immune response. Specifically, the role of genetic factors in conferring PBC susceptibility has been widely demonstrated (►Fig. 4). The relative risk of a family member of a first-degree relative of PBC patients is 50- to 100-fold higher than the general population.⁹⁰ By evaluating the concordance of PBC in a genetically defined population of twin sets (including monozygotic and dizygotic twins), genetic susceptibility was further confirmed.^{91,92} In addition, the significant female preponderance is well known and the median odds ratio in case studies corresponds to a female predominance of up to 9:1 to 10:1, among the highest described in autoimmune diseases.

Human Leukocyte Antigen

The major histocompatibility complex (MHC) is located on the short arm of chromosome 6 and harbors genes encoding molecules involved in antigen presentation, and is therefore involved in distinguishing self from non-self. The MHC complex is characterized by a cluster of genes related by sequence homology and/or function: the human leukocyte antigen

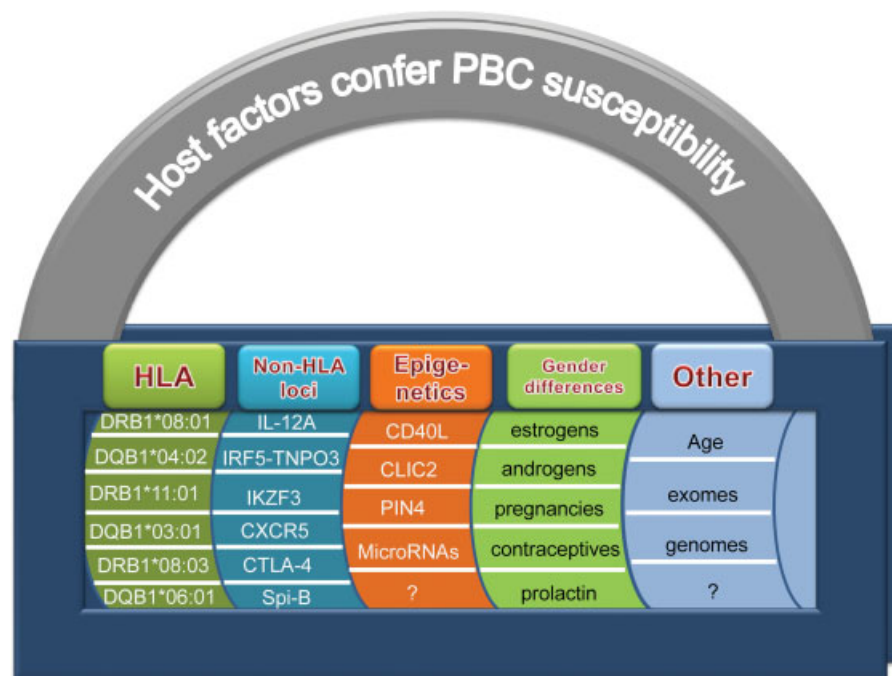


Fig. 4 Genetic factors that confer primary biliary cirrhosis (PBC) susceptibility. The relationship between genetic susceptibility and the breach of tolerance in PBC is complex, involving human leukocyte antigens (HLA), nonhuman leukocyte antigens, antigen risk loci, epigenetics, and sex-associated factors.

(HLA) class I and HLA class II genes encoding proteins involved in antigen presentation. HLA class III genes encode several other immune proteins, such as tumor necrosis factor- α (TNF- α).⁹³ Unlike other autoimmune diseases, there are only weak and regional associations between PBC and HLA molecules.^{90,94–96} In HLA candidate gene studies based on populations of European origin, PBC is associated with the risk haplotypes, DRB1*08:01-DQA1*04:01-DQB1*04:02 and DRB1*04:04-DQB1*03:02.^{97,98} Primary biliary cirrhosis is also associated with the protective haplotypes DRB1*11:01-DQA*05:01-DQB1*03:01 and DRB1*15:01-DRA1*01:02-DQB1*06:02. In Japan, DRB1*08:03-DQB1*06:01 and DRB1*04:05-DQB1*04:01 have been identified as risk haplotypes, whereas DRB1*13:02-DQB1*06:04, DRB1*11:01-DQB1*03:01, and DRB1*15:01 act as protective haplotypes.⁹⁹ In a study by Invernizzi et al, conditional analysis showed that these haplotype associations in Italians are most likely driven by the respective DRB1 alleles, the risk alleles DRB1*08 and DRB1*14, and the protective allele DRB1*11.¹⁰⁰

No consistent and reproducible associations have been detected between specific DRB1 alleles and clinical features of the disease. In Chinese PBC patients, significant association exists between HLA-DRB1*08:03, DQ2, and DQB1*06:01 alleles and PBC. DRB1*08:03-DQB1*06:01 and DRB1*07:01-DQB1*02:02 haplotypes were also associated with PBC susceptibility. On the other hand, DRB1*03:01 alleles and the DRB1*12:02-DQB1*03:01 haplotype were significantly decreased in Chinese PBC patients compared with controls.¹⁰¹

Genome-Wide Association Studies

Genome-wide association study (GWAS) analysis demonstrates that there is less of a difference in the intensity of risk association with PBC between HLA and non-HLA loci. This finding suggests that although HLA is a very important contributor of risk, the non-HLA loci, as a group, are at least equally important. So far, 27 genome-wide significant non-HLA risk loci for PBC have been identified,¹⁰² including 2q32 (STAT1, STAT4), 3q25 (IL12A, SCHIP1), 7q32 (IRF5, TNPO3), 11q23 (CXCR5), 12p13 (TNFRSF1A, LTBR), 16p13.13 (SOCS1, CLEC16A), 17q12 (IKZF3) and 19q13.3 (SPIB).^{98,103} Risk loci for PBC appear to be enriched for gene products involved in innate or adaptive immune responses, consistent with an autoimmune component to pathogenesis. Genome-wide association studies now play a significant role in evaluating a genetic role in many autoimmune hepatic diseases.¹⁰²

In 2009, Hirschfeld et al identified three susceptibility loci, HLA, IL12A, and IL12RB2, and their observations were confirmed by other GWAS studies.¹⁰⁴ IL-12, acting as a T-cell stimulating factor that is involved in the differentiation of naive T cells into Th1 cells, has been previously implicated in autoimmunity and is important for the development of antigen-specific (PDC-E2) autoreactive T cells in PBC.¹⁰⁵ IL-12 is also involved in the activity of natural killer (NK) cells and its cytotoxic activity. In addition, the binding of IL-12 to its receptor is thought to modulate autoimmune responses by evoking IFN- γ production, which may in turn alter IL-23-driven induction of IL-17-producing Th17 lymphocytes.

