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Mechanisms of Tissue Injury in Autoimmune Liver Diseases

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Abstract

Autoimmune diseases affecting the liver are mainly represented by autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). The characteristic morphologic patterns of injury are a chronic hepatitis pattern of damage in AIH, destruction of small intrahepatic bile ducts in PBC and periductal fibrosis and inflammation involving larger bile ducts in PSC. The factors responsible for initiation and perpetuation of the injury in all the three autoimmune liver diseases are not understood completely, but are likely to be environmental triggers on the background of genetic variation in immune regulation. In this review, we summarize the current understanding of the mechanisms underlying the breakdown of self-tolerance in autoimmune liver diseases.

Introduction

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are the three major forms of autoimmune liver disease, which differ in the pattern of inflammation, clinical phenotype and the focus of autoimmune injury (1). In AIH, the targets of autoimmune injury are hepatocytes, leading to the histological picture of predominant interface hepatitis. In PBC and PSC the autoimmune injury affects cholangiocytes; however in PBC, small, interlobular bile ducts are targeted, causing the typical appearance of non-suppurative destructive cholangitis (2), and in PSC, the medium-sized intra- and/or extra-hepatic bile ducts are affected, causing concentric and obliterative fibrosis and multifocal bile duct stricturing (3). Notably, the cholangiopathies can also be characterized by varying degrees of interface hepatitis and inflammatory bile duct lesions can also occur in some AIH patients (4). Moreover, all of the three major autoimmune liver diseases are associated with bowel disease; PBC with celiac disease, and most strikingly AIH and in particular PSC with inflammatory bowel disease (IBD) (5). The common characteristic the three autoimmune liver diseases share is that unchecked inflammation will cause progressive liver fibrosis eventually leading to cirrhosis (3).

AIH, PBC and PSC are disorders involving a complex interaction between genetic and environmental factors. The factors that initiate and perpetuate inflammation in these diseases are poorly understood, but are likely to be environmental triggers on the background of genetic defects in immune regulation, allowing persistent inflammation and breakdown of self-tolerance (6). Herein, we summarize the current understanding of the pathogenic mechanisms involved in liver injury in autoimmune liver diseases.

Genetics

Epidemiologic studies have revealed a strong heritability of PSC and PBC conditions. A strong genetic predisposition is evident in PSC, with first-degree relatives having a 9- to 39- fold increased risk to develop the disease (7). Similarly, 6% of PBC patients have a first-degree relative that also suffers from PBC (8), and studies in monozygotic twins have demonstrated a 63% concordance of PBC disease (9). Familial risk of AIH has not been rigorously studied, but there is also a likely clustering of autoimmune diseases in families (10, 11).

In the last decade, there have been major efforts in Europe, North America and Japan to establish large, well-characterized patient cohorts for high-throughput genetic studies of PBC and PSC, and to a lesser extent of AIH. Four genome-wide association studies (GWAS) and two iCHIP-association studies of PBC (12-18) have been published. Two GWAS of PSC followed by a number of replication studies undertaken in large, independent cohorts (19-25) and an iCHIP association study, have also been reported (26). A GWAS of AIH in European and Japanese cohorts are underway and results from these studies will be reported by 2014 (27). These high-throughput genetic studies have highlighted the shared genetic basis of these complex and diverse autoimmune diseases. GWAS analyses have clearly demonstrated that the major component of the genetic architecture of PBC and PSC is within the HLA region; similar findings are expected for AIH. Additional non-HLA risk loci in both PSC and PBC appear to be enriched for gene products involved in innate or acquired immune responses, which are consistent with an autoimmune component to the pathogenesis.

HLA and non-HLA associated loci

HLA associations

In PSC, an association with the HLA complex on chromosome 6p21 has been well documented. Key susceptibility haplotypes include HLA-B*08 and DRB1*03:01 alleles, and particularly HLA-DRB1*1501-DQB1*0602, HLA-DRB1*1301-DCB1*0603, and HLA-A1-B8-DRB1*0301-DQB1*0201 (28, 29). A strong protective influence of the DRB1*04-DQB1*0302 and DRB1*0701-DQB1*0303

haplotypes has been also reported (30). The fact that PSC can re-occur after liver transplantation suggests that the target organ has common or genetic features that predispose to the immune attack (6). In European populations, PBC is associated with the risk haplotypes, DRB1*08:01-DQA1*04:01-DQB1*04:02 and DRB1*04:04-DQB1*03:02, and the protective haplotypes DRB1*11:01-DQA1*05:01-DQB1*03:01 and DRB1*15:01-DQA1*01:02-DQB1*06:02 (31).

