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Review article

Cholangiocyte death in ductopenic cholestatic cholangiopathies: Mechanistic basis and emerging therapeutic strategies

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ABSTRACT

Among hepatic diseases, cholestatic ductopenic cholangiopathies are poorly studied, and they are rarely given the importance they deserve, especially considering their high incidence in clinical practice. Although cholestatic ductopenic cholangiopathies have different etiologies and pathogenesis, all have the same target (the cholangiocyte) and similar mechanistic basis of cell death. Cholestatic cholangiopathies are characterized, predominantly, by obstructive or functional damage in the biliary epithelium, resulting in an imbalance between proliferation and cholangiocellular death; this leads to the progressive disappearance of bile ducts, as has been shown to occur in primary sclerosing cholangitis, primary biliary cholangitis, low-phospholipid-associated cholelithiasis syndrome, cystic fibrosis-related liver disease, and drug-induced ductopenia, among other biliary disorders. This review summarizes the features of the more common ductopenic syndromes and the cellular mechanisms involved in cholengiocellular death, with focus on the main forms of cholangiocyte death described so far, namely apoptosis, autophagy, necrosis, and necroptosis. It also emphasizes the importance to study in depth the molecular mechanisms of cholengiocyte death to make possible to counteract them with therapeutic purposes. These therapeutic strategies are limited in number and efficacy at present, and this is why it is important to find complementary, safe strategies to stimulate cholangiocellular proliferation in order favor bile duct replenishment as well. Successful in finding appropriate treatments would prevent the patient from having liver transplantation as the only therapeutic alternative.

1. Introduction

Cholestatic diseases are frequently dismissed as compared to others liver diseases, such as viral hepatitis, nonalcoholic steatohepatitis, and hepatocellular carcinoma, among others. However, most of chronic hepatopathies arise from, or progress towards cholestatic cholangiopathies. In the first three months of 2018, 4798 liver transplants were carried out in USA, with cholestatic cholangiopathies being one of the main causes of this treatment (United Network for Organ Sharin Website; https://unos.org).

Cholagiopathies result from a set of disorders associated with the biliary tract. They represent an actual therapeutic challenge, due to the complex function and anatomy disposition of bile ducts. Actually, there are no appropriate experimental models to study these disorders excluding, perhaps, the bile-duct ligation model [1,2] and the Mdr2-deficiente mouse model [3], which mimic obstructive cholelithiasis and low phospholipid-associated cholestasis secondary leading to sclerosing cholangitis, respectively.

The biliary tree is a highly dynamic structure with cells specialized in bile secretion, bile acid reabsorption, drug metabolism, and immune regulation [4–6]. It is composed of bile ducts, which extend from the small canals of Hering to the large extrahepatic bile duct (Fig. 1) [4].

The biliary epithelial cells form a physical barrier, and act as the first line of defense against the potentially cytotoxic components of bile [7]. Therefore, bile formation and modification require not only functional but also structural integrity of hepatocytes and cholangiocytes [5].

Biliary tree homeostasis is achieved through a balance between cell

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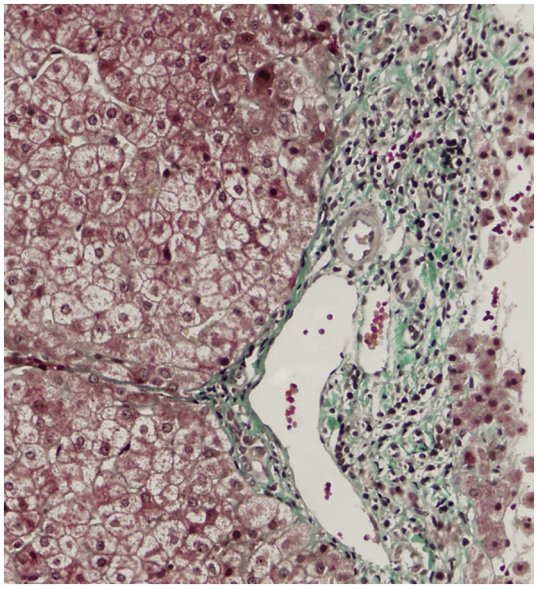


Fig. 1. Liver biopsy specimens of a patient with ductopenia. Portal trial showing absence of interlobular biliary ducts and lymphoplasmocytic infiltrate in a patient with PBC. Piecemeal necrosisis is also observed (right, low corner). Under normal conditions, biliary ducts should be observed in a space lower than two artery diameters. Masson, x40.

death and regeneration [8,9]. When this balance is lost due to exacerbation of cell death or impaired proliferation, the biliary system is affected in several ways that may lead to ductopenia and development of certain hepatopathies, collectively called "ductopenic cholangiopathies". These chronic and progressive liver disorders, characterized by the clinical syndrome of cholestasis due to an affected biliary tree, are responsible for the significant morbidity and mortality rates among pediatric and adult populations.

In this review, we describe the most frequent ductopenic cholestatic cholangiopathies by analyzing their general clinical characteristics and physiopathogenesis mechanisms, and explain in greater detail the mechanisms of cholangiocellular death that lead to these liver diseases. Finally, some hints for therapeutics to limit the ductopenic injury are provided, based either on cholangiocyte protection against these mechanisms of cell death or in the stimulation of cholangiocyte proliferation.

2. Cholestatic cholangiopathies

Cholestasis (literally, "a standing still of bile") is a syndrome originated from a disruption in bile production or flow through the biliary tree, resulting in a decreased amount of bile reaching the duodenum. The cause for this alteration can be a primary impairment in the capacity of hepatocytes or cholangiocytes to generate bile (functional or metabolic cholestasis), or a mechanical impairment hindering or impeding the bile transit (obstructive cholestasis) [10].

Cholestatic cholangiopathies are associated with a functional, obstructive, or mixed damage to the biliary tree, resulting in cholestasis. This disorder is the major determinant of most chronic cholestatic disorders.

All cholestatic cholangiopathies share, either as a primary or a secondary cause, an imbalance between cholangiocyte proliferation and death rate, associated with portal inflammation and fibrosis. Such alterations may be the main primary cause of the cholestatic disorder or a secondary manifestation of a primary hepatocellular dysfunction, which exacerbates the initial cholestatic phenomenon by extending the hepatocellular dysfunction to the cholangiocyte [11].

Cholestasis may arise from a primary obstruction in the bile ducts, but it also develops after the onset of ductopenia [8,9], which may induce loss of functional cholangiocellular mass and a biliary obstruction secondary to cellular detritus build-up and to the fibrosis caused by the inflammatory response thereto [12]. This damage induces the release of cytokines and pro-inflammatory mediators by cholangiocytes or other cell types, which stimulates cell death and proliferation responses, fibrogenesis, and impairment of the biliary epithelium transport function [11].

Chronic cholestatic cholangiopathies are the main cause of liver disease, cirrhosis, and progressive failure, eventually requiring liver transplant for the patient to survive. Some cases of cholestatic diseases are related to mutations in genes encoding proteins that are expressed in the bile canaliculus, and that are important for normal bile flow generation [13].

Cholangiopathies develop when the bile epithelium is damaged, either by infectious agents, autoimmune or genetic disorders, toxic compounds, or ischemia, which gives rise to several abnormalities in the bile duct, as observed in prototypical cholangiopathies such as primary biliary cirrhosis, cystic fibrosis, or biliary atresia [11].

The damage in the bile duct can be attributed to different causes, namely (1) the direct toxic effect of unmetabolized drugs on cholangiocytes (or reactivity of one of their metabolites) [14], (2) the immediate immune response triggered by cholangiocellular damage (e.g., due to an infectious agent or toxin), or autoimmunological causes associated with loss of tolerance to cholangiocellular antigens [6], and (3) a disruption in the protection mechanisms of the cholangiocyte against potentially toxic biliary compounds (e.g., due to a decrease in biliary phospholipids or an alteration in ductular bicarbonate excretion, two protective factors against cytotoxicity of tensioactive bile salts) [7]. Biliary complications, usually of ischemic nature [15], also constitute one of the main causes of morbidity and mortality after liver transplantation [16].

3. Ductopenic cholestatic cholangiopathies

Accelerated chollangiocellular death causes ductopenia, which is defined as the absence of interlobular bile ducts in no less than 50% of the portal triads. Its progression leads to vanishing bile duct syndrome (VBDS) when the bile ducts have virtually disappeared (Fig. 2) [8].

Among the different ductopenic cholestatic cholangiopathies, primary sclerosing cholangitis, primary biliary cirrhosis, biliary atresia, phospholipid decrease-associated cholestasis, cystic fibrosis, and drug-induced cholangiopathy are the more relevant ones [17], and they will be briefly described below.

3.1. Primary sclerosing cholangitis (PSC)

Primary sclerosing cholangitis is a chronic cholestatic autoimmune disease of unknown etiology, in which bile ducts are progressively disorganized due to the permanent presence of fibrosis and dilation of the intrahepatic and extrahepatic bile ducts due to multifocal stenosis, eventually leading to the development of biliary cirrhosis and/or liver failure [18]. It has a prevalence of 0–16.2 cases per 100,000 inhabitants [19].

The characteristic histological changes of PSC are concentric periductal fibrosis (also known as onion skin), and fibro-obliteration of medium-sized or larger bile ducts [6,20]. PSC can develop in 4 stages. At stage 1, or portal stage, lesions are minor; portal edema, lymphocyte infiltration into bile ducts, and non-destructive cholangitis appear in this stage. At stage 2, periportal fibrosis develops. At stage 3, there is septal fibrosis with major damage to the bile ducts, inducing their progressive disappearance (ductopenia). Lastly, cirrhosis is developed at stage 4 [21,22].

