



## Review

## Animal models of cholestasis: An update on inflammatory cholangiopathies<sup>☆</sup>

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## ABSTRACT

Cholestasis is a frequent clinical condition initiating or complicating chronic liver diseases, particularly cholangiopathies, where the biliary epithelium is the primary target of the pathogenetic sequence. Until a few decades ago, understanding of cholestasis relied mostly on the experimental model of bile duct ligation in rodents. However, a simple model of biliary obstruction cannot reproduce the complex mechanisms and networks leading to cholestasis in cholangiopathies. These networks are underpinned by an intricate dysregulation of pro-inflammatory and pro-fibrotic signals involving besides cholangiocytes, multiple cell elements of both innate and adaptive immunity. Therefore, in the last years, a wide range of animal models of biliary injury have been developed, mostly in mice, following three main approaches, chemical induction, immunization and genetic manipulation. In this review, we will give an update of the animal models of the two main cholangiopathies, primary sclerosing cholangitis and primary biliary cholangitis, which have provided us with the most relevant insights into the pathogenesis of these still controversial diseases.

### 1. Introduction

The term cholestasis defines a liver dysfunction characterized by an impairment of the bile flow and/or secretion, with subsequent modification of the bile composition, which may initiate or worsen a liver disease process. The bile accumulation in the hepatobiliary system together with its altered composition exerts a toxic effect on both hepatocytes and cholangiocytes, leading to cell injury and inflammation, with progressive evolution to fibrosis, cirrhosis and eventually liver failure and malignant transformation, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) [1,2]. Clinically, a wide range of liver disorders express a cholestatic phenotype, encompassing molecular defects of both hepatocytes and cholangiocytes caused by single genetic mutations or drug-induced injury, structural changes of the biliary tree due to congenital malformations or acquired obstructive processes, and autoreactive damage mainly targeting the biliary epithelium.

The pathomechanisms promoting the cholestatic damage are still enigmatic, and consequently, pharmacological treatments against cholestasis have been limited to ursodeoxycholic acid (UDCA), a hydrophilic bile acid which is the only FDA-approved drug for the treatment

of human cholestatic liver diseases [3,4]. UDCA efficacy in cholestasis is widely supported by convincing beneficial effects on both biochemical and clinical parameters. Originally, the rationale behind the use of UDCA laid in its ability as hydrophilic molecule to stimulate the hepatocellular secretion of bile acids and organic anions coupled with the generation of a bicarbonate-rich choleresis, whose protective effects on the biliary epithelium hamper the perpetuation of liver damage [5]. It is now recognized that UDCA effects are mediated by additional, more complex mechanisms of action, going well beyond the simple hydrophilicity of the molecule. Several lines of evidence indicate that bile acids are potent signaling molecules which affect the metabolism and secretory activity of cells at the transcriptional and post-transcriptional level after entry via specific bile acid transport proteins (e.g. hepatocytes, cholangiocytes, ileocytes) or via membrane receptors like TGR5, the first identified G-coupled protein receptor specific for bile acids expressed by colon epithelia, adipocytes, Kupffer cells, among other cell types [6].

Since the early '90s a main goal of the hepatology research has been the development of different animal models to unveil novel pathogenetic concepts and, consequently, to test in vivo efficacy of new treatment strategies. Among them, bile duct ligation (BDL) has been the first

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and most extensively used experimental model of cholestasis [7,8]. First developed in 1932 by Cameron and Oakley [9], BDL is a surgically created animal model, performed in rodents, consisting of ligation/excision of the common bile duct. BDL was initially described in rats, but since then it has been more suitably adapted to mice. BDL has many advantages, such as the simple technical procedure, which can be easily reproduced, the low cost, and the rapid liver damage development, which makes experimental protocols shorter and thus, more convenient. In BDL, liver phenotype is characterized by well-established features of cholestatic damage, such as cholangiocyte proliferation resulting in exuberant ductular reaction, portal inflammation and brisk establishment of biliary fibrosis [8,10,11], which however contrasts with the slow progression of cholestatic liver diseases, in particular if cholangiocyte is the primary site of injury, as occurring in cholangiopathies [12]. Rather, liver lesions featuring BDL are more consistent with an acute obstruction, a condition quite uncommon in human pathology, typically observed in choledocholithiasis, or in other causes of extrahepatic cholestasis [11,13–15]. Moreover, these models fail to recapitulate the much more complex mechanisms underlying chronic cholestasis in primary cholangiopathies, such as primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC), which evolve to ductopenia and biliary fibrosis usually over decades, in absence of overt biliary obstruction. To meet these needs, in the last few years, there has been a tremendous burst of studies aimed at developing novel animal models of cholestasis, able to capture more faithfully the fundamental steps unleashed by a selective cholangiocyte injury. The objective of the present review is to give an outline of the more recent animal models of PBC and PSC, highlighting the novel pathophysiology concepts we can draw from them. The animal models of genetic cholangiopathies, a subject examined by a recent review, which the reader can refer to [16], will not be discussed here. Before going through the different animal models, we will briefly describe the mechanisms of bile formation by emphasizing the role played by cholangiocytes, since perturbations in the biliary electrolyte transport often contributes to the pathogenesis of cholestasis in primary cholangiopathies.

## 2. Mechanisms of bile formation

The bile consists of 95% of water in which several exogenous compounds, including drugs, xenobiotics and environmental toxins, are dissolved to be discharged together with endogenous components, like bile acids, conjugated bilirubin, lipids (mainly phosphatidylcholine and cholesterol), amino acids, steroids, enzymes, vitamins, heavy metals, glutathione (GSH), and ions, like  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{HCO}_3^-$  [17].

The final bile composition is the result of several transformation steps that originate in the hepatocytes and then continue in the bile ducts, where secretory and absorptive functions of cholangiocytes modify the primary ‘canalicular’ bile. Hepatocytes are polarized epithelial cells endowed with a rich apical excretory domain equipped with microvilli that delivers biliary constituents and bile acids into the bile via active processes mediated by specialized transport proteins belonging to the ATP-binding cassette (ABC) super family. The bile salt export pump (BSEP, or ABCB11) is the main route of bile acid efflux from the hepatocytes into bile acting in a uni-directional, ATP-dependent manner. The multidrug resistance-associated protein 2 (MRP2, ABCC2) is the major ABC conjugate export pump in the canalicular membrane. MRP2 functions by transporting a large number of different amphipathic, usually multivalent organic anion conjugates, such as bilirubin diglucuronides, GSH disulfide (GSSG) and conjugates, leukotrienes, heavy metals, bile salts, as well as a variety of different drug conjugates from the hepatocyte into bile. Another ABC super family transporter named multidrug resistance protein 3 (MDR3, ABCB4), mediates the biliary translocation of phosphatidylcholine, a phospholipid essential for the formation of lipid micelles in bile. This is critical to prevent the detergent effects of bile acids on biliary epithelial cell membranes [1,17,18]. On the basolateral membrane, the organic anion

transporting polypeptide type 4 (OATP4) or solute carrier organic anion transporter family 21, member 10 (SLC21A10) [19] regulates uptake and transport of bile salts and solutes in exchange for intracellular anions including GSH and  $\text{HCO}_3^-$ . The primary bile released by the hepatocytes from the canalicular pole is then modified by the intense secretory activities of cholangiocytes, that regulate its alkalinity, fluidity and volume [20], mostly through  $\text{HCO}_3^-$  and water secretion to the ductal lumen.

