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TOPIC HIGHLIGHT

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Immune-mediated bile duct injury: The case of primary biliary cirrhosis

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Abstract

Autoimmune cholangitis would be the appropriate name to define the immune-mediated bile duct injury following the breakdown of tolerance to mitochondrial proteins and the appearance of serum autoantibodies and autoreactive T cells. Nevertheless, the condition is universally named primary biliary cirrhosis (PBC). The disease etiology and pathogenesis remain largely unknown despite the proposed lines of evidence. One twin study and numerous epidemiology reports suggest that both a susceptible genetic background and environmental factors determine disease onset while a recent genome-wide association study proposed highly significant associations with several common genetic polymorphisms in subgroups of patients. Specific infectious agents and chemicals may contribute to the disease onset and perpetuation in a genetically susceptible host, possibly through molecular mimicry. Importantly, several murine models have been proposed and include strains in which PBC is genetically determined or induced by immunization with chemicals and bacteria. From a pathogenetic standpoint, new exciting data have demonstrated the unique apoptotic features of bile duct cells that allow the mitochondrial autoantigens to be taken up in their intact form within apoptotic blebs. We are convinced that the application of the most recent molecular techniques will soon provide developments in PBC etiology and pathogenesis with likely implications in diagnostics and therapeutics.

Key words: Autoimmune cholangitis; Anti-mitochondrial antibody; Epithelial cell apoptosis; Innate immunity

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INTRODUCTION

The intrahepatic biliary tree is the only target of the immune-mediated injury associated with primary biliary cirrhosis (PBC). In fact, PBC is an autoimmune cholangitis characterized at histology by the non suppurative inflammation and destruction of intrahepatic bile ducts while serology is characterized by the highly specific antimitochondrial autoantibodies (AMA). From a clinical standpoint, PBC is considered a peculiar, yet representative, autoimmune disease[1]. It affects women more frequently than men with a female to male ratio of 10 to 1, mostly at post-menopausal age with anecdotal

cases described in younger subjects[2]. The diagnosis of PBC is made when 2 out of 3 criteria (i.e. presence of serum AMA, increased serum enzymes indicating cholestasis (i.e. alkaline phosphatase) for longer than 6 mo and a compatible or diagnostic liver histology) are fulfilled[1,3]. Clinical symptoms commonly found in reference textbooks include fatigue, pruritus and jaundice; yet the changing disease scenario has now led jaundice to be a very rare sign at presentation[4] while the impact and specificity of fatigue remains a hot topic for debate[5]. The increasing availability of serological tests for routine AMA has significantly changed the spectrum of disease presentation[6]. The need for a liver biopsy at the time of PBC diagnosis remains debatable and it is currently indicated only in patients lacking one of the other criteria, patients requiring accurate staging (although the possibility of sampling errors should be accounted for) or in patients enrolled in clinical trials. Discriminating PBC from other autoimmune or inflammatory liver diseases is usually easy, mostly based on serum autoantibody profiles. Liver histology can be classified into four stages[7]. At earlier stages, bile duct obliteration and granulomas (possibly found at all stages) are strongly suggestive for PBC. Stage Ⅲ demonstrates septal or bridging fibrosis with ductopenia (over half of the visible interlobular bile ducts having vanished) while stage Ⅳ corresponds to frank cirrhosis virtually undistinguishable from end stage liver diseases of different etiologies.

The complete pathways of PBC immunopathogenesis remain unknown yet several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC with the disease onset recognizing two necessary components in a permissive genetic background and an environmental trigger[8].

ETIOLOGY

As in most complex diseases, it is now widely accepted that PBC results from an environmental stimulus intervening on a genetically susceptible background. We will first discuss the somehow overlooked role of female predominance in autoimmunity in general and in PBC. We will then review what is known of the genetic bases of PBC susceptibility and the environmental causes of its development.

Female preponderance

Among autoimmune diseases, PBC, Sjogren's syndrome, SLE, autoimmune thyroid disease and scleroderma manifest the highest female predominance with 80% of patients being women. Based on this long-established observation, three main research directions have been addressed and will be now discussed.