PSC etiology is currently unknown, but several pathophysiological mechanisms have been suggested. Defects in the mechanisms that protect against the toxicity of tensioactive bile salts have been proposed

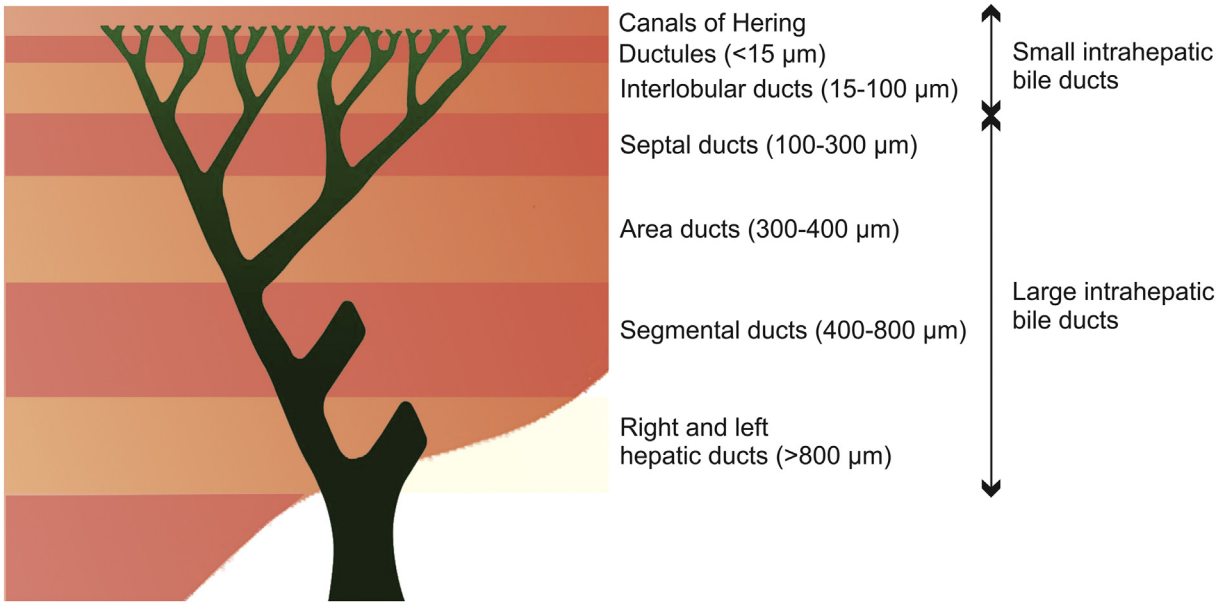


Fig. 2. Schematic representation of the intrahepatic biliary track. The intrahepatic biliary tree is a network of ducts that initiates in the canals of Hering, and gradually merges into an arrangement of interlobular, septal, and major ducts that finally coalesces to form the extrahepatic bile ducts.

as key actors in its development [7]. Release of intestinal pro-inflammatory microbial components into portal circulation (e.g., lipopolysaccharides), capable of triggering an innate immune response, has also been proposed, as well as the possibility of an antigenic factor of intestinal microbial origin [18]. Hepatic recruitment of T lymphocytes derived from intestine, due to overlapping in the pattern of adhesion molecules between intestine and bile ducts, has also been suggested [23]. T cells would then reach the portal area and the peribiliary space, thus inducing focal, fibro-obliterative lesions. Progressive periductal fibrosis, chronic inflammation, and ischemic atrophy of the biliary epithelium cause ductopenia, cholestasis, and obstructive stenosis, thus leading to secondary biliary cirrhosis [24].

Antibodies, such as perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), anti-nuclear antibodies (ANA), and smooth muscle antibodies (SMA) have been reported to be present in this disease [21]. This may reflect, in part, the response of B lymphocytes to antigens of intestinal origin (for pANCA) [25] or genetic causes associated with mutations in the *major histocompatibility complex* (MHC) responsible for antigen presentation, which are predominantly connected to autoimmune diseases related to the adaptive immunologic function; this may also explain the abnormal presence of several other autoantibodies (e.g., ANA and SMA) [26,27].

3.2. Primary biliary cholangitis (PBC)

Formerly known as 'primary biliary cirrhosis', PBC is an autoimmune disease [28] that selectively destroys small, interlobular, and septal bile ducts [6,20,29]; this leads to cholestasis, with increased levels of serum alkaline phosphatase, γ-glutamyltransferase, and bilirubin [30]. This hepatopathy is associated with progressive ductopenia and fibrosis, potentially leading to cirrhosis and, eventually, liver failure [28].

PBC predominantly affects middle aged or elderly women (1:9) [6,28], and it is the leading cause of ductopenia in adults [17]. PBC prevalence rates range from 1.91–40.2 per 100,000 inhabitants, and these values are increasing with time [19].

Histopathologically, PBC destroys intrahepatic bile ducts [6], with focal obliterations of the ducts and granuloma formation [21], inflammation due to infiltration of lymphocytes (CD4+ and CD8+) [17], macrophages, and other inflammatory cells [6]. Vacuolar degeneration and apoptosis of epithelial cells and disruption of the basement membrane is also observed [20]. This leads to the subsequent development of portal fibrosis and, eventually, cirrhosis [31]. More than 40% of PBC patients display ductopenia at their baseline biopsia [32].

Besides PBC, there are other forms of biliary cholangitis, which are secondary in nature. For example, it can represent a late manifestation of extended events of intrahepatic and/or extrahepatic bile duct obstruction (obstructive biliary cirrhosis) [33], or it may be due to other secondary causes of alteration of the bile ducts, e.g., those of infectious or ischemic nature [31], which are usually associated with cholangitis and acute pericholangitis [34].

Regarding pathogenesis, PBC is a prototypical autoimmune liver pathology. It is characterized by the presence of antimitochondrial autoantibodies (AMA) against the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC-E2) [6,35]. These autoantibodies evoke an inflammatory response characterized by infiltration of CD4+ ("helper") and CD8+ ("cytotoxic") T cells specific of mitochondrial antigens, in small and medium-sized bile ducts (i.e., larger interlobular and septal ducts). These autoantibodies are produced as a consequence of the systemic exposure of the PDC-E2 mitochondrial intracellular antigen caused by its release into the extracellular medium after cholangiocyte apoptosis [36]. In most tissues, PDC-E2 conjugates its sulfhydryl groups with glutathione (GSH) during apoptosis, rendering it antigenic in nature. However, the high levels of Bcl2 proapoptotic proteins that constitutively the cholangiocytes express inhibit this conjugation, thus exacerbating its antigenic effects [37,38]. PBC also has high serum IgM levels [28], possibly as a consequence of a decreased commutation of this kind of immunoglobulin into an IgG with similar antigenic specificity [38].

3.3. Biliary atresia (BA)

BA is a severe idiopathic neonatal cholangiopathy characterized by the rapid progression of fibrosis, obliteration, and destruction of the extrahepatic bile ducts during the first weeks of life [6,39–41]. The intrahepatic bile ducts loss their ramifications, a feature of mature ducts, resulting in cholestasis and neonatal hepatitis [42]. Histologically, the extrahepatic bile ducts exhibit extended fibrosis with inflammatory foci, whereas the intrahepatic ducts are hyperplastic, with variable inflammation and fibrosis, surrounded by giant multinucleated

hepatocytes. Approximately 30% of cases also suffer ductopenia, with narrowed interlobular ducts [43].

This pathology has a female predominance, especially in patients who also have spleen malformation [39]. During the course of the pathology, typical signs and symptoms of cholestasis appear, such as jaundice, pale stools, dark urine, portal hypertension, ascites, splenomegaly and, in certain cases, bleeding [42]. Biliary atresia is the most frequent hepatic cause of child death [44]. The main treatment for atresia is liver transplantation [39], which has improved patient survival [40,41].

Several factors are involved in the development of this disease, such as embryogenesis defects, abnormal fetal or prenatal circulation, viral infections, abnormal inflammatory response, genetic factors, autoimmunity, and environmental toxins [39]. It has been described that, akin to Alagille syndrome (a congenital ductopenic cholestatic cholangiopathy with similar characteristics to BA), a defect in the Notch signaling pathway, which controls transdifferentiation of hepatoblasts and mature hepatocytes into cholangiocytes, alters the expression of hepatic nuclear factor-1β (HNF-1β) [45,46]; this affects the repair mechanisms in postnatal life, due to the absence of reactive ductular cells and accumulation of hepatobiliary cells lacking HNF1β that cannot differentiate into a biliary phenotype [47].

3.4. Low-phospholipid-associated cholelithiasis syndrome (LPAC)

This pathological entity encompasses a group of liver diseases characterized by a variable defect in the biliary excretion of phospholipids. It is causally associated with mutations in the *multidrug resistance protein 3* (MDR3), a floppase encoded by the *ABCB4* gene that enables excretion into bile of phospholipids, such as phosphatidylcholine, by flopping them from the inner to the outer leaflet of the hepatocyte canalicular membrane, from where they are removed by action of luminal detergent bile salts [48–50]. The biliary excretion of phospholipids is crucial to protect both the canalicular membranes of hepatocytes and the apical membranes of cholangiocytes, as the phospholipids present in bile allow for the formation of mixed micelles composed of phospholipids, cholesterol and bile salts, thus protecting the epithelium against the detergent effect of free, monomeric bile salts in the biliary lumen [7,50]. Actually, hydrophobic bile salts have been demonstrated to cause necrosis and apoptosis in immortalized mouse cholangiocytes, and the effect is avoided by luminal phospholipids [51].

Since MDR3 mutations are varied in nature and have different functional impacts, they produce a wide spectrum of severity, depending on the degree of functional alteration [50,52]. In the most benign forms associated with mutations of limited functional impact, bile lithogenicity and cholesterol crystallization increase due to defects in cholesterol micellization, thus inducing obstruction of small bile ducts because of the formation of “biliary sludge”; even in its asymptomatic forms, this entity could be a predisposing factor for cholestatic conditions when potentially harmful factors are added, such as cholestatic hormones (e.g., endogenous estrogens and progesterone in cholestasis of pregnancy) or MDR3-inhibiting drugs (e.g., cyclosporine A, sirolimus, verapamil, and vinblastine) [50,52]. In its more aggressive forms associated with significant functional defects of MDR3, an overt, severe cholestatic disease develops, referred to as “progressive familial intrahepatic cholestasis type 3” (PFIC-3). This hereditary cholestasis occurs at a very young age, with a spectrum ranging from neonatal cholestasis to biliary cirrhosis in young adults. Its progression rate is associated with continuous exposure to detergent bile salt monomers. In homozygotes, a nonsense mutation in the MDR3 gene has been associated with ductopenia [53]. This pathology develops during childhood and adolescence, when the patients show typical cholestasis signs and symptoms, including jaundice, pale stools, hepatomegaly, and pruritus. Gastrointestinal bleedings and cirrhosis with portal hypertension may also occur. It has even been reported that MDR3 deficiency is a high-risk factor for developing hepatocarcinoma (HCC), and cholangiocarcinoma (CCA) has also been described in these patients [7,13,54].