The net amount of water and  $\text{HCO}_3^-$  secretion is determined by the balance between pro-secretory effects, mediated by the secretin receptor activation, upon glucagon and vasoactive intestinal polypeptide (VIP) stimulation, and the anti-secretory actions promoted by somatostatin, insulin and endothelin-1 [21]. These opposite regulatory activities converge at the level of the adenylyl-cyclases (ACs), which are transmembrane enzymes, expressed in the basolateral membrane of cholangiocyte, able to regulate the intracellular levels of cyclic adenosine monophosphate (cAMP), by its conversion from ATP. Among the nine different types of AC reported so far, the intrahepatic biliary epithelium expresses the isoforms AC from 4 to 9 (AC4-9), with a heterogeneous pattern dependent on the specific cholangiocyte subpopulation [22]. In cholangiocytes lining the large bile ducts, for example, interaction between AC8 and AC9 with secretin leads to increased intracellular levels of cAMP, activation of the protein kinase A (PKA) and phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel. This sequence induces  $\text{Cl}^-$  secretion into the ductal lumen coupled with the  $\text{HCO}_3^-$  efflux operated by the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger, also known as anion exchanger (AE)-2, or SLC4A2. Moreover, cholangiocytes express other  $\text{Cl}^-$  channels involved in bile formation, such as: a) a  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel, a  $\text{Ca}^{2+}$  and cAMP-intensive high conductance anion channel; b) a basolateral barium-sensitive  $\text{K}^+$  conductance, modulating the  $\text{K}^+$  efflux critical for the maintenance of membrane hyperpolarization driving  $\text{Cl}^-$  exit [23]; c) a bumetanide-inhibitable  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  co-transport, cooperating to the basolateral  $\text{Cl}^-$  uptake [24]. All these ions are essential for creating the osmotic gradient, maintained by the ionic efflux gradient, that leads to the passive secretion of water through paracellular transport in concert with specific water channels, namely aquaporin-1 and 4 (AQ1-AQ4) [25]. On the other hand, thanks to additional transport proteins localized both at the apical and basolateral membrane, the biliary epithelium is involved in the reabsorption of some bile constituents, as glucose, GSH and bile salts, directing them to the cholehepatic circulation [21]. Specifically, cholangiocytes possess apical  $\text{Na}^+$ -dependent bile acid transporter (ASBT) to take up bile acids from the bile and the p-glycoprotein MRP3 to secrete them into the vessels nourishing the bile duct wall (peribiliary vascular plexus).

## 3. Primary sclerosing cholangitis (PSC)

PSC is a fibro-inflammatory cholangiopathy of unknown etiology, but likely sustained by immune-mediated mechanisms. Apart from liver transplantation in the advanced cases, there are no effective treatments in PSC, and therefore, it is recognized as ‘orphan’ disease. PSC is most common in young individuals, with a slight predominance in male, and an estimated prevalence in Western countries of about one per 10,000, which makes PSC a ‘rare’ disease [26,27]. Clinically, PSC evolution is unpredictable, and the most feared complication is CCA, which may occur anytime along the course of the disease [28]. In PSC, liver phenotype is characterized by inflammatory and fibrotic lesions that may affect bile ducts of any size, and generally extend to the large extrahepatic bile ducts, resulting in irregularities with strictures and dilations [28]. Histologically, an onion skin-type periductal fibrosis accompanied by an intense ductular reaction is the hallmark of the disease. However, PSC alterations are focally distributed showing variable degrees of fibroinflammatory changes in different areas of the liver, and thus, involvement can be limited only to scattered portal tracts in the initial stages of the disease. A clinical variant of PSC with

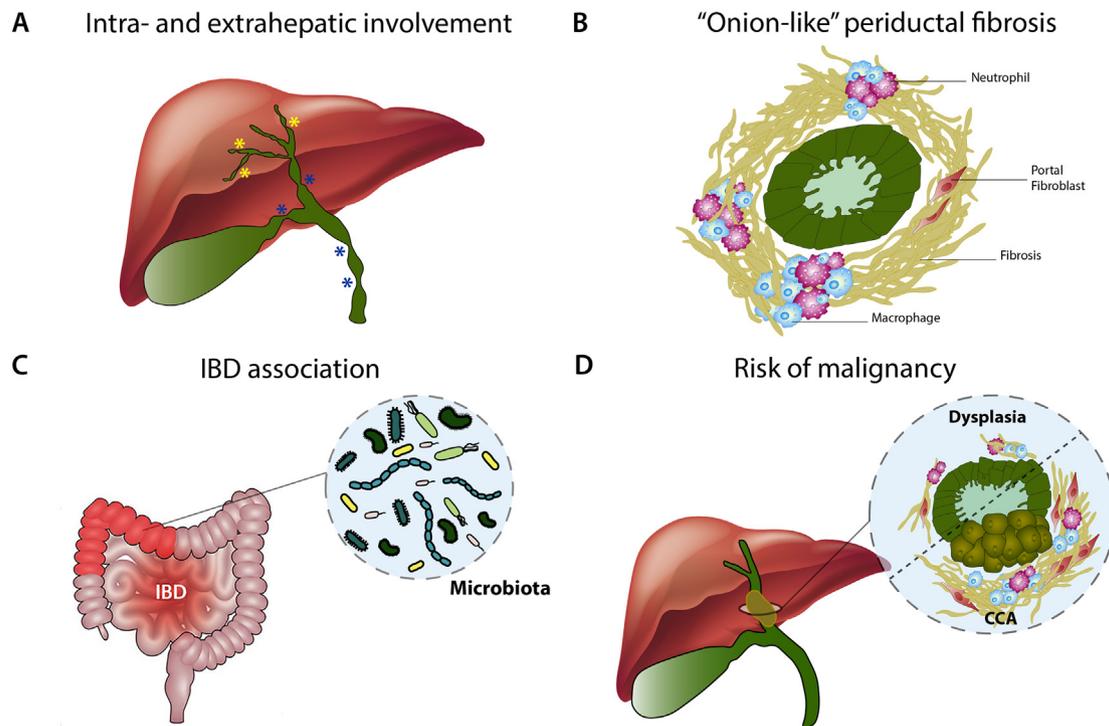
involvement restricted to the small intrahepatic bile ducts ('small-duct' PSC, accounting for 10% of total PSC), which may progress to large-duct PSC in 20% of cases and with a low risk of CCA, has been recently identified [26].

Furthermore, a distinctive clinical trait of PSC is its association with inflammatory bowel disease (IBD), mostly ulcerative colitis. To explain this association, a genome-wide analysis has indicated a linkage disequilibrium of the human leukocyte antigen (HLA) locus for IBD and PSC [29,30]. Furthermore, it has been suggested that the intestinal microbiome playing a central role in the etiology of IBD may also contribute to the pathogenesis of PSC [31–33]. Thus, failure of the intestinal barrier function may link IBD with PSC, in a way in which the 'leaky' gut allows bacterial products to reach the liver where they cause a chronic peribiliary inflammatory response [31,32]. In fact, the rich equipment of Toll-Like Receptor (TLR) constitutively expressed by cholangiocytes, in particular TLR4, the receptor for lipopolysaccharide (LPS), and TLR2, renders them particularly prone to endotoxin stimulation [34,35]. One of the major hypothesis further explaining the close PSC-IBD association postulates that memory T lymphocytes, once primed in the gut may reach the liver where they stimulate an immune-mediated response centered on the bile duct leading to pericholangitis accompanied by a progressive recruitment of collagen-producing myofibroblasts [33,36]. PSC association with IBD has indeed a strong clinical relevance. In fact, those cases with onion skin-like biliary lesions confined to the small intrahepatic bile ducts, but without accompanying IBD, should better not be called 'small duct' PSC. Ideally, immune-genetically predisposed experimental models of PSC would develop fibrous-obstructive cholangitis of both intra- and extrahepatic bile ducts in conditions of gut inflammation, with a marked propensity to develop CCA over time (Fig. 1). Given these prerequisites, unfortunately not fulfilled by a single animal model, herein we will describe the animal models of human PSC that, variably reproducing these fundamental traits, fall into three main groups, knockout mouse models, models involving enteric bacterial cell wall components or

colitis, and models of chemically induced cholangitis (Table 1).