Moreover, signals from IL-12R are mediated by TYK2 and STAT4, and negatively regulated by SOCS1.^{106,107}

IRF5-TNPO3 (encoding interferon regulatory factor 5 and transportin 3) plays a key role in the innate immune response as part of the Toll-like receptor (TLR) signaling pathway and mediates apoptosis induced by TNF related apoptosis-induced ligand.¹⁰⁸ IRF5 also contributes to the development of dendritic cells, and promotes inflammatory macrophage polarization and Th1-Th17 responses.¹⁰⁹

CXCR5 (Chemokine (C-X-C Motif) Receptor 5), acting as a multipass membrane protein, is mainly expressed in mature B cells and follicular helper-T cells (Tfh). Expression of CXCR5 affects the migration of B cells into splenic follicles, and may have a regulatory role in B-cell differentiation. Moreover, CXCR5 also affects the function of Tfh cells, which have been reported to play an important role in multiple autoimmune diseases.¹¹⁰

The transcription factor IKZF3 gene encodes IKAROS family zinc finger 3 (also known as Aiolos) and plays a critical regulation role in B-cell differentiation, proliferation, and maturation. It also controls apoptosis of T and B cells in an IL-2-dependent way, and has been implicated in the pathogenesis of autoimmunity.¹¹¹ Spi-B (Spi-B transcription factor [Spi-1/PU.1 related]), a member of the ETS transcription factor family, is an important mediator of both early T-cell lineage differentiation and B-cell receptor signaling. Spi-B also induces the development of plasmacytoid dendritic cells and NK cells.

Other new candidate genes associated with disease at GWAS thresholds include SIAE, TNFSF15, POU2AF1, CTLA-4, IL-1, IL-10, vitamin D receptor (VDR), DENND1B, CD80, IL7R, CLEC16A, PTPN22, and NFKB1,^{112–118} all of which play compelling roles in the development of autoimmunity that require further confirmation or investigation.

Epigenetics

Epigenetics is defined as stable and heritable patterns of gene expression that do not involve any alterations to the original DNA sequence. There are four known types of epigenetic mechanisms: (1) methylation of DNA on certain cytosine residues that generally silences genes, (2) posttranslational modifications of histone tails of nucleosomes (acetylation, methylation, ubiquitination, etc.) that may either render genes active or inactive depending on the histone modified and the nature of the modification, (3) active remodeling of chromatin by protein machines called remodeling complexes that can also either enhance or suppress, and (4) the silencing of gene expression by small noncoding RNA transcripts.¹¹⁹ The lack of concordance in monozygotic twins in autoimmune diseases strongly suggests that besides environmental factors, epigenetic factors may also be important in determining the susceptibility to autoimmunity.^{9,120,121} The relationship between epigenetics and the pathogenesis of autoimmune diseases, including PBC, has been described extensively.^{91,122–125}

The interaction of CD40 and CD40L plays a key role in CD4+ T cell priming, B-cell terminal maturation, and immunoglobulin class-switch recombination. No gene mutations

were detected in cDNA of CD40L from PBC patients by RT-PCR-SSCP technique.¹²⁶ Lleo et al has demonstrated significantly lower levels of DNA methylation of the CD40L promoter in CD4 + T cells from PBC patients, as compared with controls, and this decreased methylation inversely correlated with levels of serum IgM in PBC patients.¹²⁷

The reason for female predominance is unclear, although multiple theories have been proposed.^{128–138} Because the female has two X chromosomes, one is silenced to varying degrees in a process known as *X-chromosome inactivation* (XCI) or *lyonization*. Most genes on the inactive X chromosome are silenced by promoter methylation during X-chromosome inactivation (XCI).¹³⁹ Two genes (CLIC2 and PIN4) were identified as consistently downregulated in an affected cohort of monozygotic twin sets discordant and concordant for PBC. Both CLIC2 and PIN4 demonstrated partial and variable methylation

of CpG, but the possible mechanisms by which epigenetic factors influence PBC onset are likely much more complex than a simple X-linking of candidate genes.^{120,140–142}

MicroRNAs play a vital role in the regulation of various aspects of immune function and in the development of autoimmune disease. In 2009, it was shown that PBC is associated with altered expression of 35 hepatic microRNAs.¹⁴³ Subsequently, 17 microRNAs differentially expressed in PBMCs from PBC patients were also identified.¹⁴⁴ Other microRNAs, such as miR-506,¹⁴⁵ miR-let-7b, miR-505–3p, and miR-197–3p have also been reported to be associated with PBC.^{146,147} However, the precise mechanisms related to the role of microRNA in the maintenance of the breach of immune tolerance in PBC have yet to be elucidated.