Histologically, portal fibrosis and variable periportal fibrosis may be present, with progression to micronodular biliary cirrhosis, as well as ductular proliferation and infiltration of inflammatory cells, mostly lymphocytes; hepatocellular, canalicular, and ductular bilirubinostasis are also observed [13,54–56].

3.5. Cystic fibrosis (CF)-related liver disease

CF is a recessive autosomal disorder in the gene encoding the *cystic fibrosis transmembrane conductance regulator* (CFTR), an ATP-dependent cholangiocyte Cl^- channel. CFTR insertion into apical membrane of cholangiocytes is mediated by secretin-induced cAMP elevations; the high luminal-to intracelular Cl^- electrochemical gradient thus formed drives HCO_3^- ductular excretion via the *anion exchanger 2* (AE2), which exchange HCO_3^- by Cl^- in an equimolar manner. Since AE2-mediated HCO_3^- excretion is a chief determinant of ductular bile flow, dysfunction of CFTR may induce ultimately a decrease in ductular bile flow. Impairment of ductular bile flow provokes mucus plugging and further luminal obstruction in those bile ducts that cannot be drained normally [57,58]. Bile viscosity has been also verified to increase due to the high mucin content, and this may contribute to cholestasis [59], in part by promoting cholelithiasis [60]. The prevalence of CF-related liver disease ranges from 26% to 45% of CF patients. Portal hypertension without hepatocellular insufficiency is the main presentation of CF-related lived disease, and ductopenia in small portal tracts is observed frequently, in part associated with a diffuse obliterative portal venopathy [61].

In this pathology, production of the “HCO_3^- umbrella,” formed by the ductular secretion of HCO_3^- promoted by secretin, is also affected. HCO_3^- provides an alkaline environment along the apical surface of the bile epithelium that maintains bile salts in their ionic forms, thus preventing them from passively entering the cholangiocyte in their acidic, uncharged forms by non-ionic diffusion [7,62]. Since HCO_3^- excretion is also a driving force of the ductular bile flow, failure to excrete this anion in CF also increases the intraluminal concentration of bile salts, thus elevating their cytotoxicity further [7,62]. A deficit of luminal protecting factors leading to cholangiocyte overexposure to bile salts may result in cholangiocellular damage, due to the pronecrotic and proapoptotic effects of these biliary compounds [63]. Actually, it has been proven that extracellular pH is critical for the hydrophobic bile salts to cause the cellular death of immortalized human cholangiocytes; in these cells, bile salts are slightly toxic when exposed to the bile pH (7.4), but the toxicity increases dramatically with small decreases in pH values (from 7.1 to 6.4), which is in the order of that expected to occur after a failure in ductular HCO_3^- excretion [62,64]. In response to the ductular damage induced by these pathological mechanisms, a release of inflammatory cytokines occurs that leads to chronic portal inflammation; depending on the individual's immunogenetic basis and other concurrent factors, this may progress to focal biliary cirrhosis [64].

Neonatal cholestasis is an early manifestation of CF-related liver disease, which results in obstruction of the extrahepatic bile ducts; this could be one of the first manifestations of this pathology [57,58]. Up to 10% of CF patients develop cirrhosis in their first decade of life, progressing from focal biliary cirrhosis of the non-uniform portal tract to multilobular cirrhosis and portal hypertension [65], which could lead to liver failure and encephalopathy [58].

3.6. Drug-induced cholangiopathy

Many toxic drugs or their reactive metabolites can cause ductopenia and VBDS (Table 1).

These compounds may cause ductopenia mainly through the following pathomechanisms:

Table 1
Drugs that may induce ductopenic cholangiopathies.

Acepromatezine	Co-trimoxazole	Norandrostenolone
Ajmaline	Cromolyn	Phenylbutazone
Amineptine	Cyamemazine Cyclohexyl propionate	Phenytoin
Amitriptyline	Cyproheptadine	Prochlorperazine
Amoxicillin/ clavulanic acid	D-penicillamine	Terbinafine
Ampicillin	Diazepam	Tetracyclines
Azathioprine	Erythromycin	Tiabendazole
Barbiturates	Estradiol	Tiopronin
Carbamazepine	Flucloxacillin	Trifluoperazine
Carbutamide	Glibenclamide	Tolbutamide
Chlorothiazide	Glycyrrhizin	Trimethoprim/ sulfamethoxazole
Chlorpromazine	Haloperidol	Troleandomycin
Cimetidine	Ibuprofen	Xenalamine
Ciprofloxicin	Imipramine	
Clindamycin	Methyltestosterone	

1. Direct attack to cholangiocytes by drugs, or their toxic metabolites, once they have been excreted into bile.
2. Attack to cholangiocytes induced by drugs mediated by immunity, through the formation of immunogenic complexes between the drug (acting as a hapten) and endogenous proteins.

3.6.1. Direct drug attack to cholangiocytes

Toxic drugs or their reactive metabolites can cause ductopenia when they reach the cholangiocyte after being excreted into bile. Frequently, they are electrophilic or nucleophilic chemical products (or even free radicals) capable of promoting a series of chemical reactions that damage the biliary epithelium through multiple mechanisms, such as loss of antioxidant defenses, GSH depletion, and covalent binding to proteins, lipids, and nucleic acids [66].

A prototypical compound that acts via this mechanism is α-naphthylisothiocyanate (ANIT). This cholestatic agent selectively damages cholangiocytes from small and large ducts, thus causing cholangitis and intrahepatic biliary obstruction due to development of biliary sclerosis and progressive destruction of interlobular ducts. Coexistent apoptosis and proliferation are present in small and large ducts [67]. Apoptosis may stimulate cholangiocyte proliferation to compensate for the loss of mass and ductal function, but unlike hepatocytes, the limited proliferative capacity of cholangiocytes is insufficient to prevent ductopenia [68]. The bile duct damage induced by ANIT requires its hepatocellular conjugation with GSH and the subsequent biliary excretion of this metabolite; ANIT forms an adduct of labile GSH within hepatocytes, which dissociates in bile due to the medium alkalinity after being transported by Mrp2 [69]. The bile duct injury is produced mainly through apoptotic mechanisms mediated by ANIT-induced oxidative stress [70].

According to reports with animal models, the hepatotoxic compound carbon tetrachloride (CCl_4) also damages the biliary epithelium through oxidative stress-mediated apoptotic mechanisms, but it affects selectively large cholangiocytes, which produces proliferation of small duct cholangiocytes [71]. In this case, CCl_4 is converted in hepatocytes into the trichloromethyl free radical ($\cdot CC_3$) by the cytochrome P450 isoform CYP2E1, followed by excretion of free radical adducts into bile [72].

Similar mechanisms may apply for several other toxic compounds. For example, flucloxacillin, a ß-lactam antibiotic of the isoxazolyl-penicillin family, causes cholestasis associated with VBDS [73], in which toxic metabolites are generated after CYP3A4-mediated metabolism (e.g., 5′-hydroxymethyl flucloxacillin), which damage cholangiocytes after biliary excretion [74].

3.6.2. Immunologic attack to cholangiocytes by haptenization

As the liver metabolizes the drug, reactive metabolites can be produced, which may react with cellular macromolecules to form stable conjugates that trigger an aberrant immune response against self-proteins, thus forming autoantigens (haptenization) [75,76]. More commonly, haptens become immunogenic by forming adducts with proteins, either on the N-terminal domain (hydroxyalkylation) or on the C-terminal domain (oxoalkylation) [77]. Like in the molecular mimicry phenomenon, this immune response may also lead to reactivity against unmodified self-antigens, leading to immune tolerance breakdown and chronic autoimmunity [78].

4. Immunological mechanisms of ductopenic damage in cholangipathies

The mechanisms of immune-mediated ductular injury in cholangiopaties are common to most cholangiopathies. They are mainly related to the adaptive immune response mediated by CD4+ ("helper") T cells and CD8+ ("cytotoxic") T cells, damaging the cholangiocyte through highly specific T-cell receptors; the latter induce cholangiocellular death through apoptosis, by releasing perforin and granzyme B [79] (Fig. 3). For this immune response to occur, the antigenic moiety of the modified self-antigen needs to be displayed by an antigen-presenting cell (usually a dendritic cells) together with a *class-II MHC* (MHC-II) molecule, leading to a CD4+, T cell-induced immune response. Kupffer, hepatic stellate cells, and liver sinusoidal endothelial cells are all typical antigen-presenting cells in liver [80]. However, cholangiocytes themselves can also act as antigen-presenting cells in the inflammatory context [81], by aberrantly expressing MHC-I, MHC-II, and *intercellular adhesion molecule 1* (ICAM-1). This is due to the transcriptional effects of pro-inflammatory cytokines, such as interpheron-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) [82], thus increasing the possibility of activating "naïve" T cells, and initiating the cellular immune response [83].

Whatever the antigen-presenting cells are, activation of naïve CD4+, helper T cell after antigen presentation leads to CD4+ release of IL-2, followed by IL-2-induced autocrine clonal expansion. Active CD4+ lymphocytes stimulate, via IL-2 as well, the cell-mediated immunological attack of cholangiocytes by generating, activating, and allowing persistence of CD8+, cytotoxic T lymphocytes and natural killer (NK) cells, two cytolytic effector cells. These T lymphocytes bind to the cholangiocyte plasma membrane via the MHC-II/antigen complex or NKG2D-ligands, respectively [84], and release granzyme B and perforin, which induce apoptosis and necrosis in cholangiocytes [79]. In addition, soluble and plasma-membrane forms of *tumor necrosis factor-related apoptosis-inducing ligand* (TRAIL) and FasL from NK and maprophages interact with their respective receptors, TRAILR and Fas, expressed in cholangiocytes, and reinforce the apoptotic process [85]. Cholangiocytes also express constitutively low levels of leukocyte adhesion molecules, such as *class-I major histocompatibility complex* (MHC-I) and ICAM-1, but these molecules are upregulated during the inflammatory response [82]. Cholangiocytes can also secrete chemokines such as CXCL16 [86] or overexpress *monocyte chemotactic protein-1* (MCP-1) [87], which also promote inflammation by recruiting monocytes and T lymphocytes into portal tracts. MCP-1 upregulation seems to involve the epigenetic effects of micro-RNA miR-873-5p, whose circulating levels are increased in cirrhotic and cholestatic patients [88]; miR-873-5p targets glycine *N*-methyltransferase, the most abundant methyltransferase and master regulator of transmethylation flux involved in global epigenetic changes in liver [88].