### 3.1. *Mdr2* (*Abcb4*)<sup>-/-</sup> mice

In the *Mdr2* knockout (*Mdr2*<sup>-/-</sup>) mouse, cholangiopathy is caused by a primary defect of a translocase (flippase) expressed not by cholangiocytes, but by hepatocytes at the canalicular pole [37,38]. As previously mentioned *Mdr2* (MDR3 in humans) regulates the transport of phosphatidylcholine and other biliary phospholipids into the outer leaflet of the cell membrane, to be then delivered into the bile [38,39]. Lack of phospholipids in the bile determined by a defective *Mdr2* increases the biliary concentration of the non-micellar component of free bile acids, which are detergent on the cell membrane of cholangiocytes, leading to disruption of both tight junctions and basement membrane and therefore, to bile leakage inside the portal tract [37,39,40]. Bile leaked out of the ducts exerts potent harmful effects, triggering a sequence of events from peribiliary inflammation, to ductular reaction, and myofibroblast activation with intense and early deposition of fibrotic tissue around the inflamed ducts that in the long-term may play pro-tumorigenic functions. Although MDR3 defects are responsible for a range of specific cholestatic diseases different from PSC, including progressive familial intrahepatic cholestasis type 3 (PFIC-3), intrahepatic cholestasis of pregnancy (ICP), and adult biliary cirrhosis [41–43], the *Mdr2*<sup>-/-</sup> mouse has been often used as a surrogate model of sclerosing cholangitis. The main advantage of this model is its high reproducibility and easy handling, warranted by well-established, simple and extremely reliable readouts, making it particularly suitable for studies of efficacy for innovative anti-fibrotic therapies. Among them, *nor*ursodeoxycholic acid (*nor*UDCA), a shorter chain homologue of ursodeoxycholic acid (UDCA) with increased hydrophilicity, derived from the loss in a methyl group, was able to markedly revert cholangitis and biliary fibrosis in *Mdr2*<sup>-/-</sup> mouse [44]. Following passive absorption from bile by cholangiocytes, *nor*UDCA undergoes the 'cholehepatic shunting' to reach hepatocytes, by which it is resecreted in bile.



**Fig. 1.** Graphical representation of the main traits of an ideal PSC animal model.

Fibrosing cholangitis of intra and extrahepatic bile ducts (yellow and blue asterisk star, respectively) with lymphocytic infiltration and "onion skin" fibrosis around affected ducts (Panel A and B). Unique features of PSC are the close association with IBD, entailing alterations of the intestinal microbiota, and the propensity of the biliary epithelium to the sequence dysplasia-cancer (Panel C and D).

**Table 1**  
Animal models of PSC.

Models	Animal species	Major PSC features	Specific considerations	[Ref.]
<i>Mdr2</i> <sup>-/-</sup> ± DSS	FVB/N mice	<ul style="list-style-type: none"> <li>• Peribiliary inflammation, and periductal fibrosis</li> <li>• Concomitant IBD</li> </ul>	- Primary hepatocyte dysfunction	[37–40,59]
<i>fch/fch</i> + DDC	Male and female BALB/c mice	<ul style="list-style-type: none"> <li>• Biliary fibrosis with intense ductular reaction</li> </ul>	- Obstructive cholestasis due to intraductal plugs	[42,60,61]
TNBS ± BDL	Male Sprague–Dawley rats; female Lewis rats	<ul style="list-style-type: none"> <li>• Irregularities of the bile ducts, focal stricturing of the intra- and extrahepatic bile ducts</li> <li>• Portal mononuclear cell infiltrate encompassing macrophages and T-cells</li> <li>• Development of ANCA and ASMA reactivities</li> </ul>	- High mortality rate	[62–65]
LCA	Male Swiss albino mice	<ul style="list-style-type: none"> <li>• Partial bile duct obstruction, destructive cholangitis, and periductal fibrosis</li> </ul>	- No tolerable long-term protocol	[66–70]

This process stimulates a HCO<sub>3</sub><sup>-</sup>-rich hyperchloresis, which inactivates toxic bile acids, a protective mechanism regarded as ‘biliary HCO<sub>3</sub><sup>-</sup> umbrella’ [45,46]. *Mdr2*<sup>-/-</sup> mouse has been recently used to test the anti-fibrotic potential of vitamin D receptor (VDR) stimulation, starting from the observation that VDR influences several key processes related to cholestasis, including innate immunity activation, bile acid homeostasis, bile duct integrity and biliary fibrogenesis [47,48]. In *Mdr2*<sup>-/-</sup> mice vitamin D deficiency associated to an increased collagen deposition, and animals fed with a high vitamin D diet showed lower expression levels of profibrogenic genes and amelioration of liver injury [49]. *Mdr2*<sup>-/-</sup> mice have been also used to test druggability of the transforming growth factor (TGF)-β1 pathway. TGFβ1, the main profibrogenic cytokine in the liver, is secreted in a latent form, which is locally activated by integrin αvβ6, a membrane receptor up-regulated in ductal epithelia in response to injury and inflammation. Genetic ablation or pharmacological targeting of integrin αvβ6 by blocking antibodies has been pinpointed as an effective approach to prevent progression of biliary fibrosis in *Mdr2*<sup>-/-</sup> mice. The reduced peribiliary collagen deposition was dependent upon the attenuation of the ductular reaction due to the inactivation of the hepatic progenitor cell (HPC) compartment. In fact, both ductular reaction and HPC express integrin αvβ6 and rely on TGFβ1 activation for their function [50,51]. An alternative target therapy of fibrosis is lysyl oxidase-like2 (LOXL2), an enzyme member of a family inciting progression of fibrosis by mediating collagen crosslinking and stabilization. In *Mdr2*<sup>-/-</sup> mice, selective anti-LOXL2 antibodies inhibited pre-established and advanced biliary fibrosis in conjunction with a striking reduction in ductular reaction sustained by the activation of HPC, addressing a further role of LOXL2 in the regulation of biliary repair mechanisms [52]. In the current year, two studies have taken advantage of *Mdr2*<sup>-/-</sup> mice to test novel anti-fibrotic strategies, such as antagonists of H1/H2 histamine receptors (HRs) [53] and the bile acid sequestrant colestesvelam [54]. Inhibition of H1/H2HR reversed biliary fibrosis and decreased cholangiocyte proliferation, a mechanism that can be relevant to hinder the pro-tumorigenic functions in this model [53]. Colestesvelam feeding increased faecal bile acid excretion and enhanced bile acid conversion towards secondary, more hydrophilic, bile acids, which stimulate TGR5 signaling in enteroendocrine L-cells favoring their secretion of glucagon-like peptide 1 (GLP-1) in the portal system [54]. GLP-1 is known to stimulate cholangiocyte proliferation and to protect biliary epithelia against apoptosis [55]. This sequence led to an improvement in hepatic inflammation, fibrosis and ductular proliferation in the *Mdr2*<sup>-/-</sup> mice, highlighting the concept that interruption of the enterohepatic circulation with reduced output of potentially toxic bile acids may be protective against cholestatic liver injury [54]. These innovative treatment strategies evaluated in the *Mdr2*<sup>-/-</sup> mouse are summarized in Table 2.