In Europe and North America, susceptibility to AIH type 1 is conferred by the possession of DRB1*03:01 and DRB1*04:01, and protection with the allele DRB1*15:01 (30). In Chinese, Japanese and Mexican populations, type I AIH (see below) susceptibility is linked to DRB1*04:04 and DRB1*04:05, whereas in Latin American populations, is linked to DRB1*13:01 (32, 33). Susceptibility to AIH type 2 (see below) is conferred by the possession of alleles DRB1*03, DRB1*07 and DQB1*02:01 (34).

Non-HLA associations

To date, 27 non-HLA risk loci for PBC (13-18) and 12 genome-wide significant non-HLA risk loci for PSC (19, 20, 25, 26) have been identified. In PBC, candidate genes are potentially involved in regulation of the immune system, from the development and differentiation of the myeloid cell compartment (*SPIB*, *IRF5*, *IRF8*, and *IL-7R*) to antigen presentation and T cell differentiation (*HLA class II*, *CD80*, *IL-12A*, *IL-12RB*, *TYK2*, *STAT4*, *SOCS1*), up to B cell function and differentiation to plasma cells (*SPIB*, *IRF8*, *PLC-L2*, *IKZF3*, *CXCR5*) (35).

The strong association of PSC with IBD suggests a common pathway for liver and gut inflammation, and this overlap is further reflected by the presence of shared non-HLA genetic risk loci (36). The non-HLA findings in PSC to some extent indicate that the proposed hypotheses on PSC pathogenesis related to autoimmune mechanisms (*IL2* and *IL2RA*), bile acid toxicity (*GPBAR1*) and mechanisms related to the concomitant IBD (*IL2/IL21*, *ILR2A*, *CARD9*, *MST1*, *Fut2*, *SIK2*) might operate in concert to cause the disease (37).

Epigenetics

A plethora of information derived from genomic data has contributed in our slightly better understanding of these complex autoimmune liver diseases. Although they are necessary, they are still insufficient to explain the development of disease. Studies on DNA methylation in monozygotic twins discordant for PBC have shown for example a possible differential expression of two X-linked genes (*PIN4*, *CLIC2*) that are diversely methylated (38).

Environmental Factors

The development of autoimmune diseases requires a complex interaction of genetic and environmental factors. Some of these factors are highlighted by identification of concordance in identical twins or in individuals with karyotype abnormalities and predisposition to autoimmunity (39). A recent report of an expert panel workshop of the National Institutes of Health on the mechanisms of environmental influences on human autoimmunity is noteworthy (40, 41).

Antibiotics, smoking, caffeine and hormones

Studies have identified several environmental factors as potential predisposing and several as protective against the development of autoimmune liver diseases. Examples include the use of antibiotics, which has been identified as a risk factor in AIH (42), and urinary tract infections, vaginal infections, cigarette smoking and frequent use of nail varnish that have been identified as potential risk factors in PBC (8, 43). An increased risk for PBC with the use of hormonal replacement therapy (43) has been reported, although other studies have shown a protective association with oral contraceptive use (9), a finding in keeping with the age of onset being close to menopause. In PSC, hormonal factors are reported to influence the disease, since fewer female PSC patients reported ever use of hormonal contraception, than control subjects (44). In PSC smoking and coffee consumption have been proposed to be protective against development of disease (44-46).

Molecular mimicry

One of the mechanisms that have been suggested to play an important role in initiation and/or exacerbation of autoimmune diseases is molecular mimicry, whereby a foreign antigen shares sequence or structural similarities with self-antigens. Type 2 AIH is defined by antibodies to liver-kidney microsomal type 1 (anti-LKM1) and/or antibodies to liver cytosol (anti-LC1). The LKM1 autoantibodies recognize conformational epitopes on cytochrome P450 IID6 (CYP2D6), but they also cross-react with homologous regions of HCV, HSV and CMV, further suggesting a potential “multi-hit” mechanism for the generation of these antibodies (47). It can be speculated that multiple exposures to common viral pathogens, may render the immune system permissive by priming a cross-reactive subset of T cells, in a genetically susceptible host. Depending on the level of exposure and the degree of genetic susceptibility, a minority of recurrently infected individuals may progress to autoimmune disease.