Subsequently, an innate immunological response is produced, induced by the extracellular release of *danger-associated molecular patterns* (DAMPs) by the damaged cholangiocytes. DAMPs activate granulocytes and "natural killer" (NK) cells and target directly the cholangiocyte, via TLR receptors present in all these cells, able of recognizing and being activated by DAMPs [82,89]. DAMPs mimic immunological effects of

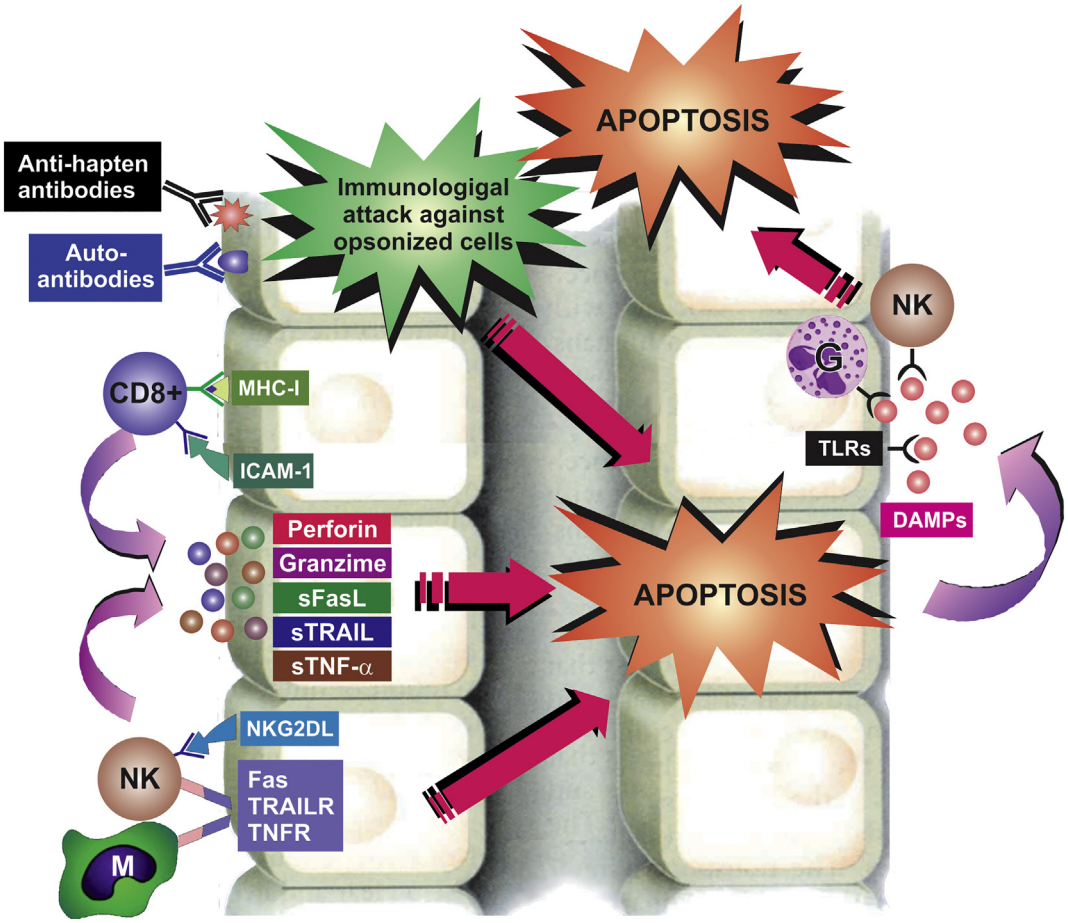


Fig. 3. Immunological mechanisms of ductopenic damage. Cytotoxic T lymphocytes (CD8 +), "natural killer" T cells (NK), and macrophages (M) induce cholangiocyte apoptosis by releasing perforin, granzyme B, and soluble forms of FasL (sFasL), TRAIL (sTRAIL), and TNF-α (sTNF-α), or by binding the membrane-associated forms of these cytokines to their plasma mambrane receptors in cholangiocytes. CD8+ binding to bile ducts is facilitated by the expression in cholangiocytes of the *major histocompatibility complex* (MHC) class I antigen (MHC-I) and T cell adhesion molecules, such as the *intercellular adhesion molecules 1* (ICAM-1). NK bind to the cholangiocyte through the NKG2D ligand. Release of *danger-associated molecular patterns* (DAMPs) by death cholangiocytes activates the innate immunity, which involves DAMP recognition by *toll-like receptors* (TLRs) located in cholangiocytes, NK cells and granulocytes, and the subsequent release of inflammatory/proapoptotic mediators that induce apoptosis in intact cholangiocytes.

pathogen-associated molecular patterns molecules (PAMPs), which alert the immune system against microbial infections. DAMPs are nuclear and cytoplasmic molecules, including nucleosides, uric acid, and the proteins *chromatin-associated protein high-mobility group box 1* (HMGB-1), S100A8/S100A9 and heat shock proteins, among others [90]. Like PAMPs, DAMPs activate NK cells and granulocytes, and the further release of inflammatory/proapoptotic mediators, such as cytokines, chemokines, and reactive oxygen and nitrogen species, thus exacerbating the inflammatory response and contributing significantly to the severity of the cellular injury [91]. Human cholangiocytes express a variety of TLR isoforms, and at least TLR-2 and TLR-4 are functional to mediate the development of a pro-inflammatory phenotype after DAMP release [92]. B cells are hyper-responsive to innate stimuli, and therefore may contribute to the perpetuation of the autoimmune process [93].

In addition to stimulate the immunological cellular response, IL-2 released by CD4+ T cells also stimulates the humoral response, by triggering B-cell proliferation and immunoglobulin synthesis [94]. B cells produce IgG antibodies against either the haptenized proteins (anti-hapten antibodies) or the native ones (auto-antibodies), able to react against cholangiocyte-specific antigens expressed in the bile-duct surface. Opsonization of cholangiocytes may lead to direct attack by immune cells bearing IgG FCγ receptors against the FC moiety of these antibodies, such as NK cells, macrophages, and granulocytes, and to trigger complement-mediated cholangiocellular lysis [78,95].

All this severe inflammation accounts for cholangitis and cholangiolitis, which results in atrophy and progressive periductal fibrosis, bile duct degeneration followed by obstructive cholestasis and, eventually, loss of interlobular bile ducts [96]. This may even coexist with small bile duct proliferation, a phenomenon known as "ductular reaction," due to maturation of intermediate hepatobiliary cells originated from a proliferative behavior in the porto-hepatic interface [97].

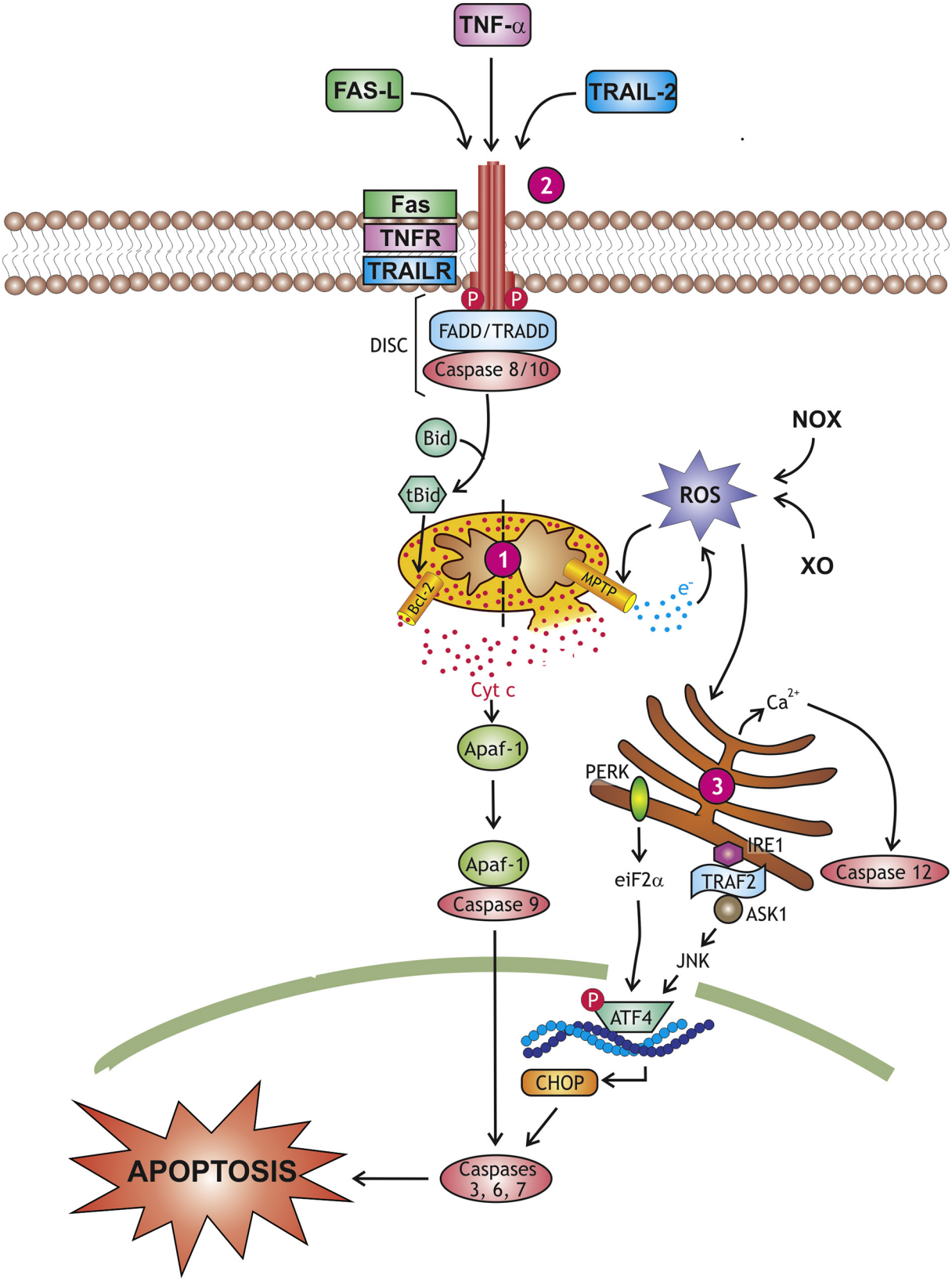
5. Mechanisms of cholangiocyte death

The mechanisms of cell death in cholangiocytes are common to the different ductopenic cholangiopathies. Cholangiocyte death is not as well characterized as hepatocyte death, but we know they comprise the three more important forms of cell death, namely apoptosis (type I), autophagy (type II), and necrosis (type III) [8,9].