Sex hormones (i.e. estrogens, androgens and prolactin) have been the first proposed candidates in the sex bias observed in autoimmunity due to their modulatory functions within the immune response, particularly acting on the development of immune cells. Sex hormones may also directly influence the homing of lymphocytes to a target organ and the process of antigen presentation, thus influencing the organ specificity of AID as well as the breakdown of tolerance. The effect of estrogens is different in normal conditions and in autoimmunity with a biphasic effect; lower levels facilitate the immune response while higher levels suppress it. These data indicate that estrogen is capable of modulating both pro- and anti-inflammatory activities of CD4+ T cells and thus has the potential to influence the outcome of CD4+ T cell-mediated immune responsiveness. Estrogens may thus be central to the regulation of the balance of Th1/Th2 cytokines within sites of inflammation and to the appropriate or inappropriate termination of the inflammatory response in infections, tolerance development or autoimmunity. Several authors have attempted to study sex hormone changes in women with PBC. These have included epidemiological studies in which a negative association with parity was first denied and ultimately confirmed[9]. Of interest, taking hormonal replacement therapies following menopause was found in this latter study to be significantly associated with PBC although this may be secondary to the proposed enhanced rate of bone loss in chronic cholestasis. Furthermore, the differences in plasma estrogen levels between women with PBC and controls observed in earlier studies may be secondary to long-standing cholestasis or may account for the wide variability of their measurements during the reproductive cycle and should be considered as non conclusive.

A second hypothesis on female predominance is the persistence of fetal genome parts in women. It has been hypothesized that the pathogenesis and female predominance of autoimmunity may be secondary to the presence in affected women of allogenic male fetal cells se veral years after pregnancy (i.e. fetal microchimerism). Microchimeric cells were first found in peripheral blood mononuclear cells from patients with scleroderma and it was suggested that non-autologous cells may be mediating a graft-versus host disease-like reaction in these patients but other studies have failed to recapitulate these findings. Several studies found no significant difference in frequency of male microchimerism in female PBC and controls[10]. We are convinced that available data on the role of fetal microchimerism in autoimmunity in general are still controversial while these should be considered as negative in PBC.

A new fascinating hypothesis on the female predominance of autoimmunity is based on major defects of sex chromosomes[11] and supported by data in other fields[12-15]. X chromosome inheritance displays a peculiar pattern compared to autosomal chromosomes since women are functional mosaics for X-linked genes. In females, most genes on one X chromosome are silenced as a result of X-chromosome inactivation (XCI). The result of XCI is to achieve equivalent levels of X-linked gene products between males and females. More recent data have undermined this dogmatic view by de monstrating that at least 15% of X-linked genes are capa

ble of escaping XCI in healthy women and are thus expressed from both X chromosomes. Up to 10% of total X-linked genes manifest variable XCI patterns in different individuals[16]. We recently proposed a role for X chromosome based on experimental evidence that women with autoimmune diseases have a significantly higher frequency of peripheral blood cells with a single X chromosome (i.e. X monosomy) compared to healthy women (Lancet). Importantly, this was observed in diseases with different organ specificities including PBC[17], scleroderma and autoimmune thyroid disease[18]. X chromosome loss is indeed preferential and involves more frequently a parentally inherited one[19], suggesting a possible critical involvement of X chromosome gene products defects in female preponderance of PBC and other autoimmune diseases while new factors such as microRNAs[20] or epigenetics[21,22] should not be overlooked. Other authors have suggested that women affected with specific female-preponderant autoimmune diseases, i.e. scleroderma, manifest a skewed XCI pattern in their peripheral white blood cells[23]. In PBC, however, we failed to demonstrate such preferential inactivation[19].

Genomics

The genetic bases of PBC are not related to a single gene or Mendelian compatible with the complex etiology previously discussed. Variable rates of familial PBC are seen in different geographical areas, possibly due to different methods of case definition, but generally 1%-6% of PBC cases have at least one family member manifesting the disease while our most recent data obtained interviewing 1032 patients throughout the US indicate 6% of cases with a first-degree relative also affected[9]. Such familial prevalence rates are significantly higher than general population prevalence estimates, thus indicating a genetic predisposition to the disease. However, the difficulty in evaluating these data is that prevalence rates in the general population are still uncertain and control groups are not always included in the family studies. On the other hand, concordance rates in monozygotic twins for late-onset female-predominant autoimmune diseases range on average well below 50%. We first reported that concordance rates for PBC are 63% in 8 monozygotic sets and null in dizygotic twins[24]. The phenotypical discordance observed in some twin pairs could be caused by epigenetic factors, differences in exposure to environmental factors or mere serendipity.

Studies on polymorphisms associated with PBC are based on case-control designs[25] and these approaches are limited by poor control matching criteria and sample size or selection while very few proposed associations have been independently confirmed in other populations. Of interest, a recent multi-center study reported the first genome-wide association study and identified interleukin 12 and its relative receptor as susceptibility genes for PBC as well as STAT4 and HLA genotypes[26]. These data were significantly strengthened by our most recent independent study and meta-analysis[27].