However, a number of caveats limit suitability of the *Mdr2*<sup>-/-</sup> mouse to mirror some pivotal PSC features and they must be underlined. First is the proclivity of fibrotic liver damage to progress at the age of 4–6 months, to macroscopic tumor nodules, histologically compatible with HCC, rather than CCA, at odds with the natural history of

human PSC [56–58]. Second is the absence of concomitant IBD, an issue deserving careful consideration by future studies since currently there are no established PSC-IBD models combining cholestatic liver injury with gut inflammation. However, though *Mdr2*<sup>-/-</sup> mice do not display overt colonic inflammation at baseline, PCR analysis of whole colon tissue reveals elevated levels of pro-inflammatory cytokines (keratinocyte chemoattractant (KC)/C-X-C motif ligand (CXCL)1, tumor necrosis factor (TNF)α, interleukin (IL)-1β, and IL-6). Based on this assumption, Battista and coll. subjected *Mdr2*<sup>-/-</sup> mice to dextran sulfate sodium (DSS), a polysaccharide with potent colitogen activity and anticoagulant properties, as a novel model of PSC-IBD [59]. Signs of IBD are well exemplified by this experimental condition, and are characterized by animal weight loss, bloody diarrhea, decreased colon length, and evident histologic damage in the colon mucosa. Taken together, baseline elevations in colonic levels of pro-inflammatory cytokines in *Mdr2*<sup>-/-</sup> mice along with increased colitis susceptibility in the *Mdr2*<sup>-/-</sup>/DSS model suggests that interdependent signaling pathways originating from liver-gut cross-talk mechanisms are important in promoting intestinal injury.

### 3.2. Mice fed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)

This model is the prototype of progressive cholestatic liver injury induced by toxicants, relying on the ability of DDC to stimulate bile secretion of porphyrins, which in chronic feeding (4 weeks), precipitate leading to the formation of intraductal plugs in the small bile ducts. Liver phenotype captures several key features of human PSC, such as brisk ductular proliferation and intense pericholangitis associated with ‘onion skin-type’ periductal fibrosis evolving to porto-portal fibrosis. Interestingly, these biliary lesions are preceded by specific transporter abnormalities at the hepatocyte canalicular level, in particular down-regulation of Oatp4 and Mrp2, responsible for reduced biliary excretion of GSH and phospholipids [42]. Similarly, mice carrying a point mutation in the ferrochelatase gene (*fch/fch*) develop pronounced biliary fibrosis associated with intense ductular proliferation as the result of porphyrinogenic compound accumulation in bile [60]. DDC administration to these mice further enhances the biliary secretion of porphyrins with rapid formation of intraductal plugs, causing obstructive cholestasis in a shorter time. This process is histologically accompanied by accelerated peribiliary fibrosis led by activated portal fibroblasts after only 4 weeks. Interestingly, in contrast with *Mdr2*<sup>-/-</sup> mouse, bile flow and biliary excretion of cholesterol and phospholipids remain unchanged following DDC feeding. Furthermore, DDC feeding does not change bile acid composition, suggesting that toxic bile acids do not play directly a pathogenic role in this model [42]. Interestingly, there is a considerable variation among the different mouse strains regarding the development of liver phenotype in response to DDC feeding, in particular with respect to ductular proliferation and biliary fibrosis. A critical difference of the DDC-fed mouse from human PSC, is the lack of biliary strictures and dilations of the large extrahepatic bile ducts, as elegantly demonstrated by bile duct platination, despite the typical

**Table 2**  
Treatment strategies for biliary fibrosis tested in the *Mdr2*<sup>-/-</sup> mouse.

Compound	Therapeutic target	Mechanism of action	[Ref.]
<i>nor</i> UDCA	HCO <sub>3</sub> <sup>-</sup> secretion	Toxic bile acid inactivation	[44]
Vitamin D	VDR	Innate immunity activation, bile acid homeostasis, bile duct integrity and biliary fibrogenesis	[49]
Anti- $\alpha$ v $\beta$ 6 antibodies	Integrin $\alpha$ v $\beta$ 6	Reduced peribiliary collagen deposition, reduced ductular reaction and HPC activation	[50,51]
Anti-LOXL2 antibodies	LOXL2	Reduced collagen cross-linking and ductular reaction	[52]
Anti-H1/H2HR blockers	H1/H2 histamine receptors	Reduced cholangiocyte proliferation and collagen deposition	[53]
Colesevelam	Faecal bile acid sequestration	Enhanced bile acid conversion towards secondary bile acids, stimulating secretion of GLP-1 by enteroendocrine L-cells	[54]

‘onion skin-type’ periductal fibrosis affecting the small intrahepatic bile ducts: thus this condition should serve as a model of the ‘small-duct’ PSC [61].

### 3.3. Rats fed with 2,4,6-trinitrobenzene sulfonic acid (TNBS)

TNBS elicits cell-mediated immune responses acting as hapten with strong affinity for the lysine moieties of the membrane proteins of the intestinal epithelial cells [62]. Since the early 90th, TNBS has been recognized as a potent inducer of transmural inflammation in the gut, and thus used as model of Crohn's disease. Later on, Mourelle and coll. found that, in rats, a single intracholedochal injection of TNBS resulted in significant increased levels of serum cholestatic indexes (alkaline phosphatase and total bilirubin) as well as inflammatory cell infiltrates in the portal areas and around the bile ducts, indicating pericholangitis [63]. In some rats, ductal proliferation and thin porto-portal fibrotic septa were observed in association with dilation of the extrahepatic bile ducts. Orth and coll. studied the effects of TNBS intraductal injection in BDL rats, resulting in chronic, fibrosing cholangitis accompanied by a mild biliary stenosis in 8-week-old rats [64]. The TNBS-related models showed several features consistent with PSC, including irregularities of the bile ducts, diffuse focal structuring of the intra- and extrahepatic bile ducts, portal mononuclear cell infiltrate predominantly populated by macrophages and T-lymphocytes, cytokine production and development of anti-neutrophil cytoplasmic antibodies (ANCA) and smooth muscle autoantibodies (SMA) autoreactivity [65]. Unexpectedly, despite the well-recognized toxicity of TNBS for the intestinal epithelium, the rat model is biliary-specific and occurs in absence of IBD. Furthermore, a major limitation of the model is the high mortality rate caused by complications derived from the combined surgical/chemical approach.

### 3.4. Lithocholic acid (LCA) fed mice

As a general concept, mice are less sensitive to bile acid toxicity than humans are, since they tend to replace the bile acid pool with more hydrophilic bile acids, including muricholic acid and atypical bile acid species, which instead are not produced in humans [42,66,67]. Thus, to understand the potential hepatotoxicity of hydrophobic bile acids in the pathogenesis of cholestatic liver injury, several studies have adopted the strategy to feeding mice with the monohydroxy bile acid LCA, as prototype of toxic bile acids. The pronounced cholestatic effect exerted by LCA can be explained by a number of mechanisms, including alterations of the biochemical properties of the bile canalicular membrane, and the formation of crystalline plugs in bile canaliculi due to the scarce solubility of LCA [68,69]. Importantly, potentially toxic bile acids may affect not only hepatocyte but also cholangiocyte membrane integrity, and similarly to what described in the *Mdr2*<sup>-/-</sup> mouse, LCA fed mice develop bile duct injury and cholangitis even in the presence of normal phospholipid secretion in bile. Histologically, partial bile duct obstruction, bile infarcts, destructive cholangitis, and periductal fibrosis develop within a few days following LCA feeding [70]. However, since animals do not tolerate the diet in the long-term, this is not an appropriate model to study the chronic evolution of biliary injury.