The most common death mechanisms in biliary epithelial cells involved in ductopenic cholangiopathies are apoptosis and necrosis [63]. Autophagy and necroapoptosis has not been widely studied, but its role in ductopenic cholangiopathies is emerging [9].

5.1. Apoptosis

Apoptosis is the most widely studied death mechanism in



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cholangiopathies, and the main cause of ductopenia associated with them [98]. It results in characteristic morphological changes in cholangiocytes, including decreased cell size, chromatin condensation, and nuclear fragmentation; this leads to formation of apoptotic bodies, which are eliminated by phagocytosis of macrophages or neighboring epithelial cells [99,100]. Apoptosis is carried out through three pathways, namely extrinsic, intrinsic, and endoplasmic reticulum stress pathways (Fig. 4).

Fig. 4. Mechanisms of apoptosis in cholangiocytes: 1) Intrinsic or mitochondrial pathway of apoptosis, mediated by cytochrome *c* (Cyt *c*) release from the mitochondrial intermembrane space to the cytosol; Cyt *c* interacts with the adaptor protein *apoptotic protease activating factor-1* (APAF-1) to form the "apopsome" complex, which further recruits and activates procaspase-9 by proteolysis; once activated, caspase-9 activates the downstream executioners caspases 3, 6, and 7. Cyt *c* release is facilitated by a) the insertion in the outer mitochondrial membrane of pore-forming proapoptotic proteins of the Bcl-2 family (e.g. Bax, Bad), or b) the formation of mitochondrial permeability transition pores (MPTPs), which is triggered by reactive oxygen species (ROS) from different sources, including the pro-oxidative enzymes NADPH oxidasetrans (NOX) and xanthine oxidase (XO); the mitochondrial permeability transition also exacerbates electron leakage from the respiratory chain, thus promoting the generation of cytosolic ROS from mitochondrial origin. 2) Extrinsic pathway, initiated by a) binding of the pro-inflammatory cytokines *Fas ligand* (Fas-L), *tumor necrosis factor-α* (TNF-α), and *tumor necrosis factor-related apoptosis-inducing ligand-2* (TRAIL-2) to their respective plasma membrane receptors (Fas, TNFR, and TRAILR), b) autophosphorylation of these receptors, c) association of activated receptors with the death domains, *Fas-associating protein with death domain* (FADD) or *TNFR1-associated death domain protein* (TRADD), and with the procaspases 8 and 10 to form the DISC death complex, d) proteolytic activation of these caspases, and e) excision of Bid to truncated Bid (tBid), thus promoting formation of mitochondrial pores dependent of Bcl-2. 3) Endoplasmic reticulum (ER) stress apoptosis, which is induced by a ROS-mediated increase in Ca^{2+} release and executed by caspase 12, or by the activation of the ER stress sensors *inositol-requiring transmembrane kinase/endoribonuclease 1* (IRE1) and *double-stranded RNA-dependent protein kinase* (PKR)*-like eukaryotic initiation factor 2α* (eIF2α) *kinase* (PERK). Both eIF2α, activated by PERK, and *cJUN NH2-terminal kinase* (JNK), activated by *apoptosis signal-regulating kinase 1* (ASK1) after IRE1-induced association of ASK1 with *TNFR-associated factor 2* (TRAF2), induce translocation of the transcription factor *eIF2α-activating transcription factor-4* (ATF4) to the nucleus, and the further expression of the proapoptotic transcription factor *C/EBP homologous protein* (CHOP). Regardless the pathway involved, endoplasmic reticulum stress-related apoptosis is the result of the downstream activation of the executioner caspases 3, 6, and 7 by caspase 12 and CHOP.

Both apoptosis pathways has been reported to be common mechanisms of cholangiocellular death in ductopenic cholangiopathies [29,101,102], either due to an autoimmune or infectious liver disease, or because of permanent exposure of the cholangiocytes to toxic drugs or endogenous damage by bile salts [8]. In the case of PBC, apoptosis of cholangiocytes of small ducts was shown to be secondary to the invasion of inflammatory cells [103]. As for PSC, a role for apoptosis in ductopenia is more controversial. Unlike what happens with PBC, staining for apoptosis was reported to be negative in liver samples of PSC patients [103]. However, further studies demonstrated that patients with PSC increases serum markers of apoptosis [104,105], and that they correlates with disease activity and prognosis [104].

5.1.1. Extrinsic pathway

It is triggered by external activation of death receptors present in the plasma membrane, such as TNFR, Fas, and TRAILR. Activation of these receptors requires binding to their corresponding ligands, i.e., TNF-α, FasL, and TRAIL, all of which have cytokine activity [106]. The aforementioned ligands are transmembane proteins from immune cells, and their soluble forms in plasma are due to the proteolytic cleavage of their extracellular domains by Zn-dependent metalloproteinases (for TNF-α and FasL) [107–109] or cysteine proteases (for TRAIL) [110]. Binding of any of these cytokines is required for apoptosis to take place. First, binding-dependent homo-oligomerization of the receptor is needed. Then, the *death-inducing signaling complex* (DISC) must be formed by association of Fas or TRAILR with the *TNFR-associated death domain* (TRADD) protein. Finally, DISC associates with procaspases 8 and 10 to activate them, and these active caspases activate, in turn, apoptosis executioner caspases, such as 3, 6, and 7 [106].

In the liver, the cytokines capable of activating the extrinsic pathway of apoptosis pathway are mainly produced by Kupffer cells. However, in bile ducts, the major sources of this type of cytokines are inflammatory lymphocytes and macrophages infiltrated in the biliary epithelium [111]. In addition, in several cholangiopathies, cholangiocytes themselves acquire a secretory phenotype associated with senescence, which aberrantly expresses pro-inflammatory receptors, chemokines, cytokines, and other growth factors that may sensitize surrounding cholangiocytes to cell death, as has been shown in PSC [111] and acute post-transplant cellular rejection [112]. Genetic and epigenetic factors may contribute to perpetuate this cholangiocellular phenotype, and the subsequent development of chronic effects, such as fibrosis, cholestasis, VBDS, and even cholangiocellular carcinoma [82].

Fas is a membrane receptor highly expressed in damaged cholangiocytes in PBC [98,113]. It is activated by its ligand, FasL, a cytokine expressed by macrophages and the cytotoxic T lymphocytes surrounding them or localized within the biliary epithelium, as as been shown in PBC [98]. Fas expression has been positively correlated with cholangiocyte apoptosis in PBC [114], with CD68+ monocytes surrounding the damaged bile ducts as a FasL source [115]. Its expression in PSC is much less pronounced, so its proapoptotic role could be considerably less relevant in this cholangiopathy [116].

The TNF-α/TNFR system is commonly involved in immunity-mediated ductopenia, by activating the extrinsic apoptosis pathway [117]. Moreover, cholangiocytes are the main source of TNF-α in the liver, and expression of TNF-α and its receptor are increased in several ductopenic cholangiopathies [118,119]. CD28+-inflammatory T cells located around the liver bile ducts of PSC patients are also an important source of TNF-α [120]. However, antibodies against TNF-α have been inefficient in patients with this pathology [121].

Regarding the TRAILR/TRAIL system, human cholangiocytes constitutively express TRAIL receptor type 2 (TRAILR2). In ductopenic cholangiopathies such as PSC and PBC, expression of this receptor is increased [28,119,122], an effect likely induced by the bile salts accumulated during the cholestatic process [123], via activation of the nuclear factor Sp1 by the *c-Jun N-terminal kinase* (JNK)-dependent pathway [124]. Serum levels of soluble TRAIL are also elevated in patients with PBC [119,125]. Systemic administration of TRAIL to mice induces a sclerosing cholangitis lesion, suggesting that endogenous TRAIL may contribute to human syndromes with these features, e.g., PSC [126,127]. Finally, in patients with BA, TRAIL levels are elevated, in association with an increase in activation of the pro-inflammatory transcription factor-κB (NF-κB) [6]. Nevertheless, none of the inflammatory cells that infiltrate the bile ducts in BA expressed other cytotoxic markers, such as perforin, granzyme B, and FasL [128].