A representation of smaller studies on candidate genes is to be subdivided into two separate groups of genes. Significant associations with specific MHC alleles have been reported in most autoimmune diseases[28-30], in some cases constituting a clinical marker[31]. The scenario in PBC is quite different with limited evidence provided thus far. We contributed in 2003 and 2008 to this issue and reported that PBC is significantly associated with various HLA-B alleles in a small proportion of the patients studied[32,33]. The association between PBC and HLA genes should be ultimately seen as weak, if any. The challenge to identify susceptibility gene(s) that predispose for the development of PBC is still open. The majority of such studies not only have been derived solely from case-control designs[25] but were also limited by poor control matching criteria and sample size or selection. A plethora of association studies have been conducted, mainly focused on immune genes that affect the immune system belonging to both the HLA family and non-HLA immune modulators genes, including CTLA-4, IL-1, IL-10 and vitamin D-receptor. The discussion of these data goes beyond the aims of this article and details have been reviewed in dedicated articles[25,34,35].

Environmental factors

An environmental insult (more likely not harmful in the general population) is believed to result in tolerance breakdown and PBC onset in the presence of a susceptible genetic background. Epidemiological data combined with experimental evidence on infectious agents and xenobiotics strongly support this view.

Our 2005 epidemiological study on 1032 patients with PBC and 1041 rigorously matched controls[9] demonstrated that a high risk of developing PBC is associated with a positive family history for PBC, a history of urinary or vaginal infections, co-morbidity with other autoimmune diseases, lifestyle factors such as smoking and previous pregnancies. We also observed that the frequent use of nail polish also slightly increased the risk of having PBC. In most cases, these factors had been previously suggested in smaller studies. Based on these observations, two main classes of environmental factors have been suggested in PBC and include infectious (bacteria, viruses) and chemical (xenobiotics) factors. The ability of infectious agents, particularly bacteria, to induce autoimmune responses has been supported by animal models and molecular mimicry in several autoimmune diseases and remains the most widely studied mechanism. The molecular mimicry hypothesis states that microbes contain peptides sharing different degrees of similarity with self-proteins, thus leading to a promiscuous immune response (antibody- and cell-mediated) in turn capable to recognize both microbial and self-epitopes. This cross-reactivity is not particularly surprising given the conserved sequence of mitochondrial enzymes across all species, from eubacteria to mammals[36]. Mitochondria originated following uptake of bacteria into the precursors of eukaryotic cells and maintenance as intracellular

symbionts and this makes it difficult to determine a causal role for microbial proteins in pathogenesis given their phylogenetic relationship to the human autoantigen. One line of argument that we have taken is that the breaking of tolerance and induction of autoimmunity would be more likely to occur when the microbial protein is extremely similar in sequence while it would not be necessary for tolerance breakdown to take place in the disease target organ[37]. In this scenario, T-cell activation produces cross-reacting T-cells leading to self-tissue destruction and this ultimately perpetuates the autoimmune injury, possibly through the degeneracy of the T-cell receptor and cross-priming. Of the bacterial strains suggested to lead to PBC through molecular mimicry[38], most evidence has been gathered for *Escherichia coli*(*E. coli*), primarily based on the reports of the increased incidence of recurrent urinary tract infections in patients with PBC[9]. Conflicting evidence has been obtained on the role of *Chlamydia pneumoniae* in the pathogenesis of PBC but original data were not independently recapitulated. Finally, our group has provided serological and molecular data suggesting that a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, *Novosphingobium aromaticivorans*(*N. aromaticivorans*), is the best candidate yet for the induction of PBC as it elicits a specific antibody-reaction (up to 1 000-fold higher than against *E. coli*) and its 16S rRNA specific sequences can detected in 25% of human fecal samples[39]. Most recently, Mattner *et al*[40] were able to induce serum autoantibodies and PBC-like liver lesions following immunization with *N. aromaticivorans*. Whether the bacterial impact should be regarded as based solely on cross-reactivity or on the presentation of mimicry antigens remains to be largely investigated. Similarly, we cannot but hypothesize a connection between the bacterial infection and the xenobiotic theory at the present status of knowledge.