Finally, a considerable number of sex-related genes appear crucial in the maintenance of physiological sex hormone

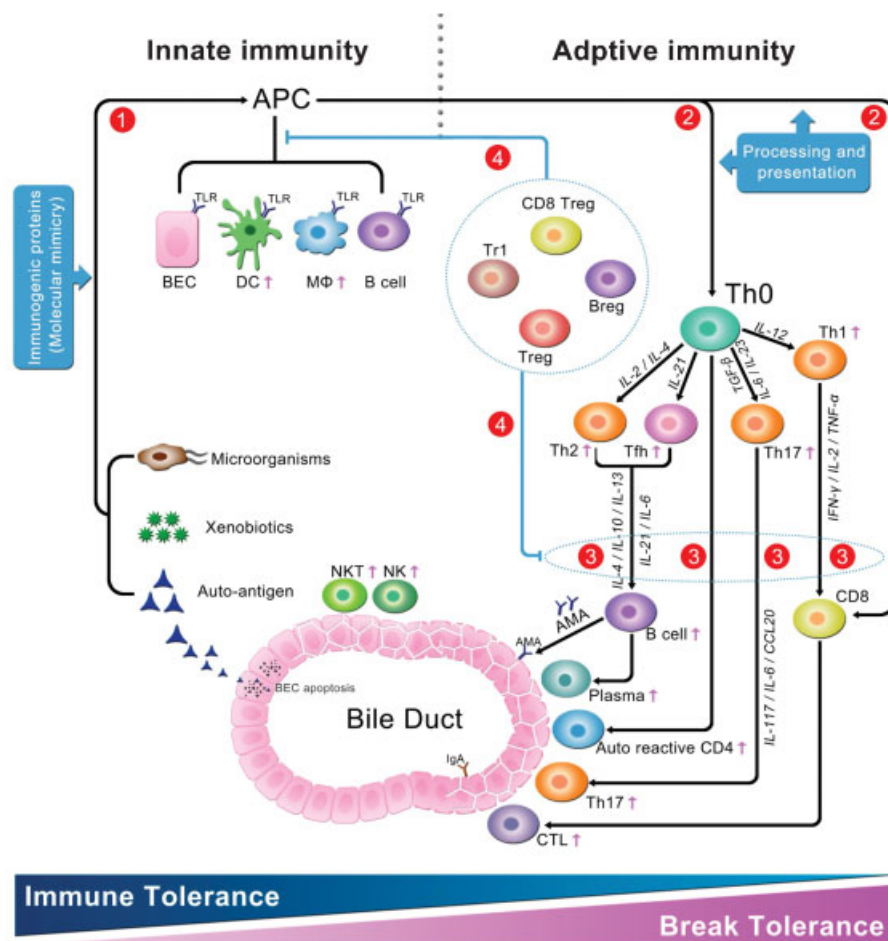


Fig. 5 Innate and adaptive immunity in primary biliary cirrhosis (PBC) patients. (1) Microorganism proteins, xenobiotics, and apoptosis of biliary epithelial cells (BEC) can be recognized and endocytosed by antigen-presenting cells (APCs), which subsequently activate innate immune cells such as Toll-like receptors (TLRs), DCs, macrophages, natural killer (NK) and natural killer T (NKT) cells, and others. (2) After being processed by APCs, some T-cell immunogenic peptides were generated and presented to uncommitted T helper (Th0) lymphocytes and CD8 T cells. (3) Activated Th0 cells then differentiate into Th1, Th2, Tfh, and Th17 cells. Furthermore, Th1 cells secrete cytokines such as interleukin-2 (IL-2) and interferon- γ , which stimulate development of cytotoxic T lymphocytes (CTL). Th2 cells or Tfh cells secrete IL-4, IL-10, IL-13, or IL-21, and may stimulate autoantibody (e.g., AMA) production by B lymphocytes. Finally, CTL (autoreactive CD8 + T), B lymphocytes, Th17, autoreactive CD4 + T, NK, and NKT infiltrate and gather around the small bile duct, and participate in the development of autoimmunity. (4) Simultaneously, the number and function of immunosuppressive cells (Treg, Breg, Tr1, and CD8 Treg) decrease significantly, which indirectly promotes overactivation of immune responsiveness. Throughout the process, the balance between immune tolerance and break tolerance is a constantly changing process, which finally leads to the breach of tolerance in PBC.

levels. Often, these genes have a pleotropic function in immune system balance.¹⁴⁸ Sex-associated hormones, such as estrogens, androgens and prolactin, which not only differ between males and females but also can vary according to age, can be central to the T helper 1/T helper 2 balance. They may therefore play a significant role in determining an appropriate or inappropriate inflammatory response and thus play a role in the balance between immune tolerance and autoimmunity.¹⁴⁹ Invernizzi et al¹⁵⁰ first determined a significantly higher frequency of monosomy of the X chromosome in peripheral leukocytes (particularly T and B cells) in female PBC subjects as compared with age-matched female controls. Moreover, several sex-related factors (pregnancies,¹⁵¹ contraceptives, estrogen-replacement treatments,⁷⁵ and recurrent vaginitis¹⁵²) appear to increase the risk of developing PBC. The availability of high-throughput efforts to sequence exomes and genomes and the advent of deep-sequencing should advance these data.

Innate and Adaptive Immunity

Defects in immune regulation that govern components of both innate and adaptive immunity contribute to the abnormal perpetuation of the immune response (►Fig. 5; ►Table 2). Innate immune cells physiologically resident in the liver constantly provide a defense against pathogens while maintaining tolerance to food antigens and commensal bacteria. The evidence for a role of innate immunity in PBC includes the following immunological features: presence of granulomatous inflammation, elevated levels of polyclonal IgM, hyperresponsiveness to CpG, increased levels of NK cells, and aberrant cytokine responses.^{153,154}

Toll-Like Receptors

Thirteen Toll-like receptors (TLRs) have been described in mammals of which 10 are found in humans.¹⁵⁵ Toll-like receptors are part of a system of pattern-recognition