In PBC the dominant autoepitope is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) recognized by the antimitochondrial antibodies (AMA). PDC-E2 has a conserved sequence across all species, from eubacteria to mammals, thus it is not surprising that AMA cross-reacts with a number of microbes. *E. coli* has proteins closely resembling the PDC-E2 (48), and several experiments have demonstrated that serum from PBC patients, and particularly AMA, reacts with *E. coli* sequences (49). Mycoplasma expresses similar proteins (PDC-E1a and PDC-E1b) on its cells surface, and immune reactivity to *M. pneumonia* antigens has been observed in large numbers of patients when compared with controls (50). The *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, is the best microbial candidate yet for the induction of PBC (51).

Xenobiotics

Another source of antigenic mimicry is xenobiotics, foreign compounds that may either alter or complex to defined self or non-self proteins, causing the native protein to change its molecular structure and inducing an immune response (52). Studies in PBC have elegantly demonstrated that the PDC-E2 lipoyl domain

is highly vulnerable to modifications by environmental xenobiotics. Notably, such chemicals include widely used compounds in perfumes, lipstick and food flavorings. 2-nonynoic acid, a cosmetic component, is such a xenobiotic able to chemically modify PDC-E2, resulting in induction of autoimmune response and initiation of an AMA response (53). Moreover, acetaminophen or similar drugs can cause electrophilic modification of lipoic acid in PDC-E2, facilitating the loss of tolerance and development of PBC (54).

Autoantibodies

AIH is an archetypal autoimmune condition, with a female:male disease incidence ratio of 7:1, and presence of autoantibodies and autoreactive T cells. AIH is classified based on its serology into two types: type I AIH defined by antinuclear antibodies (ANA) and/or anti-smooth muscle antibodies (SMA), and type 2 AIH defined by liver-kidney microsomal type 1 antibodies (anti-LKM1) and/or liver cytosol antibodies (anti-LC1). SMA antibodies are mainly active against filamentous actin but the molecular target is not well defined. Conversely, the molecular targets of anti-LKM1 and anti-LC1 have been characterized as cytochrome P450 IID6 (CYP2D6) and formiminotransferase cyclodeaminase (FTCD), respectively (55). The role of autoantibodies in the pathogenesis of autoimmune liver damage has been suggested by the finding that hepatocytes, isolated from patients with AIH, are coated with immunoglobulins and are susceptible to cytotoxicity when exposed to autologous Fc receptor bearing mononuclear cells (56). Moreover, CYP2D6 is expressed on the surface of hepatocytes, therefore is susceptible to recognition by anti-LKM1 autoantibodies; collectively suggesting these autoantibodies could be directly involved in the pathogenesis of autoimmune liver damage in AIH type 2 (57).

In PBC, similar to AIH, there is a high female prevalence (8:1), which has also led to the suggestion that X chromosome defects may play a significant role in disease, although this has not been easily confirmed or replicated (58). A hallmark of PBC patients is the presence of antimitochondrial antibodies (AMA), which can be detected in nearly 100% of patients (59). AMA antibodies are

directed against members of the 2-oxoacid dehydrogenase complexes (2-OADC) that exist in the inner membrane of mitochondria. Among them the major autoantigen is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). In addition to AMA, PBC sera can present other disease-specific autoantibodies, particularly anti-nuclear (ANA) antibodies, which react to nuclear pore glycoproteins of the inner nuclear membrane, gp210 (60) and p62 (61), with a detection rate up to about 30%. These autoantibodies show a higher prevalence among AMA-negative PBC patients and seem to correlate with disease severity and progression (62). Other PBC-specific nucleoprotein reactants include the Sp100-promyelocytic leukemia (PML) autoantigen (63) and anti-centromere antibodies (64).

PSC, cannot be considered a “classical” autoimmune disease, as it occurs with a 2:1 male predominance and lacks characteristic response to immunosuppressants (65). Serum atypical perinuclear antineutrophil cytoplasmic antibodies (pANCA) are frequently found in PSC patients (66). The pANCA appear to cross react with β -tubulin isotype 5 and the bacterial cytoskeletal protein FtsZ, which is expressed by intestinal flora (53)(67). PSC patients have a particularly high prevalence of anti-*Saccharomyces cerevisiae* antibodies (ASCA) even in the absence of advanced disease and irrespective of IBD phenotype (67). Some autoantibodies detected in PSC also bind to biliary epithelial cells (68) and induce expression of TLR4 and TLR9, the activation of which results in the secretion of pro-inflammatory cytokines and chemokines (69).