Xenobiotics can be defined as foreign compounds that are believed to alter or complex to defined self or non-self proteins and induce a change in the molecular structure of the native protein sufficient to induce an immune response[41]. Similar to molecular mimicry, therefore, such immune response may lead to the cross-recognition of the self form and ultimately chronic autoimmunity. The main detoxifying organ is the liver, thus potentially exposing hepatocytes and biliary epithelial cells to chemical byproducts. Earlier serological data obtained in our laboratory by Long *et al*[42] in 2001 demonstrated that specific halogenated organic compounds attached to the major mitochondrial epitope backbone were recognized by sera from PBC patients with a higher affinity than the native forms. These results were of critical importance supporting for the first time an organic compound serving as mimic for an autoantigen. Afterwards, the same halogenated compound was found capable to induce autoantibody production in animal models, albeit no liver lesions were observed in the short follow-up[43]. More recently, Leung *et al* reported the induction of PBC-like liver lesions following longer follow-ups in guinea pigs exposed to a specific halogenated compound which was also capable to induce liver lesion in a specific strain of non obese diabetic (NOD) mouse[44,45]. Finally, the use of a multiplex approach led to determine that 2-nonynoic acid is recognized by PBC sera with high affinity[46]. This is particularly interesting since this compound does not occur naturally and is found in several cosmetic products, including nail polish[9]. One should note that data on molecular mimicry in PBC were mainly obtained from the study of autoantibodies in sera from patient or animal models while the study of cellular autoimmunity is limited.

PATHOGENESIS

The etiology and pathogenesis of PBC remain largely enigmatic. The disease should be regarded as multifactorial and we are convinced that the numerous lines of evidence provided by clinical and experimental research will ultimately provide an answer to the several questions remaining on the table. Prior to illustrating the available evidence on the immune mechanisms involved in PBC pathogenesis, it should be clear that the etiology of the bile duct injury and in particular the strict organ-specificity of the immune-mediated tissue damage remains to be elucidated and current hypotheses will be discussed. The lines of evidence should not be regarded as mutually exclusive from any causative factor such as susceptibility genes but rather as terminal mechanisms of the pathway leading to the clinical manifestations[47] ultimately orchestrated by a specific cytokine pattern[48-50].

First, the pathogenetic role of AMA is not yet clear, as discussed in further details below. AMA belong mainly to the IgG isotype, in particular IgG3 subclasses, and thus are potentially pathogenic through different mechanisms, e.g. complement activation, antibody-dependent cytotoxicity. There are no direct experimental evidence, however, supporting the involvement of these mechanisms in the pathogenesis of PBC as serum AMA are elicited in animal models following several types of immunization; yet PBC-like liver damage is caused only in selected cases[51]. More importantly, AMA can be also of IgA isotype. The role of such AMA-IgA has been ignored for long time but may be critical in the pathogenesis of PBC. It is now well established that AMA-IgA, indeed, can be detected not only in sera but also in bile, saliva and urine of patients with PBC, in some cases correlating with disease severity[52]. Moreover, IgA represents the principal Ig isotype in epithelial surfaces, including biliary epithelia. AMA-IgA has been reported to co-localize with the major AMA autoantigen (i.e. the E2 subunit of pyruvate dehydrogenase, PDC-E2) both inside the cell cytoplasm as well as the apical membrane of cholangiocytes in PBC but not in controls. Thus, AMA-IgA and in particular AMA-IgA bound to mitochondrial antigen could be able to disrupt cell metabolism and may also induce cellular dysfunction and damage thus leading to a tissue specific injury. We cannot preclude the possibility that the apical staining obtained with anti-PDC-E2 monoclonal

antibodies may be secondary to the presence of immune complexes formed by secreted IgA and AMA antigens, as some line of evidence seems to suggest[53].

Over the past decade one hypothesis for the selective destruction of biliary epithelial cells was proposed implying that the immunodominant autoantigen PDC-E2 should be aberrantly exposed on cholangiocytes cell surface where it may be recognized by AMA and/or antigen-specific T cells[52-54]. Studies based on in situ hybridization of PDC-E2 mRNA failed to demonstrate significant differences in its amount in PBC liver compared with other liver diseases. PDC-E2 may be selectively over-expressed in small bile duct cholangiocytes as indirectly suggested by early experimental evidence showing a positive staining of a murine anti-PDC-E2 monoclonal antibody selectively on the surface of biliary epithelial cells in the liver of patients with PBC but not in normal controls. On the other hand, co- or post-translational modifications of PDC-E2 may cause its abnormal turnover leading to its accumulation. Chemicals (i.e. xenobiotics) disposed by the liver may have a role in this scenario by accumulating in the biliary epithelial cells and modifying PDC-E2 locally. Solid data to support these fascinating mechanisms are lacking or weak and we cannot rule out that the molecules expressed and identified on the ductular surface and recognized by AMA may not be PDC-E2 itself but possibly unrelated PDC-E2 mimics cross-reacting with human PDC-E2.