Table 2 Innate and adaptive immune in patients with primary biliary cirrhosis

Immune type	Function	Reference
Innate immunity		
TLR	TLR signaling; contributes to the breach of tolerance	154,157
DC	↑ Number (peripheral and intrahepatic) ↑ Function (presenting and cross presenting)	163,164,166
Macrophage	↑ Hypersecretion of proinflammatory cytokines	168,253
BEC	Act as antigen presenting cells; defense against pathogenesis; induces IgA-related damage	173,175,180
NK	↑ Number (peripheral and intrahepatic) ↑ Function (cytotoxic activity)	184,185
NKT	↑ Number (peripheral and intrahepatic) ↑ Function (cytotoxic activity, perforin expression) ↑ Lymphoid cell infiltration	187,188
Adaptive immunity-Cellular immunity		
CD4	↑↑ Autoreactive CD4 T cells (100- to 150-fold) ↑ Ratio of Th1/Th2	190,191,203
Th17	↑ Number (intrahepatic) ↑ IL-17 secretion	192-194
Treg	↓ Number (peripheral and intrahepatic) ↓ Function (maintenance self-tolerance)	195,196
Tfh	↑ Number (peripheral and intrahepatic) ↑ Function (provides help to B cells)	
CD8	↑ Autoreactive CD8 T cells (10-fold) ↑ Function (cytotoxic activity)	59,208
- Humoral immunity		
Antibodies	Indicates a breach of immune tolerance (AMA, AMA-IgA, ANA, anti-Gp120 and anti-Sp100)	211,212
B cells	↑ Number (peripheral and intrahepatic) ↑ Intrahepatic CD38+ plasmablast ↑ Function (promote antibody production) Suppressive B cells exist (Breg)	38,214,215,254

Abbreviations: AMA, antimitochondrial antibody; ANA, antinuclear antibody; BEC, biliary epithelial cell; DC, dendritic cell; IgA, immunoglobulin A; NK, natural killer; NKT, natural killer T; TLR, Toll-like receptor.

receptors (PRRs) that act by recognizing unique, highly conserved structural components of bacteria, fungi, viruses called *pathogen-associated molecular patterns* (PAMPs), as well as *damage-associated molecular patterns* (DAMPs). The binding of PAMPs or DAMPs to TLRs leads to signaling pathways that activate and propagate innate and adaptive inflammatory responses.¹⁵⁶

The expression of TLRs is normally regulated by a negative signaling pathway in the liver, which prevents inappropriate activation of inflammation. Peripheral blood mononuclear cells in PBC patients produce higher amounts of inflammatory cytokines in response to the ligands of TLR2, TLR3, TLR4, TLR5, and TLR9 than PBMCs in healthy subjects.¹⁵⁷ In PBC patients, peripheral B cells exposed to CpG motifs express increased amounts of TLR9 and CD86, which enhance their production of intracellular IgM and AMA.^{154,158}

Furthermore, the cytotoxicity of liver NK cells in PBC patients is more profound than cells in control subjects when incubated with poly I:C and LPS-primed liver macrophages.¹⁵⁹ These results indicate that patients with PBC exhibit hypersensitivity to TLR signaling.

Antigen-Presenting Cells

Dendritic Cells

Dendritic cells (DCs) occupy a unique position at the interface between innate and adaptive immunity, orchestrating a large panel of responses. The presentation of antigens by immature DCs should result in immune tolerance, while activated and mature DCs are capable of priming robust adaptive immune responses.¹⁶⁰ In 1989, Demetris et al first identified dendritic cells that localize inside the basement membrane between biliary epithelial cells of septal bile ducts in livers of early stage PBC.¹⁶¹ Using immunohistochemical methods, Tanimoto et al reported that there exists activated CD83-positive DCs in PBC patients.¹⁶² In 2001, Akbar et al stimulated peripheral blood T cells from PBC with either PDC alone or DCs plus PDC. They reported that peripheral blood T cells from 100% of AMA-positive PBC patients reflected PDC-specific proliferation when cultured with PDC-pulsed DCs.¹⁶³ This finding demonstrated the effectiveness of antigen-pulsed DCs.

In 2002, Kita et al also found that PDC-E2-specific cytotoxic T lymphocytes (CTLs) could also be generated by pulsing DCs with full-length recombinant PDC-E2 protein, which indicated that CTL activation could be augmented by immune complexes cross presented by dendritic cells.¹⁶⁴ Hiasa et al analyzed the phenotypes of DC1 and DC2 from patients with primary biliary cirrhosis.¹⁶⁵ A phenotype of DC2 with reduced expression of HLA D2 and CD123 in PBC may have relevance to the breakdown of tolerance to self-antigen. In addition, Langerhans cells (LCs) existing around or within biliary epithelial layers are important as periductal antigen-presenting cells in PBC. The migration of LCs into bile ducts is closely associated with the periductal cytokine milieu in patients with PBC.¹⁶⁶

Monocytes

Monocytes have also been implicated in the pathogenesis of PBC and other autoimmune diseases.¹⁶⁷ Peripheral-blood

monocytes in PBC are more sensitive to infectious stimuli, which lead to hypersecretion of proinflammatory cytokines. The expression of TLR4 in circulating monocytes increased significantly after stimulation with lipopolysaccharide (LPS) in PBC patients, and circulating monocytes produce higher proinflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF- α ¹⁶⁹, that can amplify adaptive T-cell-mediated immune responses against pathogens. At the same time, the level of RP105, which is involved in the negative regulation of TLR4 signaling, is decreased in PBC monocytes.¹⁶⁸ In the liver of PBC patients, TLR3 is highly expressed on macrophages surrounding the portal tract and on hepatocytes, and these macrophages produce type-I interferon through TLR-3 signaling.¹⁶⁹ The complexity of the immune response is illustrated by the fact that the proinflammatory cytokine IL-6 has been found to play a role in activation of IL-10 producing Tr1 cells in suppressing autoimmune tissue inflammation.^{170,171}