5.1.2. Intrinsic pathway

This apoptotic pathway is activated in response to a large number of cellular stress conditions, such as DNA damage, extracellular matrix detachment, hypoxia, loss of survival factors, and oxidative stress, among others. In this pathway, the mitochondrial internal membrane is permeabilized by formation of *mitochondrial permeability transition pores* (MPTPs), causing colloid-osmotic swelling of the mitochondrial matrix due to entry of small solutes; this causes rupture of the mitochondrial external membrane, release of cytochrome *c* into the cytosol, formation of the apoptosome along with caspase 9 and *apoptosis protease-activating factor-1* (APAF1) and, finally, the apoptosome-mediated activation of the executioner caspases 3, 6, and 7. The mitochondria can also become permeable by assembly into the external mitochondrial membrane of pore-forming proapoptotic proteins of the Bcl-2 family, such as Bax and Bak. These two proteins are also recruited to mitochondria by truncated Bid, originated from to proteolytic excision of a C-terminal fragment of Bid by the extrinsically activated caspases 8 and 10 [129]; this acts as a functional link between intrinsic and extrinsic apoptosis pathways. Nonetheless, these pro-apoptotic stimuli can be avoided by anti-apoptotic proteins of the same family, like Bcl-2, Bc-xL, and Mcl-1, since they sequester proapoptotic proteins of the Bcl-2 family, thus impairing their

membrane association and insertion, oligomerization, and pore formation [130]. Therefore, change of mitochondrial permeability depends ultimately on the balance between pro- and anti-apoptotic Bcl-2 families of proteins [131]. Loss or reduction of Bcl-2, Mcl-1, and Bcl-XL expression, thus promoting pore formation in the mitochondrial membrane by Bax/Bak, has been observed in small bile ducts upon rejection of a liver allograft [117], and in PBC [103,113,132,133]. In addition, since Bax is expressed throughout the biliary tree and Bcl-2 only in the interlobular bile ducts and bile ductules, susceptibility to pro-apoptotic stimuli is specially selective [134].

The perforin/granzyme B route is the most widely used by CD8+ cytotoxic T cells and NK cells to produce apoptosis in their target cells through the intrinsic pathway. These immune cells release granules containing these two proteins, and perforin forms pores in the plasma membrane of the target cell that enable the diffusion of granzyme B into the cytosol. Once there, granzyme B triggers apoptosis by activating executioner caspase 3, or by excising Bid, which leads to tBid formation and further activation of the intrinsic apoptosis pathway [79,135]. Finally, the immunological cells detaches from the apoptotic cell, and can further interact with another intact target cell [79].

Granzyme B-positive biliary cells are prominent in small bile ducts of PBC patients, and the transcriptional levels of perforin and granzyme B are elevated in liver tissue of these patients [136,137]. Caspase 3 activated by this pathway can potentially generate immunogenic fragments of PDC-E2, so it may contribute to the deposit of autoantigens and the production of antimitochondrial antibodies in this pathology [138].

Oxidative stress is a significant inducer of mitochondrial dysfunction, and as such, a relevant factor in the activation of the intrinsic apoptosis pathway. It has indeed been implicated in cholangiocyte apoptosis and necrosis secondary to immune aggressions, ischemia/ischemia-reperfusion, and exposure to toxic bile salts [139]. Oxidative stress induces drastic changes in the permeability of the plasma membrane, release of mitochondrial components to the cytosol, decreased production of mitochondrial adenosine triphosphate (ATP), and, ultimately, apoptosis or necrosis, depending on the number of mitochondria affected; since apoptosis but not necrosis is an energy-dependent process, the latter predominates when ATP is depleted [140]. Oxidative stress has been shown to be independently associated with more advanced stages of some cholangiopathies as for example PBC [141]. Furthermore, expression of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was preferentially detected in the nuclei of damaged interlobular bile ducts in this cholangiopathy [142,143]. In response to DNA damage, the gene encoding the cyclin-dependent kinase inhibitor WAF1 (aka p21) is induced by upregulated p53; WAF1 is a potent and reversible inhibitor of cell cycle progression at both the G1 and G2 checkpoints, and upregulated WAF1 induces irreversible G1 arrest and apoptosis. This reactive oxygen species (ROS)-mediated genotoxic mechanism has been shown to be a causal factor of cholangiocyte apoptosis in PBC [144]. In PSC, ductular lesions seem to be secondary to the production of ROS by leukocyte metabolic activation induced by antibodies against the cytoplasm of neutrophils, which is found in the serum of more than 50% of PSC patients [145].

The origin of oxidative stress in cholangiopathies is multifactorial. Once activated, lymphocytes generate an increase in ROS, and this phenomenon is involved in the apoptosis occurring in ductopenic diseases, e.g., in liver allograft rejection [139]. Mitochondrial dysfunction is another source of ROS. Cytochrome *c* release from damaged mitochondria affects the electron flow in the respiratory chain, thus inducing *i*) over-reduction of mitochondrial complexes and electron leakage into the cytosol, with the subsequent formation of ROS, and *ii*) decrease in the electron acceptor NAD^+, which results in ROS emission from the α-ketoglutarate dehydrogenase complex [146]. Oxidative stress can also be produced by enzymes that produce ROS as a consequence of their catalytic activity, such as NADPH oxidase (NOX) and xanthine oxidase (XO). NOX is a membrane-integrated enzyme that transports electrons through the plasma membrane and generates superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) from molecular oxygen inside the cells, by using NADPH as an electron donor [147]. XO, instead, produces H_2O_2 by catalyzing the oxidation of hypoxanthine to xanthine, and its subsequent oxidation to uric acid [148]. Both pro-oxidative enzymes are involved in ischemic damage to the bile ducts, e.g., in hepatic arterial stenosis, after liver transplantation, or in common bile duct compression after infusion of intra-arterial chemotherapeutic drugs [149]. Finally, the infiltrated eosinophils can be another source of ROS in PBC, since expression of eosinophil peroxidase, an eosinophilic granule protein that produces the powerful oxidant hypochlorous acid (HOCl), is increased within the portal space of PBC patients; macrophages also show evidence of the phagocytized enzyme [150]. The high sensitivity of cholangiocytes to oxidative stress has been attributed to their low GSH content, which is only 1/3 of that of hepatocytes [151], and cholangiocyte GSH levels are further decreased in cholangiopathies, such as PBC [150].

5.1.3. Endoplasmic reticulum stress

This proapoptotic pathway is triggered when the production and transport systems of proteins are altered, leading to accumulation of misfolded proteins, and eventually, apoptosis. Endoplasmic reticulum stress is triggered by several factors, such as oxidative stress, Ca^{2+} accumulation in the organelle, nutrient deprivation, and a large number of toxic aggressions, among others [152].

Endoplasmic reticulum stress is also triggered by loss of Ca^{2+} homeostasis due to its excessive release of the cation from this organelle; under this condition, Ca^{2+}-dependent calpains activate caspase 12, which in turn activates the executioner caspases 3, 6, and 7 [153,154]. Similarly to what happens in mitochondria under pro-apoptotic conditions, Bax and Bak can migrate to the endoplasmic reticulum, where they regulate Ca^{2+} channels and the inositol 1,4,5-trisphosphate receptor, thus potentiating Ca^{2+}-dependent apoptosis. Furthermore, protein misfolding can activate c-Jun through the JNK-dependent proapoptotic route, and induce expression of proapoptotic transcription factor CHOP, which favors apoptosis by modulating activity and expression of members of the Bcl-2 protein family [155].

Although there is no direct evidence of the involvement of endoplasmic reticulum stress as an apoptosis mechanism in ductopenic cholangiopathies, the machinery enabling this pathomechanism is completely functional in cholangiocytes. Furthermore, it can be activated by the high levels of cytotoxic, endogenous bile salts at which they are exposed in obstructive cholangiopathies [35]. Moreover, expression of PDI and GRP78, two endoplasmic reticulum stress markers, was significantly higher in small bile ducts, which are the most affected ones in PBC [9,35].

A possible mechanism accounting for the oxidative and endoplasmic reticulum stress in PBC is the microRNA (miR) dysregulation that occurs in hepatocytes and in immune cells of these patients. miR-506 is overexpressed in cholangiocytes of PBC patients, through a mechanism involving proinflammatory cytokines that are usually found to be elevated in PBC livers, such as IL-8, IL-12, IL-17, IL-18, and TNF-α [156]. Experimental overexpression of miR-506 in cholangiocytes affected mitochondrial energetic metabolism, with increased oxygen consumption, glycolysis, and proton leakage; this indicates uncoupling of respiration, which may have in turn accounted for by the increase in oxidative and endoplasmic reticulum stress [156]. Overexpression of miR-506 also sensitized cholangiocytes to bile acid-induced apoptosis, via a caspase 3-dependent mechanism [156]. Impairment of the HCO_3^- umbrella is a likely mechanism explaining the increased vulnerability to bile acid-induced apoptosis, since overexpression of miR-506 also decreases the Cl^-/HCO_3^- exchanger AE2 expression in cholangiocytes from PBC patients [157], and impaires type III inositol 1,4,5-trisphosphate receptor [158]; this is the major isoform of the receptor in cholangiocytes, where it regulates HCO_3^- and Cl^- secretion into bile [159].

5.2. Autophagy

Autophagy is regarded as a cellular protective process that eliminates damaged or aged proteins and organelles [9]. It also play a protective role during acute cellular stress (e.g., nutrient deprivation), thus helping surrounding cells to maintain cellular energy homeostasis [35]. Nevertheless, under chronic stress conditions, it represents an alternative mechanism of cell death to apoptosis; actually, these two self-destructive processes share common executer pathways, and are functionally linked [63,160,161]. The first step of autophagy is the nucleation of a membrane of unknown origin, although it seems to come from the endoplasmic reticulum, the Golgi apparatus, or *de novo* synthesis. This initial membrane, referred to as "phagophore", is elongated until the edges are fused, thus producing a double-membrane vacuole, the "autophagosome", that encapsulates the material to be degraded. Once the autophagosome is formed, it fuses with the lysosome, thus creating the autophagolysosome; lysosomal enzymes degrade its content, which allows for the degradation of aged or damaged cell material [162].

There is accumulating evidence that autophagy plays a major role in PBC pathogenesis. Cytoplasmic vesicles containing the autophagy-related marker *microtubule-associated protein-light chain 3* (LC3) has been usually found in injured bile ducts in this hepatopathy [35]. Autophagy seems to be related to the autoimmune process against mitochondrial antigens, by regulating T lymphocytes [63]. In a study where cholangiocytes in culture were exposed to several forms of stress, cells showed LC3 and PDC-E2 co-localization followed by PDC-E2 expression on the cell surface, suggesting that autophagy is responsible for granular expression and subsequent cell surface expression of PDC-E2 [160].