Bacteria, Molecular Patterns, and the Innate Immunity

The close association of PSC with IBD, mainly with ulcerative colitis (UC) (75% of PSC patients have UC and at least, if not more than, 3% of UC patients have PSC as a concomitant comorbidity (70, 71), makes plausible the suggestion that PSC shares similar pathogenic mechanisms with IBD. The latter results from an abnormal innate immune response to antigens of the intestinal flora, which further activates the adaptive immune response (72). Similarly in PSC, a dysregulated response to pathogen stimulation may contribute to the immune

system activation and thus disease initiation, establishment and progression (73). Several lines of evidence further support this suggestion: i) the prevalence of PSC among UC patients is significantly higher in the patients with total colonic involvement, suggesting a strong positive association among intestinal inflammation and PSC pathogenesis (70), ii) enteric bacteria such as *E. coli* and *Candida* are often found in the bile of PSC patients (74) and iii) the expression of genes involved in innate immune pathways is significantly increased at the late stages of PSC (75).

Enterohepatic circulation brings potential mediators of inflammation, such as microbes or metabolites of enteric microbiota (e.g. endotoxins, metabolites), from the gut to the hepatic sinusoids, where sinusoidal endothelial cells, Kupffer cells, hepatocytes, hepatic stellate cells, dendritic cells (DCs) and monocytes through their pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), can recognize and respond to pattern associated molecular patterns (PAMPs), causing a pro-inflammatory response that will contribute to repair processes (76) (Figure 1). Cholangiocytes (also called biliary epithelial cells) express multiple TLRs as well, which upon pathogen recognition can initiate signalling cascades that alter their physiology and the extracellular cytokine milieu (77). *In vitro* studies have shown that upon pathogen recognition cholangiocytes produce and release pro-inflammatory mediators, TNF α , IL-6 and IL-8, promoting further the recruitment and activation of T cells, macrophages, neutrophils, NK cells and resident and recruited mesenchymal cells, thus initiating biliary repair responses (78). Notably, cholangiocytes from PSC liver explants show high TLR expression, nucleotide-binding oligomerization domain, MyD88/IRAK complex, TNF α , IFN γ and IL-8 production and a lack of tolerance to repeated endotoxin exposure (79). IFN γ and TNF α expression could mediate such hyperresponsiveness, since in the absence of these inflammatory mediators, PSC cholangiocytes can revert to a phenotype capable of initiating endotoxin tolerance (80). It is evident therefore, that the inflammatory milieu of the diseased liver coupled with persistent exposure and response to PAMPs, can promote a persistent inflammatory phenotype of cholangiocytes.

Cholangiocytes are also exposed to lipopolysaccharide (LPS) and lipoteichoic acid. Exposure to LPS may cause disruption of tight junctions in colonic epithelial cells, as well as cholangiocytes, via TLR4-dependent mechanisms (81, 82). Alterations of these barriers could therefore expose cholangiocytes to a variety of substances, such as bile acids, that could eventually promote injury and inflammation. In animal models, the disruption of cholangiocyte tight junctions is an important step for the development of PSC (83). In particular, mice with altered cholangiocyte tight junctions leak bile into the portal tract, which leads to an inflammatory response involving T lymphocytes, upregulation of injurious cytokines (TNF α , TGF β and IL-1 β), myofibroblast activation and fibrosis (84). Interestingly, in patients without PSC, exposure to such PAMPs does not activate the innate immune system (80), further highlighting the specificity of the disease.

In addition to microorganisms or microbial derived molecules, endogenous molecules, including several present in bile, such as products released from injured or dying cells (damage-associated molecular patterns, DAMPs) (e.g. HMGB1, S100A8/S100A9, and heat shock proteins) can activate TLRs and/or DAMP receptors (85). Moreover, other components of bile such as oxysterols, the oxygenated derivatives of cholesterol, have been demonstrated to mediate inflammatory processes (85). Treatment of cultured cholangiocytes with selected oxysterols rapidly activates both cholangiocyte NF- κ B and MAPK pathways and induces cholangiocyte expression of IL-6 and IL-8 (86). Therefore, antigens translocating to the liver via the portal circulation, may act as molecular mimics in genetically susceptible individuals to cause an immune reaction that could be responsible for initiating PSC.