### 3.5. Models involving enteric bacterial cell wall components or colitis

The close relationship between PSC and IBD that is central in the PSC pathogenesis has inspired hypothetical animal models to understand the role of gut bacteria or bacterial products in driving the chronic biliary damage. In fact, early theories on PSC pathogenesis proposed that portal bacteremia or bacterial products released from the inflamed gut in IBD promote inflammation all along the biliary tract. Originally, this concept was highlighted in a rat model of small intestinal bacterial overgrowth (SIBO). In the early 90's, Lichtman and coll. [71] showed that the surgical creation of a self-filling jejunal blind loop (SFBL) in the susceptible Lewis and Wistar rat strains caused a cholangitis involving both intra and extrahepatic bile ducts, which appeared thickened, fibrotic, and dilated. The disease was most likely caused by gut-derived bacterial products, primarily via activation of TLR pathways, consistent with the observation that cholangitis was significantly attenuated by antibiotics and peptidoglycan-degrading enzymes. In fact, selective gut microflora decontamination using a cocktail of non-absorbable broad spectrum antibiotics was shown to slow down the progression of the disease, in particular of liver fibrosis induced by BDL. Increased plasma levels of LPS were found also in experimental models of liver fibrosis induced by administration of carbon tetrachloride (CCl<sub>4</sub>), or thioacetamide (TAA) [72]. This has lent support to the notion that increased intestinal permeability and gut microflora-derived LPS contribute to the progression of liver fibrosis. Notably, in TLR4-mutant mice liver fibrosis was reduced even if LPS levels were similar to wild type mice [34], thereby suggesting that in the liver, TLR4 activation is mediated by gut-derived LPS, though this translocation is independent of the intestinal TLR4. The exact mechanism by which experimental liver injury may induce a leaky gut barrier remains elusive. Further mouse model studies performed in germfree animals or in animals with known composition of the intestinal microbiota provided new insights into the causative role of gut inflammation in cholangitis. In 2007, Garret and coll. elegantly described how transferring the microbiota from a mouse model of colitis into wild type littermates resulted in a phenotype similar to a genetically induced model of colitis [73]. More recent studies have used germ-free mice as approach for mono-colonization experiments and humanization of the gut microbiota. An important challenge in the context of biliary diseases is the different bile acid profile of mice from humans, which make hard the interpretation of findings in murine models. However, in biliary disease models, microbiota data are so far limited and some controversies exist. For example, in a recent study, Tabibian and coll. re-derived *Mdr2*<sup>-/-</sup> mouse by embryo transfer into a germ-free facility [74]. The new housing condition caused a dramatically worsened PSC phenotype, characterized morphologically by increased cholestasis, with exacerbation of ductular reaction, peribiliary fibrosis and ductopenia, and functionally by enhanced pro-inflammatory cytokine secretion and increased cholangiocyte senescence, which the authors interpreted as mechanistic determinant of the progression of biliary disease. Noteworthy, UDCA, a metabolite generated by commensal microbial activities, revoked senescence in vitro, indicating that in PSC intestinal microbiota and the secondary bile acids they produce might act as critical protective factors against biliary

injury [74].

#### 4. Primary biliary cholangitis (PBC)

PBC, whereby the term ‘cholangitis’ has recently replaced the former ‘cirrhosis’, is an inflammatory, slowly evolving cholangiopathy with features of autoimmunity, typically affecting females in the fifth/sixth decade, with a variable female:male ratio, up to 10:1, depending upon the geographical areas [75]. Incidence and prevalence of PBC vary largely worldwide, but both have increased significantly in the last 20 years, ranging from 0,33–5,8 per 100,000 inhabitants/year and 1,91–40,2 per 100,000 inhabitants, respectively, rates which are much higher than PSC [27]. The apparent increase in incidence and prevalence for PBC over time has been widely reported, and several risk factors may be implicated, including cosmetic products. PBC liver phenotype is characterized by a selective destruction of the small interlobular intrahepatic bile ducts associated with a rich lymphocytic infiltrate in the portal tract (more intense than in PSC), characteristically in a granuloma configuration (‘non-suppurative destructive cholangitis’) leading to portal fibrosis that may progress to biliary cirrhosis and eventually, to end-stage liver disease [75,76]. Unlike PSC, PBC progression is effectively halted by UDCA and malignant transformation is much less common, though some cases of HCC have been reported [77]. The diagnostic hallmark of PBC is the presence of anti-mitochondrial antibodies (AMA) directed against the lipoyl domain of the immunodominant E2 component of pyruvate dehydrogenase complex (PDC) in serum of more than 90% of patients. Noteworthy, AMA reactivity can develop years before the clinical appearance of the disease [78], suggesting it can be etiologically relevant. Consistent with the pathogenetic role of the adaptive immune system, the portal infiltrate predominantly encompasses helper (CD4<sup>+</sup>) T cells, with lesser increase in cytotoxic/effector (CD8<sup>+</sup>) T cells. Numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells reactive to mitochondrial auto-antigens are also increased in the hilar lymph nodes and in the peripheral blood of affected patients, while detected neither in healthy controls nor in patients with other liver diseases, supporting the high specificity of mitochondrial autoreactivity in PBC [79]. In the pathogenesis of PBC, the inappropriate activation of the immune response results from a complex interplay between genetic and environmental factors, addressed by recent genome-wide studies pointing towards a pivotal role of the IL-12 pathway [80].

Based on these unique phenotypic traits, a potential PBC model should include positive testing for AMA, histological evidence of interlobular bile duct injury, preferably with granuloma formation, and female predominance. The first attempts to develop a PBC animal model were performed by transferring peripheral blood mononuclear cells (PBMC) from PBC patients into severe combined immunodeficient (SCID) mice, but these first mouse models did not prove to be successful due to the lack of PBC-like morphology and the technical difficulties making them scarcely reproducible [81] (Fig. 2). Afterwards, several new murine models of PBC have been generated, and they can be clustered into spontaneous models employing genetically modified mice and inducible models, with the aid of xenobiotics harboring structural similarities to PDC-E2 (molecular mimicry) or self-biliary antigens (Table 3).

##### 4.1. NOD.c3c4 mice

The introgression of large genetic intervals on chromosomes 3 and 4 in non-obese diabetic (NOD) mouse strain leads to the development of NOD.c3c4 mice. These mice are protected from autoimmune diabetes but spontaneously develop lymphocytic infiltrates with a peribiliary localization and AMA positivity [82]. Notably, AMAs were detected in female mice, in accordance with the female predominance of human PBC. Importantly, in the liver of NOD.c3c4 mice, portal tracts appear infiltrated by CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes surrounding selectively the

interlobular bile ducts without any evidence of hepatocyte targeting [82,83]. Furthermore, intrahepatic cell phenotyping from NOD.c3c4 show increased numbers of T-cells with specific T-cell receptors (TCR), natural killer (NK), and NKT cells compared to lymph nodes or spleen. With aging, eosinophils are enriched in the portal infiltrates, reproducing the early stages of human PBC, a finding not detected in any other mouse model. However, infiltrating lymphocytes are also observed at the level of the common bile duct, a feature more consistent with PSC than PBC phenotype. Moreover, as the disease progresses, cholangitis evolves into non-suppurative cystic lesions in both the intrahepatic and extrahepatic bile ducts, with partial exfoliation of the lining biliary epithelium, and dense neutrophil infiltration, findings which typically, are not seen in PBC. Nonetheless, even with these limitations, the fundamental pathogenetic role played by the infiltrating lymphocytes has been demonstrated by the observation that disease development was hindered following treatment with monoclonal anti-CD3 antibodies [82]. Accordingly, liver disease was not observed in NOD.c3c4-SCID mice. Altogether, although morphological changes in NOD.c3c4 mice are not completely coherent with the PBC phenotype, this model is helpful for addressing the role of T cells.