Biliary Epithelial Cells

Biliary epithelial cells (BECs) are not just innocent bystanders in the pathophysiology of PBC.¹⁷² First, unlike other epithelial cells, BECs act as antigen-presenting cells that express HLA class II^{173,174} and costimulatory molecules, such as CD80 and CD86.^{175,176} After phagocytosing and processing the apoptotic BECs, BECs present novel mitochondrial self-peptides in conjunction with HLA class II.^{80,176,177} Finally, autoreactive T cells against 2-OADC infiltrate into the liver,¹⁷⁸ and gather around small bile ducts. This was observed in PBC irrespective of their serum AMA status.¹⁷⁹ These data support the notion that phagocytosis of apoptotic cells by nonprofessional phagocytes may contribute to the tissue specificity of autoimmune diseases. Biliary epithelial cells also contribute to the defense mechanisms against infectious agents by recognition of PAMPs via specific membrane TLRs, resulting in the generation of chemokines that activate both the innate and adaptive immunity systems. Biliary epithelial cells are unique in secreting immunoglobulins A of the secretory type (sIgA) through transcytosis in the biliary lumen, which may constitute organ-specific immune-mediated injury.^{180,181}

Natural Killer Cells

Natural killer cells are mainly involved in defense against infections and tumors, but also link innate with adaptive immunity. Several studies indicate that aberrancies in NK-cell-mediated immune homeostasis can lead to the onset of autoimmune diseases.¹⁸² In 2001, Panasiuk et al first reported the increase of NK cell numbers in the peripheral blood of PBC patients.¹⁸³ The finding has been confirmed by Chuang and his colleagues, and it was further demonstrated that NK cells express higher levels of perforin and decreased cytokine production in PBC patients compared with healthy controls.¹⁸⁴ At the same time, a higher frequency of CD56dim/CD16pos hepatic NK cells was present within the liver of PBC patients. Further studies have shown that hepatic NK cells have cytotoxic activity against autologous biliary epithelial cells.

Because NK cells have been reported to express CX3CR1 and CXCR3, it has been hypothesized that the higher

frequency of cytotoxic NK cells found in the liver of PBC is due to an increased migration of circulating NK cells through mechanisms that involve both CX3CR1 and CXCR3. In a mouse model, there is a marked suppression of AMA and cytokine production from autoreactive T cells after *in vivo* depletion of NK and natural killer T (NKT) cells.¹⁸⁵ The increased infiltration of cytotoxic NK cells in the liver may reflect the breakdown of NK cell immune tolerance, but further studies are needed to describe the detailed mechanisms driving the expansion of autoreactive NK cells.¹⁸⁶

Natural Killer T Cells

Natural killer T cells are regulated by self- and nonself-glycolipid antigens that are presented by the antigen-presenting molecule CD1d. This process allows for rapid NKT cell expression of effector cytokines and chemokines, thereby modulating both innate and adaptive immune responses. In PBC, the frequency and absolute number of blood and liver NKT cells are markedly increased compared with healthy controls. Moreover, cytotoxic activity and perforin expression by isolated NKT cells were significantly increased in PBC with increased IL-8 levels and expression of CD128a (IL-8 receptor) on NKT cells. In contrast, the levels of interferon- γ (IFN- γ), IL-6, and IL-8 synthesized by NKT cells were significantly decreased in PBC when compared with controls.¹⁸⁷ In 2008, Chuang et al generated a CD1d(-/-) dnTGF-nRII mice and reported decreased hepatic lymphoid cell infiltration as well as milder cholangitis than that seen in controls.¹⁸⁸ CD1d-restricted NKT cells in the liver exhibit increased IFN- γ production after exposure to α -galactosylceramide. In both *N. aromaticivorans* induced and 2-OA-BSA induced PBC mouse models, NKT cells are involved in disease exacerbations, which include signs of portal inflammation, bile duct destruction, and liver fibrosis.^{58,189}

Adaptive Immunity: Cellular Immunity

Excessive T-Cell Helper Function

An enhanced ratio of Th1 to Th2 cells is an important factor in the onset of PBC.^{190,191} Th17 cells, a subset of CD4 T cells, accumulate around damaged bile ducts in liver and in a mouse model of PBC.¹⁹² In IL-2R α (-/-) mice, marked aggregation of IL-17+ cells within portal tracts compared with the periphery has been demonstrated. Interestingly, CD4+ T cells from the livers of normal C57BL/6J mice can secrete higher levels of IL-17 compared with those from spleens, indicating a role of the liver microenvironment in Th17 induction. Finally, Th17 cells involved in the pathogenesis of various autoimmune diseases are also constituents of the periductular infiltrates in human PBC.^{193,194} In recent studies, our preliminary data demonstrated that the frequency of T follicular helper cells are more highly expressed in PBC patients, and that Tfh accumulates around the damaged bile ducts in liver tissue.

T-Cell Regulation

CD4 + CD25+ T regulatory cells (Tregs) play an important role in the maintenance of peripheral self-tolerance as well as

downregulation of various immune responses.^{24,25} In PBC patients, FoxP3+ Tregs can be identified in the lymphoid infiltrates localized to portal tracts, and significantly lower proportions of circulating CD4 + CD25^{high}Tregs are observed in patients and family members.¹⁹⁵ In addition, the dnTGF β RII and IL-2R α knockout murine models support a role of Tregs deficiency in loss of immune tolerance. The selective deficiency of the TGF β R-signaling pathway exclusively in T lymphocytes accounts for major impairments of peripheral tolerance as Treg cells depend on TGF β for their regulatory activity. The result is the triggering of tissue-specific autoreactive effector T cells.³⁹ A mouse deficient for IL-2 receptor α (IL-2R α), which is highly expressed on Tregs, developed 100% AMA positivity against PDC-E2, 80% antinuclear antibody (ANA) positivity, and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury.³⁸