In addition, a high proportion of PSC patients have non-specific antibodies as well as autoantibodies that bind to cholangiocytes (68, 87). These autoantibodies have been shown to bind to biliary epithelial cells and activate the innate immune system by inducing expression of ERK1/2 transcription factor and upregulating TLRs, leading to inflammatory cytokine production (69). The autoantibodies are also able to increase expression of IL-6 and adhesion

molecules such as CD44, thereby promoting lymphocyte proliferation, immunoglobulin production and cell adhesion (68, 69).

During the course of PSC an increased number of NK cells in the peripheral blood and in the colonic mucosa has been reported but not in the liver of PSC individuals (88). Interestingly, the NK cells present in the PSC liver microenvironment have decreased cytolytic activity, likely because of the high levels of local TNF α production (89). It has been suggested that this poor cytotoxicity of NK cells may be counteracted by induction of TRAIL on activated hepatic NK cells and further cell-mediated destruction of cholangiocytes via TRAIL-TRAIL receptor 5, which is increased in cholangiocytes of human PSC patients (90).

An increased response to pathogen-associated stimuli is also observed in PBC, as indicated by higher levels of pro-inflammatory cytokines secreted *in vitro* by monocytes after exposure to microorganisms (91). In PBC, a marked increase in the frequency and absolute number of NK cells in blood and liver has been demonstrated. Isolated NK cells have showed a significant increase in their cytotoxic activity and perforin expression, which were associated with increased levels of plasma IL-8 and the expression of IL-8R on such cells. In contrast, the levels of IFN γ , IL-6 and IL-8 synthesized by NK cells were significantly decreased in PBC compared to controls (92).

Adhesion Molecules and Lymphocyte Recruitment

The recruitment of mucosal lymphocytes, previously activated in the gut, to the liver via interaction with ectopically expressed adhesion molecules and chemokines, has emerged as an important step in the pathogenesis of PSC (93). Expression of the adhesion molecule MAdCAM-1 and chemokine CCL25 is normally restricted to the gut; but in PSC, MAdCAM-1 is also found on portal endothelium and CCL25 on sinusoidal endothelium, and ~20% of liver-infiltrating lymphocytes express their cognate receptors $\alpha 4\beta 7$ integrin and CCR9, respectively (94, 95). We have shown that activation of VAP-1, which is

constitutively expressed in human liver, and further up-regulated in the presence of inflammation, can lead to NF- κ B activation in hepatic endothelial cells and in the presence of pro-inflammatory TNF α , in the expression of MAdCAM-1 (96), thereby promoting the recruitment of α 4 β 7 + mucosal effector cells to the liver (Figure 1). Notably, VAP-1 can catabolize a broad range of substrates, and several gut commensals and enteric pathogens such as *Bacteroides fragilis*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *E. coli* and *Clostridium perfringens*, secrete other branched chained amines which may be putative substrates of VAP-1, thus providing another potential link between the microbiota, mucosal immunity and the pathogenesis of PSC (97).

Most of CCR9+ cells are IFN γ producing long-lived memory cells (CD45RA-CCR7-CD11a^{hi}) (94). Apart from CCL25, PSC patients also show altered expression of CCL28, CXCL12 and CXCL16 chemokines. CCL28 and CXCL12 trigger α 4 β 7-mediated adhesion of human lymphocytes to MAdCAM-1 *in vitro* (98, 99), and CCL28 can also activate α 4 β 1 integrin thus increasing its adhesion to VCAM-1, which is primarily expressed in the portal and sinusoidal endothelial cells of the liver, and further upregulated during development of PSC. Once lymphocytes have entered the liver, mucosal lymphocytes may use other chemokines such as CXCL12 and CXCL16 to localize to biliary epithelium where they can destroy bile ducts. Notably, some of the recruited α 4 β 7+ T cells may undergo local differentiation to express α E β 7 integrin providing another pathway to bind biliary epithelium (95). Previous studies have demonstrated the inability of human hepatic DCs and stellate cells to induce α 4 β 7 integrin and CCR9 expression on T cells, which clearly shows that the ability to imprint naïve lymphocytes with gut tropism is restricted to intestinal CD103+DCs (100). In a recent murine study, hepatic endothelial cells, which also possess the ability to present antigen, were able to prime naïve T cells and induce α 4 β 7 and CCR9 expression on CD4+ T cells in a retinoic acid dependent manner (101).