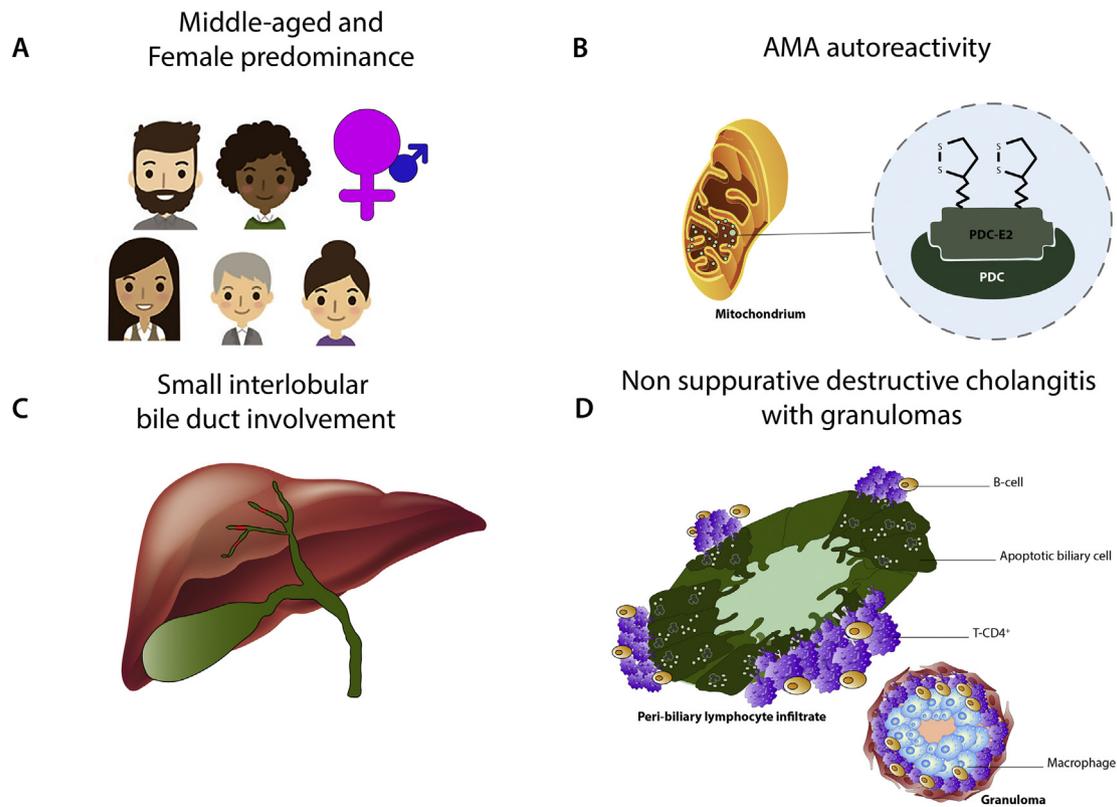
##### 4.2. IL-2R $\alpha^{-/-}$ mice

IL-2R $\alpha^{-/-}$  mice, as well as mice deficient in other components of the IL-2R complex, develop severe anemia and lymphoproliferative autoimmune disorders as well as IBD, with appearance of autoimmune cholangitis and serum AMAs [84,85]. Furthermore, these mice develop lymphocytic liver infiltrates and biliary inflammation that come to full development only after treatment with broad-spectrum antimicrobial therapy. The antibiotic therapy stimulates the generation of hepatic lesions in association with a significant decrease in the gut-specific inflammation. CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes represent the major cell types and are predominant in the portal spaces, while NKT or NK cell populations are not increased. However, granuloma formation and portal fibrosis are undetectable at 24 weeks [84].

Surprisingly, these animals show concomitant severe intestinal inflammation, which is not typical of PBC. To decipher whether and to what extent IL-12, a cytokine consisting of a p40 and a p35 subunit, drives bile duct damage in PBC, IL-2R $\alpha^{-/-}$ IL-12p40 $^{-/-}$  double-knockout mice were generated. Deletion of the IL-12p40 chain in IL-2R $\alpha^{-/-}$  mice led to more severe portal inflammation and splenomegaly, due to liver fibrosis and portal hypertension, in association with an amelioration of colitis compared to IL-2R $\alpha^{-/-}$  mice [85]. Notably, in double-KO mice, lymphocytic portal infiltrate comprised increased CD8<sup>+</sup> T cells, mainly effector memory cells, thus indicating that in liver, IL-12 acts as negative regulator of inflammation by inhibiting Th1 response.

##### 4.3. dnTGF $\beta$ RII mice

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a cytokine with pleiotropic effects on cell growth and differentiation, including the immunological cell compartment, where it modulates the activation of the regulatory CD4<sup>+</sup> T cells. Ishigame and coll. generated the dnTGF $\beta$ RII mouse, harboring a dominant negative TGF- $\beta$  receptor that was expressed under a CD4 promoter, thus abrogating TGF- $\beta$  responsiveness selectively in T cells. The dnTGF $\beta$ RII mouse is characterized by diffuse autoimmune manifestations affecting several organs associated with the production of multiple self-reactive antibodies [86]. In the liver, dnTGF $\beta$ RII mouse develop PBC-like features with lymphoid cell infiltration of the portal spaces with 100% of AMA positivity, including PDC-E2. However, even in this case, the drawback of the model is the concomitant association of autoimmune cholangitis to colitis. According to human PBC, and unlike IL-2R $\alpha^{-/-}$  mice, phenotyping of the lymphocytic infiltrates in the liver showed a quantitative increase of the NK cell subset compared with the circulating pool, as well as the



**Fig. 2.** Graphical representation of the main traits of an ideal PBC animal model. Requirement for the ideal model of PBC must include incidence in middle-aged females and development of AMA autoreactivity found in about 90% of patients (Panel A and B). Morphologically, disease process should target the interlobular bile ducts (C) resulting in a peribiliary lymphocyte infiltration and granulomas formation (D).