In 2009, Zhang et al studied Scurfy (Sf) mice, which have a mutation in the gene encoding the Foxp3 transcription factor resulting in a complete abolition of Foxp3+ Tregs. At 3 to 4 weeks of age, 100% of animals manifested high titers of serum AMA of all isotypes. Furthermore, these mice had moderate to severe lymphocytic infiltrates surrounding portal areas with evidence of biliary duct damage. These data illustrate that lacking normal Treg function is a major predisposing feature for breach of tolerance that leads to PBC.^{196,197}

Other regulatory cells, such as CD8 Tregs have also been reported to contribute to a significant phenotypic alteration in PBC patients characterized by increased expression of CD127 and reduced CD39 compared with normal controls. Furthermore, *in vitro* induction of CD8 Tregs by incubation with IL-10 is significantly reduced in PBC.¹⁹⁸ The immunomodulatory effects of the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) have been studied at a cellular level and implicated in the pathogenesis of several complex diseases. Impaired IDO production has been reported and postulated to contribute to the development of disease pathogenesis in PBC patients.¹⁹⁹

Expansion of the Autoreactive T-Cell Pool

Under normal circumstances, lymphocytes are scattered throughout the liver parenchyma, as well as in the portal tracts. These lymphocytes belong to several subpopulations.²⁰⁰ T-helper (CD4 +) TCR $\alpha\beta$ + and CD8+ T cells are present in portal tracts and around damaged bile ducts, strongly supporting their role in the development of biliary damage.²⁰¹⁻²⁰⁷ Autoreactive CD4 T cells that specifically target PDC-E2-self-antigen are present in peripheral blood and liver. There is a 100- to 150-fold increase in the number of PDC-E2-specific CD4 T cells in the hilar lymph nodes and liver versus peripheral blood in patients with PBC.²⁰³ The HLA class I restricted epitope for CD8 T cells, namely the 159-167 aa sequence, maps in close vicinity to the epitopes recognized by CD4 T cells as well as by AMA.⁵⁹ Notably, the autoepitope for both CD4 and CD8 T cells overlaps with its B-cell (AMA) epitope, including the inner lipoyl domain. Similar to CD4 autoreactive T cells, there is a 10-fold higher frequency of

Table 3 Novel approaches that may restore immune tolerance in primary biliary cirrhosis

	Treatment target	Possible mechanisms	Treatment efficacy
Traditional			
UDCA (13–15 mg/kg/d)	Bile acid pool	Not clear (involving choleresis, antiapoptosis, anti-inflammation)	About 60% efficacy
Nonspecific immunosuppression			
Budesonide ^{219,255} Methotrexate and colchicine ²²⁰ Cyclosporine ²²¹ Azathioprine ²²²	Whole activated immune systems (autoimmune or nonspecific immune)	Immunosuppression	Transitory biochemical response in some patients, but with side effects
Specific target therapy			
Rituximab ^{216,217}	Total B cells	Depletion B cells	Limited efficacy in patients
Ustekinumab ²²⁶	IL-12/IL-23	Block the signal pathway of IL-12	In progress (NCT01389973)
NI-0801	CXCR10 (IP-10)	Inhibits T-cell migration and inflammation	In progress (NCT01430429)
CTLA4/immunoglobulin ²³⁰	CTLA4	Inhibits T-cell activation	In preclinical studies
Novel therapeutic methods			
Mesenchymal stem cells ^{234,235}	Whole activated immune systems and damaged bile ducts	Potential immunomodulatory capacity; tissue regeneration and repair	Pilot data in PBC patients; significant benefits in mouse models
Tolerogenic dendritic cell (tolDCs) vaccine	DC	Induce immune tolerance to PDC	In theoretical stages
Oral tolerance ²³⁸	Immune system	Induce immune tolerance to PDC	failed
siRNA or microRNA	Key molecular	Restore immune tolerance	In theoretical stages

Abbreviations: DC, dendritic cell; IL, interleukin; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; UDCA, ursodeoxycholic acid.

PDC-E2 159–167 specific CD8 T cells in liver compared with blood. Moreover, the frequency of precursor of PDC-E2-specific autoreactive CD8 T cells is significantly higher in early-rather than late-stage disease.²⁰⁸ The autoreactive CD8 T cells in PBC produce IFN- γ rather than IL-4/IL-10 cytokines. Autoreactive CD8 T cells have also been implicated in hepatocyte damage seen in autoimmune hepatitis.²⁰⁹

Adaptive Immunity: Humoral Immunity

A high titer of serum AMA can be detected in up to 95% of patients with PBC. Antimitochondrial antibodies can be detected even before clinical symptoms or biochemical anomalies. However, most studies have shown that there is no correlation between the level of serum AMA and the severity of PBC. Antimitochondrial antibody positivity alone also does not predict the patient's response to treatment with ursodeoxycholic acid (UDCA). However, AMA-IgA can be detected not only in sera, but also in bile, saliva, and urine of patients with PBC. In one study, AMA-IgA correlates with disease severity.²¹⁰ In addition to AMAs, ANAs are detected in nearly 50% of patients with PBC, and their presence may be clinically significant.^{211,212}

Although B cells are well known for their antibody production, they also participate in antigen presentation, modeling of the spleen architecture, and Th1/Th2 polarization of T cells.²¹³ Specific substrates of B cells can act as negative regulators and facilitate immune tolerance. In the liver of PBC patients, the number of infiltrating B cells is higher than that of PSC patients and normal controls. Moreover, the proportion of CD19 + CD69+ activated B cells is markedly higher in liver than in peripheral blood in PBC, and the number of AMA-producing cells is five-fold greater in liver than in PBMCs.²¹⁴ In 2012, Takahashi et al reported a unique coronal arrangement of CD38+ cells around the intrahepatic bile ducts in PBC, but not in controls; there was an association with AMA titer and an inverse association with serum γ -glutamyltranspeptidase levels.²¹⁵