Autophagy may also precede biliary epithelial senescence in cholangiopathies, a process that seems to be involved in bile duct lesions [163]; almost 90% of hepatobiliary cells express senescence markers in cholestatic cholangiopathies [164]. Cholangiocyte senescence can be triggered by different cellular stresses occurring in these hepatopathies, such as oxidative stress and DNA damage [165]. Senescence is a state of irreversible growth arrest in the G1 phase of the cell cycle [166], and therefore, accumulation of senescent cholangiocytes may contribute to loss of functional biliary mass [164]. Furthermore, senescent cholangiocytes express various cytokines and chemokines that may exacerbate the inflammatory microenvironment around bile ducts, thus contributing to pathogenesis [163]. Cholangiocyte senescence during cholestatic liver injury has been linked to the epigenetic regulation of the expression of the positive regulator of biliary development FoxA2, as has been shown for PBC, PSC, and BA [164,167]; the proto-oncogene N-Ras, a known inducer of senescence, has been also causally linked to senescence in PSC cholangiocytes [111].

5.3. Necrosis

Generally, necrosis is a consequence of acute and severe metabolic perturbations, such as those that take place in ischemic cholangiopathies or drug-induced toxicity. This leads to an abrupt increase in the plasma membrane permeability and, eventually, cell lysis. As a consequence, a strong inflammatory response is triggered due to the massive release of DAMPs to the extracellular medium, which activates the innate immune system and initiates a secondary cascade of damage and inflammation against bile ducts [82,89,160].

Necrosis is the main cell death mechanism when there is massive formation of MPTPs, leading to a significant decrease in cellular ATP levels [168,169]. This type of ductopenic lesion predominantly affects the middle third of the bile ducts, leading to hepatic duct confluence, with intrahepatic affectation rarely being present [170]. In PBC, focal lesions display severe inflammatory changes and necrosis around bile ducts, often referred to as "florid duct lesion" [171]. The lymphocytes and mononuclear cells that constitute the inflammatory infiltrate are in close contact with the basal membrane of cholangiocytes undergoing necrosis [171]. In addition, non-anastomotic biliary strictures and bile duct necrosis have been described as frequent complications in liver transplantation related to ischemia-reperfusion injuries [172]. A role for necrosis in PSC ductopenic lesions is doubtful, however, since biochemical markers of necrosis are not elevated in PSC patients [105].

5.4. Necroptosis

Necroptosis, a controlled and genetically regulated form of necrosis that resemble apoptosis regarding its "programmable" trait [173], also occurs in cholangiopathies [174,175]. Necroptosis is similar to necrosis in terms of the kind of cellular alterations observed, as it shows plasma membrane permeation, colloid osmotic swelling (oncosis), mitochondrial dysfunction, and release into the extracellular space of the cytoplasmic content, also inducing inflammatory reactions due to DAMP release [173].

It is induced by similar factors to necrosis (e.g., intracellular ATP depletion, loss of Ca^{2+} homeostasis, mitochondrial depolarization, proteolysis by non-apoptotic proteases, and ROS increase), but it differs in that it can be induced, like apoptosis, by activation of cell surface receptors, mainly TNFR1, although Fas and TRAILR can be involved as well [176]. Caspase 8, the initiator of this process, is normally involved in apoptosis and not necrosis, because it simultaneously inhibits the excision of *receptor-interacting serine/threonine-protein kinase* (RIP)-1 and 3, two molecules that execute necrosis [177]. However, under certain conditions where caspase 8 is inhibited, RIP1 and RIP3 form the "necrosome" with other proteins, and cause necrosis through the *mixed lineage kinase domain-like* (MLKL)-mediated mitochondrial pathway, which involves the recruitment of the mitochondrial protein phosphatases PGAM5L and PGAM5S, and the further *dynamin-related protein-1* (Drp1) activation; this latter protein triggers mitochondrial fission and ROS production, thus causing a cellular damage similar to that observed in necrosis [178].

High expression levels of RIP3 and MLKL have been detected in the liver of PBC patients [175]. Moreover, mice with a RIP3 genetic ablation subjected to obstructive cholestasis by bile duct ligation showed decreased oxidative stress levels, inflammation, and necrosis, suggesting a role of this cell death mechanism in obstructive cholangiopathies [175]. A role for the microRNA miR-21 in cholangiocyte necroptosis has been suggested by studies showing that it is particularly overexpressed in biliary cells in human, as well as in both bile-duct ligated and Mdr2 knockout mice [179]. Finally, although studies of liver transplantation are lacking, necroptosis may play a role in ischemia-reperfusion injuries and also in liver allograft rejection, as inferred from the occurrence of similar injuries after renal transplantation [180].

6. Therapeutic approaches to limit ductopenic injury in cholangiopathies

Since there is a lack of therapeutic strategies to stimulate cholangiocellular proliferation in order to replenish bile duct under ductopenic conditions, current therapeutic strategies are limited to protect cholangiocytes against cell death mechanisms operating via either the exacerbated immunological response or exposure to luminal factors associated with the occurrence of a "toxic bile" in cholangiopathies. The current therapeutic strategies to counteract cholangiocyte death include *i*) the anticholestatic drug ursodesoxycholic acid (UDCA), which may protect these cells from apoptotic and possibly necrotic death induced by deleterious bile salts accumulated during the obstructive process and by cytokines released during the inflammatory process, and *ii*) immunosuppressive and anti-inflammatory agents, when ductopenia is suspected to be predominantly associated with immune-mediated mechanisms.

6.1. UDCA

This hydrophilic, non-toxic bile salt is used nowadays as the first-choice drug for the treatment of cholestasis in general, and for most cholangiopathies in particular [181]. For example, UDCA has been approved by the Food and Drug Administration for the treatment of PBC [171]. Moderate UDCA doses has been recommended by the European Association for the Study of the Liver for PSC treatment based upon results showing improvement in liver function tests and surrogate markers of prognosis in these patients, although no improved survival was demonstrated so far [21]. As for CF-associated liver disease, even when there is no established therapy for this cholangiopathy, UDCA therapy is highly recommended, since it was suggested to reduce bile viscosity [182,183]. UDCA could be also useful as an adjuvant therapy in BA, where biochemical benefit has been shown in a single crossover trial in older children with the disease after successful surgery [184]. Finally, UDCA could be beneficial even under conditions of complete extrahepatic obstruction, at least when administered at low doses and during a short administration period [185].

A compelling body of experimental and clinical evidences points UDCA as a bile duct-protective agent due to its well recognized capability to counteract bile salt-induced apoptosis and necrosis, and due to its alleged immunosuppressive and immunomodulatory properties.

Its protective mechanisms are as follows:

1) UDCA displaces and replaces highly toxic, endogenous bile salts accumulated in cholestatic hepatopathies; UDCA comprises no more than 4% of the total endogenous bile acid pool, but its value is increased to 40–60% under a conventional UDCA therapy [186]. Since biliary bile salt composition reflects the plasmatic one, the bile salt biliary would be far more harmless to cholangiocytes in patients on UDCA.
2) UDCA may counteract hepatic bile acid-induced cell death in liver. Although there is no direct evidence in cholangiocytes, UDCA have well-documented anti-apoptotic [187] and anti-necrotic [188] properties both in hepatocytes and in other extrahepatic cell types. UDCA has anti-apoptotic effects secondary to mitochondrial injury by blocking MPTP formation induced by both bile salts [189,190] and proinflammatory cytokines, including TNF-α [191], FasL [192], and transforming growth factor-β1 (TGF-β1) [193]. UDCA also reduces the expression of the members of the Bcl-2 family of proteins that form mitochondrial pores (e.g., Bad and Bax) by inhibiting p53, a pro-apoptotic transcription factor that induces Bad and Bax expressions [194]. UDCA also counteracts *t*Bid-induced the mitochondrial pore formation [192], and the upregulation of AP-1, a pro-apoptotic transcription factor activated via the TNFR/TNF-α signaling pathway [195]. Finally, tauroursodeoxycholic acid (TUDCA), a main UDCA metabolite, inhibits endoplasmic reticulum stress-induced apoptosis both by inhibiting caspase-12 activation via modulation of intracellular Ca^{2+} levels [196], and by acting as a chemical chaperone that counteracts endoplasmic reticulum stress itself [197]. Apart from its anti-apoptotic effects, UDCA can activate survival pathways. TUDCA binds to the *epidermal growth factor receptor* (EGFR), which activates the signaling survival pathways mediated by *extracellular signal-regulated kinase* (ERK) and *phosphoinositide 3-kinase* (PI3K)-Akt [198,199]. This mechanism of UDCA protection was confirmed in a rat model of apoptosis-induced ductopenia by combined vagotomy and bile-duct ligation [200].
3) UDCA reinforces adaptive mechanisms that the liver evokes spontaneously in cholestasis to attenuate the damaging effects of accumulated bile salt on hepatocytes and cholangiocytes. This adaptive response involves *i*) decrease of intracellular (and hence intrabiliary) levels of bile salts by repression of Cyp7a1, the rate-limiting enzyme of bile salt synthesis [201]; *ii*) attenuation of bile salt toxicity by promoting formation of less harmful polyhydroxylated bile salts, via induction of sterol hydroxylases [202]; *iii*) downregulation of sinusoidal bile-salt uptake transporters, e.g., *Na^+-taurocholate cotransporting polypeptide* (Ntcp), and upregulation of basolateral bile salt extrusion pumps systems, e.g., *multidrug resistant-associated protein* (Mrp) 3 and 4, which prevents bile salts from building up in liver by diverting them to urine. Patients on UDCA might activate some of these adaptive mechanisms in part by transactivating the nuclear receptor farsenoid X receptor (FXR) [202], but this response can be reinforced by the concomitant administration of the more potent FXR ligand obethicholic acid [203], or by supplementation with rifampicin, which activates pregnane X receptor (PXR), another nuclear receptor with complementary beneficial mechanisms to FXR [204].
4) UDCA restores ductular mechanisms of defense against toxic bile salts, which are frequently altered in ductopenic cholangiopathies [7]. This is accounted for by *i*) stimulation of MDR3-mediated phospholipid biliary excretion [204], an effect that can be reinforced by peroxisome proliferator activated receptor-α (PPAR-α) ligands, such as fibrate drugs [205], and *ii*) stimulation of HCO_3^- ductular excretion, with the concomitant reinforcement of both the "bicarbonate umbrella" and the hypercholeresis-induced dilution of toxic luminal bile salts; this occurs (*1*) by the stimulation of cholangiocyte HCO_3^- secretion via AE2 by transcriptional and post-ranscriptional mechanisms [181,206], particularly when combined with glucocorticoids [207], and (*2*) by the ability of unconjugated UDCA to generate luminal HCO_3^- molecules as part of its characteristic cholehepatic recirculation. The UDCA homologue *nor*-UDCA, which suffers cholehepatic shunting even more efficiently than UDCA and has a higher osmotic choleretic efficiency [208], bore better therapeutic effects than UDCA to ameliorate sclerosing cholangitis in Mdr2-knockout mice, which have a complete inability to secrete phospholipid into bile [209]. Furthermore, *nor*UDCA has promising anticholestatic effects in patients with PSC, according to a recent phase II clinical study [210].
5) UDCA bears anti-inflammatory and immunomodulatory properties, in part due to its capability to bind and further activate the glucocorticoid receptor [211]. UDCA may inhibit humoral autoimmunity, as suggested by its ability to inhibit IgM, IgG, and IgA production by B cells exposed to bacteria [212]. UDCA also counteracts the cellular immune response by inhibiting the release of cytokines produced by mononuclear cells, such as IFN-α, IL-2, and IL-4 [212], a finding that was confirmed for IL-2 in a cholangitis experimental model [213]. UDCA may also counteract the overexpression of MHC-II [214] and ICAM-1 [215] in the apical membrane of the biliary epithelial cells, as well as of the ICAM-1 partner receptor in lymphocytes, *lymphocyte function-associated antigen 1* (LFA-1) [215].