**Table 3**  
Animal models of PBC.

Models	Animal species	Major PBC features	Specific considerations	[Ref.]
NOD.c3c4 mice	NOD.B10 Idd9.1/9.2/9.3 and NOD.B6 Idd3/17/10/18 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA (50–80%)</li> <li>● Portal infiltrates with CD4+ and CD8+ T cells and eosinophils</li> <li>● Granulomas</li> </ul>	<ul style="list-style-type: none"> <li>- No gender bias</li> <li>- Dilatation of the common bile duct</li> </ul>	[82,83]
IL-2Rα <sup>-/-</sup> mice	C57BL/6 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates enriched with CD8+ T cells</li> </ul>	<ul style="list-style-type: none"> <li>- Increased serum levels of TNF-α, IFN-γ, IL-6, IL-12p40 and IgA</li> <li>- Defective Treg cells</li> <li>- Deletion of CD8 leads to attenuated damage of the bile ducts but increased colon inflammation</li> </ul>	[84,85]
dnTGFPRII mice	C57BL/6 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates</li> </ul>	<ul style="list-style-type: none"> <li>- Inflammatory infiltration in the colon and lungs</li> <li>- Defective Treg cells</li> </ul>	[86–90]
Scurfy mice	Male Treg-deficient Foxp3s-f/Y; C57BL/6 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates enriched with CD8+ T cells</li> </ul>	<ul style="list-style-type: none"> <li>- Increased serum levels of TNF-α, IFN-γ, IL-6, IL-12p40 and IL-18</li> <li>- Defective Treg cells</li> </ul>	[91,92]
Ae2 <sup>-/-</sup> mice	BALB/c background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates enriched with CD8+ T cells</li> <li>● Spontaneous cholestasis</li> <li>● Spontaneous portal fibrosis</li> </ul>	<ul style="list-style-type: none"> <li>- Constitutive Ae2 defect results in generalized abnormalities</li> <li>- Increased production of IFN-γ and IL-12</li> <li>- Decreased number of Treg cells</li> </ul>	[93–95]
2OA-BSA injection	Young female C57BL/6 mice	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates with high number of CD8+ T cells and CD19+ B cells</li> </ul>	<ul style="list-style-type: none"> <li>- Increased serum levels of TNF-α and IFN-γ</li> </ul>	[96–98]
ARE-Del <sup>-/-</sup> mice	C57BL/6 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal infiltrates enriched with CD8+ T cells</li> <li>● Formation of granuloma</li> <li>● Female predominance</li> </ul>	<ul style="list-style-type: none"> <li>- Increased production of IFN-γ</li> </ul>	[99]
MRL/lpr mice	C57BL/6 background	<ul style="list-style-type: none"> <li>● PBC-specific AMA</li> <li>● Portal inflammatory infiltrates</li> <li>● Biliary damage</li> </ul>	<ul style="list-style-type: none"> <li>- Only 50% of mice develop PBC-like features</li> <li>- Generalized lymphadenopathy and autoimmune manifestation</li> </ul>	[100,101]

presence of isolated B cells.

Furthermore, the generation of CD1d<sup>-/-</sup>-dnTGFβRII mice allows to elucidate the central role of NKT cells in PBC pathogenesis [87]. Ablation of the NKT function caused stark amelioration of inflammation and bile duct damage with only mild ductopenia, and therefore, improvement of cholestasis, and ultimately of biliary fibrosis. Although some lymphocytic aggregates could be identified, typical granulomas were absent. The role of lymphocytes was further investigated using the recombinant-activating gene (Rag1)<sup>-/-</sup> mice, which lack mature T and B cells ('nonleaky' SCID mice) [88]. Interestingly, immuno-deficient Rag1<sup>-/-</sup> mice receiving CD8<sup>+</sup> cells isolated from dnTGFβRII developed PBC-like features, while CD4<sup>+</sup> T cell transfer had no effect on the liver phenotype but worsened colitis. These mice displayed an altered cytokine profile resembling PBC, indicating analogous cell activation patterns. Specifically, serum levels of interferon (IFN)-γ, TNF-α, IL-6 and IL-12p40 appeared all significantly increased in dnTGFβRII mice compared to wild type littermates. In particular, the contribution of IL-12 was studied by generating IL-12p35<sup>-/-</sup> and IL-12p40<sup>-/-</sup> mouse strain on the dnTGFβRII background. IL-12p40<sup>-/-</sup> mice were protected from liver inflammation [89], while in IL-12p35<sup>-/-</sup> mice, liver inflammation was similar but with delayed onset compared to the parental dnTGFβRII mice [90]. The IL-12p35<sup>-/-</sup> dnTGFβRII liver phenotype was characterized by smoldering liver fibrosis sharing several immunological and histological features with human PBC [90].

The involvement of IL-6, which gradually increased in the dnTGFβRII mouse in an age-dependent fashion, was studied by generating dnTGFβRII/IL-6<sup>-/-</sup> double KO mice. In this mouse model, whereas IBD improved, at both clinical (diarrhea) and histological (intestinal lymphocyte infiltration) level, autoimmune cholangitis worsened, with exacerbation of biliary injury sustained by increase in pro-inflammatory cytokines (IFN-γ and TNF-α) and T cell activation. Thus, in dnTGFβRII mouse cholangitis and colitis are likely underpinned by distinct mechanisms, and IL-6 antagonism might be detrimental for cholangitis.

However, in this setting, phenotypic effects of other cytokines, including IL-23 and IL-35, are worth of further investigations by cytokine supplementation or cytokine neutralization approaches.

#### 4.4. Scurfy mice

Scurfy mice have a missense mutation in the gene encoding for the transcription factor forkhead-box protein 3 (FoxP3), also known as scurf, which negatively regulates CD4<sup>+</sup> T cell function [67,91,92]. These mice lack functional CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Treg) resulting in hyper-responsive CD4<sup>+</sup> T cells and develop an autoimmune disease with multi-organ inflammation, more pronounced in the skin, lung and liver. Liver damage is characterized by serological and morphological features of peribiliary lymphocytic infiltrate, with enhanced cytokine secretion, including TNF-α, IFN-γ, IL-6, IL-12 and IL-23, and AMA production on a background of a multi-system autoimmunity. Therefore, this model highlights the significance of Treg cells in the pathogenesis of PBC. Regrettably, the extremely short life span of these mice (not exceeding the 4 weeks) seriously limits their use for longitudinal studies, and thus this model is unsuitable to evaluating disease progression, or drug testing.

#### 4.5. Ae2a,b<sup>-/-</sup> mice

The Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger AE2 mediates the electroneutral Na-independent HCO<sub>3</sub><sup>-</sup> efflux in exchange with Cl<sup>-</sup> across the apical plasma membrane of ductal epithelia, including cholangiocytes. In addition to alter bile composition by reducing its alkalinization, it has been suggested that AE2 dysfunction may eventually affect the immune system, predisposing animals to develop a PBC-like disease. This hypothesis stemmed from the observation that AE2 is also expressed by peripheral blood lymphocytes, where it regulates intracellular pH, and that mouse

Ae2-defective CD8<sup>+</sup> (cytotoxic) T cells expands more actively upon T-cell stimulation. In line with PBC onset in middle-aged women, Ae2a,b<sup>-/-</sup> mice showed in adult age, significant portal inflammation with damaged interlobular bile ducts with portal infiltrates enriched in CD8<sup>+</sup> T cells [93]. In young animals, intrahepatic CD8<sup>+</sup> T cells were activated but then effectively deleted by apoptosis via programmed cell death (PD-1)/PD1 ligand (PD-L1) interaction driven by cholangiocytes. The PD-1/PD-L1 pathway is a protective mechanism against excessive T cell response, which can initiate when necessary, but it is dampened soon afterwards to avoid chronic autoimmune damage [94]. In older Ae2a,b<sup>-/-</sup> mice, activated intrahepatic CD8<sup>+</sup> T cells could expand because PD-1 silencing by epigenetics mechanism (DNA methylation) prevented their apoptotic loss thereby leading to autoimmune bile duct inflammation. This experimental model extends the role of AE2 beyond bicarbonate biliary secretion to immune homeostasis, unveiling that deficiency of AE2 in liver-infiltrating CD8<sup>+</sup> T cells affects immunosuppressive mechanisms when age-related epigenetic changes occur [95]. However, it is worth mentioning that histological changes are variable, with some mutated mice resembling control wild type, and animal breeding is difficult. Furthermore, it is unclear whether large ducts are also involved, and eventually, if biliary fibrosis will develop later on.