AIH can co-exist with IBD (102), and ~60% of patients with chronic AIH also demonstrate MAdCAM-1 expression on portal vessels (95). MAdCAM-1 can also be detected on the portal and sinusoidal vessels in some patients with PBC and is

associated with a high frequency of circulating and intrahepatic CD8⁺ effector-memory cells expressing the gut-homing integrin $\alpha 4\beta 7$, which respond to the PBC-specific autoantigen, pyruvate dehydrogenase E2 (PDC-E2) (103). Serum from PBC patients cross-reacts with mucosal antigens and immune responses against intestinal microbes may be promoted by the finding of increased intestinal permeability and defective barrier function in PBC (104). This has further led to the suggestion that PBC may also be triggered by exposure to entero-bacterial antigens (105).

T and B lymphocytes and Adaptive Immunity

The interface hepatitis in AIH is characterized by a striking infiltrate of lymphocytes, plasma cells and monocytes/macrophages, suggestive of an autoaggressive cellular immune attack playing a key role in the pathogenesis of AIH. Studies have revealed a predominance of $\alpha\beta$ T cells (106), with the majority of them being CD4 helper T cells and a sizeable minority being CD8 cytotoxic suppressor T cells. NK cells, monocytes/macrophages, $\gamma\delta$ T cells and B lymphocytes are also detected (107). A considerable number of cells producing IL-17, which is a potent pro-inflammatory cytokine, are also present in the AIH inflammatory infiltrate (108). Reduced numbers of NKT cells are detected in AIH patients, especially during active disease, and these cells produce less immunoregulatory cytokine IL-4 (109). IL-4 is a potent inhibitor of Th17 development therefore impairment of the IL-4 pathway may be critical in favouring a pro-inflammatory milieu in which Th17 cells thrive.

The PBC liver is heavily infiltrated by CD4⁺ and CD8⁺ T lymphocytes (110); both CD4 and CD8 lymphocytes can be purified from biopsy samples of PBC patients and both subsets recognize epitopes of PDC-E2 (111). A predominant type-I cytokine pattern with high levels of IFN γ , IL-5, IL-6, IL-10, IL-12 and IL-15 in the blood and liver of PBC patients has been demonstrated (112). The portal tracts in PBC are rich in chemokines CXCL10, CXCL9 and CX₃CL1, which are responsible for recruiting CD4 and CD8 T cells that bear their cognate receptors CXCR3 (for CXCL9 and CXCL10) and CX₃CR1, respectively. Although both T cell subsets seem

to recognize similar sequences within the same epitope of the lipoyl domain, supporting a common etiological trigger (110), it is believed that CD8⁺ T cells play a role in the degeneration and death of cholangiocytes that aberrantly express PDC-E2 (113). Moreover, an increase in specific CTLs in the liver compared to the peripheral blood has been reported, which supports the role of these cytotoxic cells in the evolution of bile duct injury in PBC (114). Using recombinant fragments of PDC-E2 it has been demonstrated that there is a sequence overlap in the PDC-E2 specific T and B cell epitopes (115). Recently, high frequencies of CD8⁺ effector-memory cells expressing the gut-homing integrin $\alpha 4\beta 7$ have been detected in the peripheral blood of PBC patients (103). These T cells were shown to accumulate around the portal area and respond specifically to the MHC class I epitope of PDC-E2. However, there does not appear to be an association of PBC and IBD, aside from occasional case report (116).

In PSC, a mixed inflammatory cell infiltrate consisting of lymphocytes, plasma cells, neutrophils (particularly intense around bile ducts), natural killer cells (in portal infiltrates), Kupffer cells and perisinusoidal macrophages is detected (117, 118). However, the majority of mononuclear cells in the portal infiltrates are T lymphocytes (119) that produce high levels of TNF α , supporting PSC as a predominantly Th1-mediated disease (89). An increased proportion of $\gamma\delta$ ⁺ T cells in PSC patients has been also observed, with these cells expressing CD45RO and IL-2, suggestive of an activated memory phenotype (120).