In a dnTGF- β RII PBC mouse model, anti-CD20 administered every 2 weeks ameliorated autoimmune cholangitis if given early in the disease process.²¹⁶ In PBC, a regimen of two doses of 1000 mg rituximab (mouse-human chimeric anti-CD20 monoclonal antibody) separated by 2 weeks was found to be safe, but only marginally effective in some patients, who had an incomplete UDCA response.²¹⁷ In 2-octynoic acid-

bovine serum albumin (20A-BSA)-induced mice, treatment with anti-CD20 and anti-CD79 antibodies led to a higher number CD4+ and CD8+ T cells around damaged bile ducts in portal areas, which manifest as more severe cholangitis.²¹⁸ Adoptive transfer of CD19+ cells from dnTGF- β RII mice into recombination activating gene-1 (Rag-1)(-/-) mice resulted in decreased liver inflammation and bile duct damage.²¹⁶ Together, these findings suggest that there is a subclass of B cells that has a regulatory role by producing IL-10. However, further studies are needed to explore the functions of B cells in PBC.

Novel Approaches to Restore Immune Tolerance in Primary Biliary Cirrhosis

Currently, UDCA is the only drug approved by the Food and Drug Administration to treat PBC patients. Early treatment with UDCA at a dose of 13 to 15 mg/kg per day can delay the progression of histological changes, ameliorate long-term morbidity, and prolong life expectancy. Ursodeoxycholic acid acts by increasing the hydrophilic properties of the bile acid pool, producing bicarbonate-rich choleresis, which protects against hepatocellular damage. However, over 40% of patients have an incomplete response to UDCA, resulting in progressive disease necessitating liver transplantation or ultimately resulting in death from liver-related causes. Therefore, future studies should focus on the development of novel approaches that restore the immune tolerance in PBC (► **Table 3**).

Unlike other autoimmune diseases, nonspecific immunosuppressive medication is not recommended as a first-line strategy for PBC. But it is sometimes used in patients who fail to respond to UDCA. In 2000, Angulo et al treated PBC patients who had an incomplete response to UDCA therapy with budesonide, a corticosteroid with an extensive first-pass hepatic metabolism.²¹⁹ After 1-year treatment, there was a significant but transitory improvement in serum levels of total bilirubin, and a significant but marginal improvement in serum alkaline phosphatase. The Mayo risk score increased significantly, and there was a significant loss of bone mass of the lumbar spine. In 2010, Kaplan et al treated PBC patients who had not responded fully to UDCA with methotrexate and colchicine.²²⁰ The result showed that methotrexate and colchicine significantly improved liver enzyme tests and liver histology. Others, like cyclosporine,²²¹ azathioprine²²² were also used to treat PBC patients. However, these nonspecific immunosuppression drugs not only suppressed the underlying autoimmune disease, but also led to global suppression of the immune system. The results of this approach include side effects such as increased risk of infection, carcinogenesis, and osteoporosis. There is a clear need for more specific strategies to restore immune tolerance to the specific autoantigens implicated in disease pathology.

Ideally, autoimmune disease therapies would uniquely target the specific autoreactive lymphocyte populations, while leaving unperturbed the rest of the immune system. Primate or humanized monoclonal antibodies against pan-T cell or B cells as well as leukocyte-specific antigens or soluble

receptors have been used extensively in the treatment of autoimmune diseases.²²³ Several antibodies targeting specific cells or signaling pathways have been also applied in PBC patients.

In PBC patients, although the role of B cells in the pathogenesis is not clearly defined, B-cell depletion therapy has been shown to be of potential value. The concept of B cell depletion was first tried in a PBC mice model, Moritoki et al showed that in younger dominant-negative TGF- β RII mice aged 4 to 6 weeks, anti-CD20 treatment significantly alleviated liver inflammation and reduced bile duct damage.²¹⁶ Rituximab is a mouse-human chimeric anti-CD20 monoclonal antibody designed for B-cell depletion therapy in humans. It has been approved for the treatment of lymphoma and certain autoimmune diseases such as rheumatoid arthritis. Tsuda et al used rituximab to treat six patients with PBC who had suboptimal biochemical response to UDCA.²¹⁷ After B-cell depletion, they observed a reduction in the number of AMA-producing B cells, AMA titers, serum alkaline phosphatase levels (ALP), and plasma levels of immunoglobulins (IgA, IgM, and IgG) at week 24. As the levels of immunoglobulins, AMA titers and ALP returned to baseline levels at week 36, repeated anti-CD20 treatment was suggested to maintain the treatment effect. However, there were two patients who experienced upper respiratory infection and reactivation of varicella zoster after the first infusion. Further clinical studies targeting B cells in PBC patients are warranted.²²⁴

Interleukin-12 (IL-12) and its receptors have been identified as susceptibility genes for PBC, and play a very important role in the breakdown of immune tolerance, thereby offering another potential therapeutic target.²²⁵ Ustekinumab,²²⁶ a human monoclonal antibody directed against IL-12 and IL-23, is involved in immune regulation and has the potential to mediate certain autoimmune diseases such as active psoriasis arthritis.²²⁷ A phase II clinical trial (NCT01389973) using ustekinumab to treat PBC patients is in progress.