Does apoptosis hold the key to organ specificity?

Apoptosis of biliary epithelial cells in PBC warrants further discussion and may prove to be crucial for immune tolerance breakdown[55,56] as illustrated in other experimental settings[57]. It was first reported that PDC-E2 remains intact and retains its immunogenicity during cholangiocyte apoptosis due to a cell-specific lack of glutathionylation of biliary epithelial cells[58]. The intact PDC-E2 in apoptotic fragments could be taken up by local antigen presenting cells and transferred to regional lymph nodes for priming of cognate T cells thus initiating PBC. This is indeed an attractive possibility, however, solid data of such antigen presentation are awaited and it cannot be excluded that the reported mechanisms are not PBC specific. A major contribution came from Lleo *et al* who most recently demonstrated that PDC-E2 is found in the blebs of human intrahepatic bile duct cells undergoing apoptosis[56] and that they could be presented to local dendritic cells to initiate the immune response[59]. More importantly, this phenomenon was not observed in other epithelial cell lines and appears to confirm the importance of apoptosis in the perpetuation of the autoimmune injury[55] as well as the view that PBC bile duct cells are not unique[60].

Is PBC an autoimmune disease?

Whether PBC is indeed an autoimmune disease remains to be clearly determined, based on somehow conflicting clinical and experimental data. An autoimmune pathogenesis for PBC is accepted based on clinical and experimental findings that make this condition somehow a model and a paradox for autoimmunity. The former is represented by the PBC features that are common to the autoimmune spectrum such as the female predominance, the genetic predisposition or the presence of specific autoantibodies in the vast majority of cases as well as the frequent comorbidities[61]. Serum autoantibodies, however, in the case of PBC also represent the basis for the disease being a paradox as their direct pathogenetic role is still poorly defined[62]. In fact, serum autoantibodies are detected in approximately 90% of patients; yet seronegative cases manifest a similar disease progression[63]. Furthermore, in the autoimmunity paradigm, the passive transfer of autoantibodies should reproduce the clinical features and experimental immunization with the antigen should produce a model disease. This has been reproduced only in part for PBC, as illustrated in details below. In autoimmune diseases, the reduction of autoantibody titers will correlate with disease amelioration; this criterion is also poorly met in PBC where there is no correlation between the pattern or titer of AMA and progression or severity of the disease. Finally, it is well-established that most autoimmune diseases are responsive to immunosuppressive therapy while no such agent has proven effective for PBC. We will now discuss the major characteristics of PBC in terms of adaptive (humoral and T cell) and innate immunity as well as the emerging role of T regulatory cells. Ultimately, the putatively comprehensive animal models will be discussed.

Autoantibodies

Serum AMA are highly specific for PBC and detected in nearly 100% of patients when tested using techniques based on recombinant mitochondrial antigens (immunoblotting or ELISA) which allow higher sensitivity and specificity. In most clinical settings, however, indirect immunofluorescence remains the test used for initial screening of cases and might lead to falsely positive or negative results. AMA autoantigens have been known since 1987[64]. Antibodies react with lipoilated domains within components of the 2-oxoacid dehydrogenase (2-OADC) family of enzymes within the mitochondrial respiratory chain, most frequently the E2 and E3 binding protein (E3BP) components of the pyruvate dehydrogenase complex (PDC-E2) and the E2 components of the 2-oxo glutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes. The immunodominant epitopes in all major antigens contain the motif DKA, with lipoic acid attached to lysine (K). The necessary/sufficient role of lipoic acid in the epitope recognition by AMA is unclear based on serum reactivity studies, as briefly mentioned above. Serum antinuclear antibodies (ANA) have been detected in as many as 30% of patients with PBC and indirect immunofluorescence more specifically produce a 'nuclear rim' or 'multiple nuclear dots' pattern, based on the recognition by the autoantibodies of gp210 and nucleoporin 62 (within

Table 1 Hints towards an innate immunity involvement in primary biliary cirrhosis

	Peculiarity
Monocytes	Increase in absolute number
	Increase in pro-inflammatory cytokines upon infectious challenge
Elevated IgM	B cell response to bacterial stimuli
NKT cells	Increased NKT cells in PBC peripheral blood and liver
	Increased NKT cytotoxic activity
Liver histology	Focal duct obliteration with granuloma formation

PBC: primary biliary cirrhosis models.