6.2. Immunosuppressant agents

Despite many cholangiopathies are considered autoimmune diseases, they not usually respond to conventional immunosuppressive drugs (e.g. azathioprine, chlorambucil, cyclosporine, methotrexate, mycophenolate mofetil), including PBC [216,217] and PSC [218]. These drugs were either only marginally effective, ineffective, or even detrimental, and this is why they are not currently recommended to treat these liver diseases [219]. Similarly, the treatment of PBC with the corticosteroid budesonide is controversial, and should be reserved to non-cirrhotic patients with PBC/autoimmune hepatitis overlap syndrome [21]. A 1-year pilot trial in PBC patients treated with prednisolone showed some improvement in histology and liver function tests, but also a marked increment in bone loss [220]. Finally, a further study combining prednisolone with UDCA showed histological recovery at early PBC stages, but was not superior to UDCA monotherapy [216].

Corticosteroids have been also regarded as mostly ineffective in drug-induced VBDS by some specialists [14]. However, the often association of VBDS with drug-induced hypersensitivity reactions justify the pharmacological induction of immunosuppression as part of the

general therapeutic strategy for this particular case. Benefits have been supported by anecdotal cases of drug-induced ductopenia in Stevens-Johnson syndrome [221–223], toxic epidermal necrolysis [224–226], erythema multiforme [227], and hemophagocytic lymphohistiocytosis [228]. Corticosteroids are the most common immunosuppresors used in these cases, either alone or together with other immunosuppressive drugs when the patient is unresponsive to corticosteroids. Alternative immunosuppressants applied with some success include mycophenolate moefetil [229], tacrolimus [230], and cyclosporine [225]. Other therapeutic strategies for immunologically mediated diseases, such as administration of infliximab (a monoclonal antibody against human TNFα) and plasmapheresis, have been also successfully employed in these particular cases [224].

6.3. Stimulators of cholangiocyte proliferation

There is no current established therapy to treat ductopenia based upon stimulation of cholangiocyte proliferation.

Cholangiocyte proliferation occurs spontaneously in early stage of the cholangiopathy course as a consequence of the inflammatory process, and the mechanisms involved in this adaptive response may be useful to envisage therapeutic strategies based upon its stimulation. This phenomenon, referred to as “ductular reaction”, consist of the formation of small epithelial tubules, with cholangiocytes been produced from hepatic progenitor cells within the Canal of Hering that are activated during liver injury [231,232]. Peribiliary glands that are lined with biliary epithelial cells of large intrahepatic bile ducts and extrahepatic bile duct are also a source of multipotent stem/progenitor cells for cholangiocyte proliferation and renewal [233,234].

Regulatory aspects of this proliferative process have been intensely studied in animal models of biliary hyperplasia, such as bile duct ligation (BDL), partial hepatectomy, chronic bile acid feeding, and more relevant to our issue, in the ductopenic model of administration of CCl_4 to bile-duct ligated rats [71,235,236]. Using these models, several signaling pathways have been identified to regulate proliferation and migration of resident cells in cholangiocyte progenitor niches. After BDL, large cholangiocytes lining larger bile ducts, but not small cholangiocytes lining smaller bile ducts, proliferate through the cAMP-mediated activation of the PKA-Src-MEK-ERK1/2 signaling pathway stimulated by secretin [235,237]; the reason for this difference is that small cholangiocytes do not express secretin receptor, and are normally mitotically dormant [238,239]. However, small cholangiocytes can proliferate via activation of the *Ca^{2+}/calmodulin/calmodulin-dependent protein kinaseI* (CaMKI)/*cAMP-response element binding* (CREB)-dependent signaling pathway by certain stimuli, such as H1 histamine receptor stimulation [240]. In pathologic conditions of large cholangiocyte damage (e.g., after CCl_4 or γ-aminobutyric acid treatment), small cholangiocytes replenish the damaged biliary tree by both amplification of this Ca^{2+}-dependent signaling pathway and transdifferentiation to the large cholangiocyte phenotype [241,242]. These and other hyperproliferative signaling pathway (e.g., the PKCβ-I-dependent one) are evoked by different signaling modulators with potential to be used as coadjuvant agents for ductopenia treatment, including neurotransmitters/neuromodulators (e.g., dopamine, neural growth factor, acetylcholine, epinephrine, calcitonin gene-related peptide, acetylcholine, norepinephrine, anandamide, and histamine) and hormones (e.g., growth hormone, vascular endothelial growth factor, epinephrine, insulin-like growth factor-1, prolactin, melatonin, angiotensin, glucagon-like peptide-1, and different sex hormones) [235,243], acting either from blood and through innervations by the autonomic nervous system, respectively. Alternatively, autocrine/paracrine mechanisms have also been described, which are associated with an increased transdifferentiation of proliferating small cholangiocytes towards a neuroendocrine phenotype able to secrete many of these mediators, and bind them via expression of their receptors [244]. This greater plasticity and proliferative potential of small cholangiocytes as compared to more differentiated, large ones points the former as a functional hepatobiliary progenitor cell population that can be therapeutically targeted to replenish the biliary epithelium in ductopenia. Whatever is its nature, this treatment should be applied early during the ductopenic process, since bile ducts are only rarely reconstructed as complete epithelium-lined tubes once they have been fully destroyed [245].

Caution is warranted when designing these therapeutic strategies, due to putative adverse effects associated with exacerbated cholangiocyte proliferation. First, proliferating cholangiocytes secrete a number of mediators, such as cytokines, chemokines, and other profibrogenic factors, that act via paracrine mechanisms to stimulate myofibroblast activation, migration, and proliferation, thus promoting liver fibrosis [96]. Second, the stimuli needed to activate otherwise quiescent cholangiocytes may trigger uncontrolled cholangiocyte hyperproliferation in susceptible individuals, resulting in liver diseases associated with growth-promoting effects, such as polycystic liver diseases [246] and cholangiocarcinoma [247]. This therapeutic approach may be particularly dangerous in diseases with a high lifetime risk of developing cholangiocarcinoma, such as PSC and CF-associated liver disease, especially if associated with an adjuvant antiapoptotic therapeutic strategy [248]. The crucial question here is whether we will be able to specifically stimulate those pathways that are beneficial for bile duct reconstitution without activating those causing harmful effects, or alternatively, to antagonize the unwanted effects without affecting the beneficial ones. The better comprehension of the signaling pathways that regulate differentiation and proliferation of both stem/progenitor cells and mature cells is clearly needed to develop such novel regenerative therapies.

7. Conclusions

The important advances in understanding cell death mechanisms in general, and the search for evidence of their particular involvement in cholangiopathies, have allowed us to understand in more detail the nature of these liver diseases. This knowledge has allowed us to explain why, under certain conditions or in certain individuals, the cholangiocellular death rate exceeds the cell proliferation rate, thus leading to different degrees of ductopenia. In addition to clearing the main functional cells out of the bile ducts causing both functional and obliterative cholestasis, the exposure of immunological cells to intracellular, potentially antigenic components released by death cholangiocytes is a powerful inflammatory stimulus that perpetuates a vicious cycle by which inflammation leads to cholangiocyte death, which in turn induces even more inflammation. Therefore, to limit this process is critical to improve disease prognosis.

The studies conducted in this field have allowed us to establish that ductopenic disruption is closely related to cholangiocellular death mechanisms, with apoptosis being the major death pathway involved, and the one we know currently in greater detail. However, our knowledge of other less studied cell death mechanisms to which cholangiocytes might be exposed in the context of each cholangiopathy, as well as their mechanisms of initiation and regulation, is still largely insufficient. In this sense, more studies on the role of autophagy and necroptosis, two recently acknowledged cholangiocellular mechanisms of cell death in ductopenic cholangiopathies, are eagerly awaited to better understand their role in the physiopathology of the disease. But at the same time, we must understand more thoroughly the defense mechanisms of the cholangiocyte that allow it to survive, in order to modulate them pharmacologically. Finally, making progress that allows us to envisage repair mechanisms against the damage suffered in each hepatopathy is crucial. Unfortunately, there are no well established therapeutic strategies to stimulate cholangiocellular proliferation in order to counteract ductopenic conditions through bile duct replenishment that lack risks associated with excessive growth-promoting effects. Meanwhile, even more efforts to envisage new modes to counteract the exacerbated cell death must be made, in order to restore

the balance between cell death and proliferation, by simultaneously improving both sides of the equation. This challenge will also involve answers to the question on how gradients in cholangiocyte phenotypes along the biliary tree will be reconstituted to assure an appropriate ductal structure and function at the organ level.

Conflicts of interest

All authors declare no conflict of interest.

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