#### 4.6. Immunization of mice and guinea pigs using xenobiotics and self-antigens

Amano and coll. discovered that 2-octynoic acid (2OA), a xenobiotic, chemically synthesized compound not found in nature, widely used in cosmetic products, such as perfumes, soaps, and lipstick, as well as in many common food flavorings, had the potential to modify PDC-E2 in vivo [96]. Thus, 2OA has been regarded as an optimal candidate for antigenic modification of the PDC-E2 peptide. Starting from this observation, immunization of C57BL/6 mice with 2OA in bovine serum albumin (BSA) solution induced AMA autoreactivity characterized by production of anti-PDC-E2 antibodies accompanied by a rich lymphocytic infiltration of the portal areas around the bile ducts. Phenotypically, portal infiltrates were dominated by CD8<sup>+</sup> T cells and levels of TNF-α and IFN-γ were found to be elevated in serum. These findings give strong support to the concept that environmental factors may initiate AMA generation by xenobiotic modification of PDC-E2, and that this mechanism can be of paramount pathogenetic significance. Taking a single gene-deleting approach in mice immunized with 2OA, recent studies showed that both IL-12/Th1 and IL-23/Th17 signaling pathways were strongly associated with the pathogenesis of PBC [90]. A shift from a Th1 to a Th17 response occurs at advanced stages, suggesting therapeutic interference of the IL-23/Th17 pathway as a potential strategy for late PBC.

Other groups tested the effects of immunization with other chemical compounds with affinity to PDC-E2 on the onset of PBC. Leung and coll. demonstrated that guinea pigs immunized with 6-bromhexanoate (BH-6) coupled to BSA developed PDC-E2 autoantibodies, but with a delayed development of PBC-like features (after 18 months) and with a rather mild phenotype compared to 2OA, making this model of more limited interest [97]. Thus, 2OA can be regarded as a more relevant trigger of PBC pathogenesis.

However, all these models are based on the structural modification of PDC-E2 by cross reactive xenobiotics. To address more directly the loss of tolerance, a fundamental step in the natural history of PBC, a recent study performed mouse immunization with self-tissue, using syngeneic bile duct proteins (BDP). Mice immunized with BDP developed a liver-specific inflammatory phenotype, centered on the portal tracts, with increased number and activation state of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which also extended to the spleen, where germinal centers were hyperplastic. Of note, in this model, AMA autoreactivity was found in 100% of animals [98].

#### 4.7. *ARE-Del*<sup>-/-</sup> mice

IFN- $\gamma$  has been emerging as a key factor in the pathogenesis of PBC, and importantly, it is usually found increased in patient's serum, although its precise functions are still a conundrum. To generate a mouse model with a dysregulation of IFN- $\gamma$  signaling, the adenylate-uridylyltransferase (ARE) of the IFN- $\gamma$  3'-untranslated region was deleted resulting in the constitutive and persistent production of the cytokine (*ARE-Del*<sup>-/-</sup> mice) [99]. *ARE-Del*<sup>-/-</sup> mice spontaneously captured many typical manifestations of human PBC, including non-suppurative destructive cholangitis, AMA production, and elevated serum total bile acid levels [99]. Noteworthy, these features were predominant in female mice, likely related to the exacerbating effects of estrogens on immune cell production of IFN- $\gamma$ , as described in NOD mouse. Compared to males, female mice indeed developed a moderate-to-severe portal tract lymphoid cell infiltration at 20 weeks of ages, leading to a destruction of small interlobular bile ducts associated with granuloma formation. These findings were associated to a strong up-regulation of Th1-mediated signaling in female but not in male. Adoptive cell transfer of CD4<sup>+</sup> T cells from *ARE-Del*<sup>-/-</sup> mice to B6/*Rag1*<sup>-/-</sup> mice induced moderate inflammatory changes paralleled by up-regulation of genes potentially defining early stages of PBC. Therefore, this novel animal model is of great relevance since it provides functional evidence linking female gender dominance with the pathogenic role of IFN- $\gamma$  in the early stage of PBC [99].

On the other hand, ablation of IFN- $\gamma$  signaling prevents PBC appearance, in line with the concept that a Th1 response is critical for initiating disease activity by determining tolerance breaking.

#### 4.8. *MRL/lpr* mice

Mice harboring the homozygous mutation of the lymphoproliferative gene *lpr* (also known as *MRL*) spontaneously develop massive lymphadenopathy associated with proliferation of T cells, hyper- $\gamma$ -globulinemia, multiple serum autoantibodies, and a generalized autoimmune disease comprising of glomerulonephritis and arthritis, thus serving as a valuable model to study systemic lupus erythematosus. In the early 2000, *MRL/lpr* mouse was also proposed as a suitable animal model of PBC due to an abundant plasmacellular infiltration of the portal fields (more prominent compared to other animal models here discussed) with biliary damage and AMA production [100,101]. However, the use of *MRL/lpr* mouse as a good model of PBC was limited by the fact that only about 50% of mice actually developed in the liver PBC-like features.

### 5. Conclusion

Cholestasis is a multifaceted clinical condition resulting from multiple etiologies that for many years has been mostly investigated by means of the classic BDL rodent model. Although supported by unquestionable advantages, it was clear that BDL was insufficient to recapitulate the intricate mechanisms underpinning cholestasis as it develops in cholangiopathies, which are the most common chronic cholestatic liver diseases unrelated to biliary obstruction. In most cholangiopathies, such as PSC and PBC, bile duct damage is induced by chronic inflammation resulting from a tight dysregulation of innate and adaptive immunity. Thus, it became strong the need to generate experimental conditions able to capture the fundamental features of these peculiar diseases, going well beyond a simple model of obstructive cholestasis. Keeping this necessity in mind, in the last decade there has been a sort of 'gold rush' aimed at modeling cholestatic cholangiopathies following two main approaches, 'spontaneous' models based on genetically modified mice, and 'inducible' models, based on the administration of toxicants, targeting both biliary epithelium and colonic mucosa, or on the immunization with xenobiotics or self-antigens. Thanks to these models, a number of mechanistic insights have been

gained making much clearer the pathogenesis of cholestasis in these specific settings. For instance, in PSC we have been convinced that signals derived from the liver are critical in directing intestinal injury, while on the other side, normal intestinal microbiota may be protective on the biliary tree by generating secondary bile acids, though the strong differences in bile acid composition between mice and humans make the translatability of this observation quite difficult. Moreover, the role of IL-12 in PBC has been elucidated as negative regulator of Th1 response, which instead, is unleashed by IFN- $\gamma$ , and these events seem to be essential for initiating the disease process. Expression of AE-2 by lymphocytes and effects of its dysregulation in PBC models are paradigmatic of how mechanisms underlying cholestasis and inflammation can be interwoven. Furthermore, 2OA has been identified as a putative xenobiotic able to mimic or cross-react with self-antigens thereby triggering PBC pathogenesis. This notwithstanding, several gaps remain to bridge, such as in PSC modeling of concomitant intra and extra-hepatic involvement of biliary fibrosis, as well as of its malignant progression towards CCA, or in PBC the generation of models with a cleaner PBC phenotype or with a better characterization of fibrosis progression. All these issues will deserve deeper attention by the next future studies.

### Transparency document

The [Transparency document](#) associated this article can be found, in online version.

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