the nuclear pore complex) and Sp100 and PML (possibly also cross-reacting with small ubiquitin-like modifiers, SUMO) respectively[65-67]. Rim-like ANA, in fact, react against proteins of the nuclear pore complexes (NPC), supramolecular structures that include gp210 (a 210-kDa transmembrane glycoprotein involved in the attachment of NPC constituents within the nuclear membrane), p62 (a nuclear pore glycoprotein), and the inner nuclear membrane protein lamin B receptor (LBR). Serum anti-gp210 ANA are detected in about 25% (10%-40%) of AMA-positive and up to 50% of AMA negative patients (in both cases with high specificity). Autoantibodies reacting with p62 or LBR are found in about 13% and 1% of patients with PBC respectively. Interestingly, the presence of anti-gp210 and anti-p62 ANA in the same serum is rare. It was first supposed that ANA-positive patients are more frequently AMA-negative, possibly because of the lack of a masking effect of these latter antibodies in such sera, yet this remains to be determined. Interestingly, these PBC-specific ANA have been consistently found associated with more severe[68] and rapidly progressing disease[65,66]. Similar to AMA, the pathogenic role of ANA in PBC remains enigmatic although cross-sectional and longitudinal data demonstrate an association between ANA positivity and a poorer prognosis.

T cell autoimmunity

T-helper (CD4+) TCR+ and CD8+ T cells are most commonly seen around injured bile ducts in PBC. Autoreactive T cells have been well characterized in PBC from both the liver and in peripheral blood of affected individuals. Autoreactive cytotoxic T lymphocytes (CTL) have been well characterized in PBC and currently considered major effectors in the tissue injury encountered in PBC. The MHC class I restricted epitope for CTLs, namely amino acid 159-167, also maps in close vicinity to the epitopes recognized by CD4+ cells and by AMA. Moreover, the use of tetramer technology has shown a 10-fold higher prevalence of PDC-E2159-167 specific CTL in the liver as compared to peripheral blood of patients with PBC. PDC-E2 specific autoreactive CD4+ T cell (T-helper) clones were first isolated by in vitro stimulation of intrahepatic or peripheral lymphocytes to PDC-E2. It is of note that the autoepitope for T cells overlaps with the B cell (AMA) counterpart and includes the lipoilated amino acid of the inner lipoilated domain. Similar to CTL, there is also a specific 100-150 fold increase in the number of autoreactive CD4+ T cells in the PBC hilar lymph nodes and liver when compared with peripheral blood, regardless of the AMA status[69].

Innate immunity

Following decades in which adaptive immunity was considered self-sufficient to explain autoimmunity, the study of innate immunity has received a significant impetus over the past few years and is no longer overlooked by clinical immunologists. This interest has been growing[70] since evidence has been provided that the cellular components of the innate immune system such as monocytes, dendritic cells (DC), natural killer (NK) and NK T cells modulate the function of both the humoral and cellular adaptive immune responses. Immunological features such as elevated levels of polyclonal IgM and hyper-responsiveness to CpG, increased levels of NK cells and cytokine responses in patients with PBC compared to controls ultimately suggest a determinant role of innate immunity in the onset and perpetuation of PBC (Table 1). The liver express effective immune responses against a wide range of pathogens from viruses to multicellular parasites. In that sense, the environment of an inflammatory milieu seems to be critical for effective immunogenic signals to be delivered to intrahepatic T cells. The tolerogenic potential of intrahepatic DC and sinusoidal endothelial cells is, on the other hand, based on their hyporesponsiveness to lipopolysaccharide as a "pathogen associated molecular pattern" (PAMP) by reason of an altered expression of toll-like receptors (TLR), with deprivation of crucial upregulating signals for antigen presentation and co-stimulatory activity. Some of the innate immunity cells are known for their regulatory function in detailing the quality and quantity of subsequent adaptive immune responses including antigen-specific antibody and T cell responses. Innate immunity is involved in several aspects of autoimmunity[71,72] and in PBC these may include the presence of epithelioid granulomas. Recent data have supported a role for memory B cells, monocytes and NKT cells. It is common to observe elevated levels of IgM in PBC sera, independent of the AMA or ANA status, while their reduction during medical treatment[73] support a direct link. It has been reported that hyper-IgM is secondary to a chronic polyclonal innate immune response of memory B cells to bacterial stimuli represented by unmethylated CpG motifs that share immunostimulatory effects on human cells[74]. Following stimulation with synthetic oligodeoxynucleotides containing such motifs, cultured peripheral blood mononuclear cells from patients with PBC also secreted higher amounts of IgM compared to controls and this is reduced by ursodeoxycholic acid[75]. Alternatively, one may hypothesize that the hyper-IgM in PBC patients is the result of a failed attempt at preservation of the state of tolerance. This could follow the exposure to chemical-metabolizing bacteria as indicated in recent reports of the presence of IgG antibodies against xenobiotic modified PDC-E2 and of a