CXCL-10 has been shown to play a role in autoimmunity.²²⁸ Plasma and portal IFN- γ -inducible protein-10 (IP-10) levels were found to be increased in PBC patients compared with controls.²²⁹ NI-0801, an anti-CXCL10 monoclonal antibody that inhibits T-cell migration and inflammation, is being studied in the treatment of PBC patients in a phase II clinical trial (NCT01430429). Other specific antibody treatments, such as those targeting CTLA4/immunoglobulin,^{230,231} are also in preclinical studies in mouse models, and offer other potential areas of pharmaceutical development in the treatment of PBC.²³²

Mesenchymal Stem Cells: Re-establishing Immune Tolerance

Mesenchymal stem cells (MSCs) have been used as a therapeutic strategy for tissue regeneration and repair, and their potential immunomodulatory capacity, especially in the induction of immune tolerance, has raised significant clinical interest. Recently, autologous and allogeneic MSCs have been reported to significantly improve symptoms in patients with severe autoimmune diseases, such as Crohn disease, multiple

sclerosis, refractory systemic lupus erythematosus, and systemic sclerosis.²³³

Mesenchymal stem cells have also been used in mouse models of PBC. In 2011, Wang et al used bone marrow-derived mesenchymal stem cells (BM-MSC) in treatment of polyinosinic-cytidylic acid poly (I:C) induced C57BL/6 PBC mouse model.²³⁴ The result showed that after 6 weeks of MSC infusions, serum aminotransferase and autoimmune antibodies declined, and histological examination showed significant amelioration of monocyte infiltration around bile ducts. In addition, allogeneic BM-MSC transplantation markedly increased CD4 + Foxp3+ regulatory T cells in peripheral blood as well as lymph nodes. In our pilot studies,²³⁵ umbilical cord-derived mesenchymal stem cells (UC-MSCs) were administered three times at 4-week intervals to seven PBC patients who were suboptimally treated with UDCA. The patients were followed for a 48-week period. The result of this proof of concept study indicated that MSC treatment in PBC is safe. Fatigue and pruritus were alleviated in most patients after UC-MSC treatment. There was a significant decrease in serum alkaline phosphatase and γ -glutamyltransferase levels at the end of the follow-up period as compared with baseline. A larger, randomized controlled cohort study is warranted to confirm the clinical efficacy of UC-MSC transfusion.

Novel Vaccine Therapies

Tolerogenic dendritic cell (tolDCs) vaccines, an antigen-specific treatment that only targets the autoreactive inflammatory response, has already been proven effective in experimental animal models of various autoimmune disorders as well as with *in vitro* experiments using *ex vivo* generated human tolDCs.²³⁶ A PDC-E2 specific tolDCs is a potential strategy for the treatment of PBC.

Induction of oral tolerance by administration of protein antigens has the potential to induce antigen-specific immunological unresponsiveness (immune tolerance). High doses of orally fed antigen may lead to deletion or anergy of Th cells, whereas low-dose administration leads to induction of transforming growth factor (TGF)- β -secreting regulatory T cells.²³⁷ This approach represents a potential means to prevent or treat autoimmune disease. Suzuki et al administered 5 mg PDC in gelatin capsules to six PBC patients for 12 months, but failed to induce oral tolerance.²³⁸

Since the discovery of the RNAi pathway, there has been an increase in interest in the development of RNAi-based therapeutics for “undruggable” targets. Undruggable targets are those pathogenic molecules that cannot be targeted due to chemical, structural, or accessibility limitations. Using conventional therapy, only ~20% of molecules are targetable. siRNA therapy has the potential to break this barrier, and has been used in autoimmune mouse models for rheumatoid arthritis and Sjögren (SjS) syndrome to restore immune tolerance.^{239,240} Similarly, siRNAs or microRNA targeting special genes might be another potential therapeutic method for the treatment of PBC patients.^{241,242}

The concept of a breach of immune tolerance in autoimmune disease may change the way we think about PBC and its

treatment. In the future, novel therapeutic approaches based on restoration of immune tolerance will be developed to maintain disease remission with minimal ongoing treatment or a drug-free regimen (such as stem cell therapy) rather than the use of general immunosuppressive agents.²⁴³

Summary

In PBC, considerable progress has been made in understanding the basic mechanisms of the breach of tolerance, but our knowledge is still incomplete.

In most autoimmune diseases, the treatment generally involves immunosuppression. In PBC, most immunosuppressive agents have proven to be relatively ineffective.²⁴⁴ This indicates that the pathogenesis of PBC is not simply an over-reactive immune system directed against a self-antigen, but more likely involves an imbalance in immune tolerance that is affected by many different factors. The specific role of these factors, which may include those that are involved in innate immunity, and additionally those that are involved in cellular regulation, such as T regulatory cells, is not completely elucidated.

Primary biliary cirrhosis, being a chronic liver disease, is generally characterized by a slow progression but a highly variable clinical course. The clinical features and natural history of the disease vary greatly between patients, ranging from asymptomatic and slowly progressive to symptomatic and rapidly evolving to decompensated status. However, very little is known about the relationship between the variable clinical course of PBC and its immune tolerance state. Moreover, the exact time point of the breach in immune tolerance is unclear. Whether it occurs in asymptomatic clinical stages when AMA may be detected, or only in liver biopsy stage I and beyond where the disease has progressed to small bile duct damage needs to be further elaborated.

It is unclear where the breach in tolerance in PBC originates. Tolerance is regulated by both innate and adaptive immunity, and PBC may occur through different mechanisms. This is illustrated by the fact that some PBC patients are AMA positive, while others are AMA negative, but patients lacking AMAs present with similar disease characteristics and progression as AMA-positive patients. The quality of life and life expectancy in responders to UDCA treatment are comparable with the general population. However, the 40% of patients who are UDCA unresponders exhibit a much more aggressive disease progression that can lead to liver transplantation. The mechanisms that affect UDCA responsiveness may be a combination of genetic factors and environmental exposures.

There are other factors that may also play a role in the breach of immune tolerance in PBC patients. The majority of the patients are in their sixth decade of life at the time of diagnosis of PBC. Immunosenescence (aging of the immune system), which may result in a decline of T- and B-cell function, may be accompanied by a loss of ability to differentiate “self” and “foreign” antigens and may contribute to the development of PBC. Finally, further research is needed to decipher the extent of the involvement of various components in the pathogenesis of PBC. Novel approaches that are

based on an understanding of the detailed mechanisms may then be developed and used to improve treatment efficacy in the future.

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