Table 2 Features of spontaneous and induced murine primary biliary cirrhosis models resembling data in the human condition

	Mouse model	Adaptive immunity	Innate immunity
Spontaneous	Ae2(a.b)-deficient	AMA Lymphocytic CD8+ infiltrates Decreased T regulatory cells PBC-like liver lesions	--
	dnTGFbRII	AMA Deficient T reg function	NKT cells worsen liver injury
	$IL2Ra^{-/-}$	AMA Portal tract CD4+ and CD8+ cells	--
	NOD.c3c4	Lymphocytic infiltrate AMA, ANA	--
Induced	*N. aromaticivorans* on NOD 1101	AMA PBC-like liver lesions Disease transfer by T cells	NKT cells are required
	Xenobiotic on C57BL/6	Lymphocytic CD8+ infiltrate AMA PBC-like liver lesions	--

AMA: antimitochondrial autoantibodies; ANA: antinuclear antibodies; NOD: non obese diabetic; PBC: primary biliary cirrhosis models.

possible association of *N. aromaticivorans*. The monocyte activation by PAMP through TLR induces the release of pro-inflammatory cytokines by monocytes, characteristic of the innate immune response, including IL-1, IL-6, IL-18, IL-12 and TNF-α which mediate the amplification of T-cell mediated immune response against pathogens. Peripheral monocytes from patients with PBC and controls challenged with different ligands for TLR2, TLR3, TLR4, TLR5 and TLR9 produce a significantly increased level of all pro-inflammatory cytokines compared to healthy controls[76]. From the innate immunity perspective, these findings suggest that peripheral blood monocytes from patients with PBC are more sensitive to infectious stimuli resulting in the secretion of pro-inflammatory cytokines. The mechanisms for such increased sensitivity are currently unknown but might reflect or be secondary to the higher frequency of recurrent Gram-negative bacterial infections (e.g. urinary tract infections) in PBC. This effect is seen as a consequence of the constant exposure of monocytes and B cells to bacterially derived products from the portal blood but with inflammation compensating for such hyporesponsiveness and inducing efficient intrahepatic T-cell priming and subsequent effective cellular immune responses.

The role of NKT cells in autoimmunity is also attracting growing attention. The presence of NKT cells restricted for the α-galactosylceramide (α GalCer) in a CD1 context has been evaluated and reported a higher prevalence of these cells in the affected tissue than in peripheral blood[77]. This behavior resembled that of NKT cells in other liver diseases and healthy controls while Chuang *et al* recently demonstrated a marked increase in the frequency and absolute number of blood and liver NKT cells in PBC patients. We are well aware that the innate immune system hyper-responsiveness is likely not sufficient for the breakdown of tolerance; we can hypothesize that these alterations might play a role in the initiation and/or perpetuation of the autoimmune injury. This is particularly intriguing considering the study by Mattner *et al* demonstrating that *N. aromaticivorans* is capable of inducing autoreactive AMA and chronic T cell-mediated autoimmunity against small bile ducts in a murine model of PBC in an NKT-dependent fashion[40].

T regulatory cells

T regulatory cells (Tregs) are $CD4^+$ $CD25^{high}$ cells and play a role in the prevention of autoimmune disease as demonstrated in several clinical settings[78]. As an example, in chronic autoimmune hepatitis therapeutic attempts are ongoing in animal models based on cell expansion[79]. In fact, some studies have demonstrated that the transfer of T cells lacking the $CD4^+$ $CD25^{high}$ Tregs subset into athymic nude mice results in the development of various T cell-mediated autoimmune diseases[80]. Experimental data demonstrate that PBC patients displayed significantly lower frequencies of $CD4^+$ $CD25^{high}$ Tregs as percentages of total TCR-$\alpha\beta^+$/$CD4^+$ T cells which may contribute to the breakdown in tolerance in PBC[81], possibly through interleukin 2[82]. The recently defined field of CD8+ FoxP3+ regulatory cells has not been investigated in PBC.

ANIMAL MODELS

The development of an animal model is of obvious importance in elucidating the mechanism(s) responsible for the initiation and progression of PBC and to investigate new medical treatments. Several models, mostly murine[83], have been proposed for PBC and are illustrated in Table 2. These include both spontaneous and induced models and have subsequently been utilized to prove specific mechanistic hypotheses in PBC pathogenesis and data have demonstrated that CD8+ T cells are required to transfer disease[84] while B cell depletion has unexpected consequences on biliary disease[85].

Two animal models, i.e. dnTGFβRⅡ and IL-2Rα-knockout mouse, point out the possible crucial role of Tregs deficiency in the loss of immune tolerance with consequent development of autoimmune response against PDC-E2 in PBC. In particular, a mouse with dominant negative form of transforming grown factor β (TGFβ) receptor Ⅱ, (dnTGFβRⅡ) showed PBC-like liver disease, e.g. 100% AMA positivity against PDC-E2[86]. TGFβ receptor Ⅱ is essential for signal transduction of TGFβ that is a key regulator of lymphocytes activation[85]. A mouse deficient for IL2 receptor IL-2R which is highly expressed on Tregs developed 100% AMA positivity

against PDC-E2, 80% ANA positivity and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury[87]. As previously mentioned, animal models support our hypothesis that xenobiotics can induce tolerance breakdown. Firstly, loss of tolerance has been demonstrated in rabbits immunized with 6-bromohexonate, a xenobiotically modified hapten mimicking lipoic acid, coupled with bovine serum albumin[43]. The immunized rabbits were able to produce not only antibodies against the xenobiotic but also high titer of anti-PDC-E2 antibodies. Anti-PDC-E2 antibodies in this model were not, however, sufficient to induce specific hepatic lesions, at least in the short follow-up[43]. More recently, induction of PBC-like lesions was obtained in a NOD background by Wakabayashi *et al* and in guinea pigs by Leung *et al* exposed to xenobiotic immunization[88,89]. Furthermore, an animal model is derived from a variant of the NOD mouse model (NOD.c3c4). It has been described that NOD.c3c4 manifests autoimmune cholestasis and PBC-specific serology, showing AMA positivity of 50%-60% and ANA positivity of 80%-90%. Histologically, it presents lymphocyte infiltration around portal tracts with chronic nonsuppurative destructive cholangitis and epithelioid granuloma formations; nevertheless, the morphological features of bile ducts differ somewhat from those in human PBC[90]. Finally, we submit that the PBC-like model induced by *N. aromaticivorans* immunization[40] awaits further recapitulation.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, we recapitulate our current working hypothesis[8]. Three major events in PBC are represented, i.e. bile duct cell apoptosis, female predominance and genetic susceptibility. A mimicking microorganism (possibly *N. aromaticivorans* as discussed below) enters the human system through the digestive mucosa and its PDC-E2-like proteins are modified within the liver by xenobiotics to form immunoreactive adducts. These modifications could be then sufficient to trigger the innate immune system to initiate a cascade of local inflammatory events resulting in local dendritic cell activation and antigen processing. Mucosal antigen-presenting cells in turn could activate autoreactive T and B cells that are directed to the liver through the portal system. T cells, therefore, could participate directly, not only to the autoimmune injury, but also to its amplification and perpetuation. B cells, on the other hand, could secrete AMA, particularly of the IgA type. AMA-IgA could be then transported to the vascular side of biliary epithelial cells where they could recognize PDC-E2-like molecules located on the luminal surface cell membrane. AMA-IgA/PDC-E2-like molecules engagement could initiate apoptotic signaling cascade. Ultimately, the immune complexes of post-apoptotic PDC-E2 and IgG-AMA and the direct cytopathic effects of autoreactive T cells (and possibly AMA) lead to the selective bile duct destruction.

Recent years have had a tremendous impact on our knowledge of PBC causes and consequences, as well represented by data on apoptosis or genomic associations. We are convinced that future studies should be dedicated to overcoming conceptual and logistical obstacles. Firstly, the role of xenobiotics and bacteria in the onset of PBC should be further studied by means of new mo lecular multiplex tools (including but not limited to proteomics) and available animal models. Secondly, only the collection of large series of patients and, quite crucially, representative families and the use of genome-wide analysis on larger numbers of polymorphisms will allow the determination of the genetic bases of PBC, similar to what was recently observed in other autoimmune diseases. Thirdly and most importantly, it is time to prove the AMA pathogenic role in PBC and the proposed animal models may be a good starting point to achieve this goal. Ultimately, we are convinced that newer discoveries in the field of molecular biology will provide exciting data in PBC pathogenesis, as in the case of new cytokine paradigms[49], microRNA[20], DNA methylation[91] or copy number variations[92], with putative therapeutic implications. Among these, the use of epigenetic drugs[22], antisense miRNA[93] as well as new biologics[94] may be of seminal importance, similar to what was recently observed for new compounds targeting nuclear receptors[95].

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