Th17 cells have been linked to PBC, AIH and PSC (108, 121-123). Activated Th17 cells secrete IL-17, IL-21 and TNF α , which promote inflammation via the recruitment of leukocytes, including neutrophils, and also participate in epithelial repair by secreting IL-22. Th17 cells are abundant in the intestinal lamina propria where they are induced by commensal bacteria and provide protection against invading pathogens (124). In mice, peripheral Th17 cells can be redirected from the periphery to the small intestine via CCR6-CCL20 interactions; in humans CCL20 is expressed on inflamed bile ducts, thus suggesting that the same chemokine pathway might promote accumulation of

Th17 cells in the inflamed liver (125). CD161 expression by human T cells correlates with IL-17 secretion and CD161+ T cells include Th17 CD4+ T cells, CD8+ Tc17 cells and IL-17 producing MAIT cells (126). The implications of the Th17 biliary microenvironment in PBC have been recently emphasized (127).

In AIH, preliminary studies indicate circulating and intrahepatic Th17 are numerically expanded and that the expression of Th17 cytokines (IL-17, IL-23, IL-6 and IL-1 β) is significantly increased within the liver of AIH patients compared to chronic hepatitis B (CHB); serum levels of IL-17 and IL-23 are also reported to be significantly elevated in patients with AIH, than in CHB and healthy controls (108). IL-17 induces IL-6 expression via the MAPK signalling pathway in hepatocytes, which in turn may further stimulate Th17 cells, triggering a positive feedback loop (108). It is believed that an inflamed microenvironment, especially at the site of damage appears to favour phenotypic and functional conversion of Tregs into Th17-like effector cells (128).

Katt and colleagues (122) have recently demonstrated a very elegant study in which they showed bacterial RNA within the portal tracts of PSC patients but not in patients with chronic HCV or AIH controls. In PSC patients but not in PBC, stimulation of PBMCs with heat-inactivated bacteria led to a marked induction of Th17 responses. In particular, stimulation of PBMCs with inactivated *C albicans* led to the highest expression of IL-17A in up to 30% of CD4+ T cells, and more Th17 cells were found to co-express IFN γ after stimulation with *E faecalis* or *C Albicans*. Very interestingly, IL-17A expressing lymphocytes were found localized around bile ducts in PSC patients. Notably, cholangiocytes express the receptors for IL-17A and upon stimulation with this cytokine they produce IL-1 β , IL-6 and IL-23 pro-inflammatory cytokines, which in turn can promote the survival of Th17 cells, but also induction of periductular fibrosis (129, 130). Interestingly, polymorphisms on the genes CARD9 and REL, which encode for molecules involved in Th17 cell differentiation and transduction of signals received by TLR and dectin-1/bacterial and fungal PAMPs, have been recently identified from GWAS analyses as non-MHC loci associated with PSC (22).

Notably, Tregs and Th17 cells share the same CD4 progenitor, which in the presence of TGF β only develops into Tregs, while in the presence of TGF β and IL-6 differentiates into Th17 cells (131). To note development of Th17 cells is suppressed by IFN γ and IL-4. A decreased reactivity of CD4+CD25^{high} natural regulatory T cells appears to contribute to a number of human autoimmune diseases (132). Defects in the number and function of intrahepatic and peripheral blood T regulatory cells have been demonstrated in some studies in AIH and PBC although other studies report no functional deficit (123, 133, 134).

In AIH, Tregs are numerically decreased and compared to healthy controls, CD4+CD25^{high} cells have a lower *in vitro* ability to expand and to control CD4+CD25⁻ T cell proliferation (135). Additional functional studies have demonstrated the inability of AIH Tregs to regulate IFN γ production by CD4 and CD8 T cells, and their impaired capability to control activation of monocytes, which are abundantly present in the intrahepatic inflammatory infiltrate (136). Other studies, however, have reported no differences in the frequency and function of CD127-CD4+CD25^{high} Tregs in AIH compared to healthy subjects (134).

A relative reduction of Tregs compared with healthy controls has been detected in PBC, as the ratio of hepatic Tregs over hepatic CD8⁺ cells in PBC patients was lower than that in patients with chronic hepatitis C (137). Moreover, observations have shown a reduced ratio of intrahepatic Tregs to effector CD8⁺ and Th17 cells (123, 137). Taken together these findings certainly suggest that a lack of functional Tregs contributes to a breakdown in self-tolerance.

A reduction in the frequency of peripheral blood Treg cells, and in intrahepatic Foxp3⁺ cells has also been detected in PSC patients, with an apparent impaired suppressive capacity (138).

Transporter Defects, Defective Bile Acid Secretion and Cholestasis

Bile is a complex mixture of bile acids, bilirubin, cholesterol, phospholipids and proteins, which even under normal conditions can be toxic to the cells; thus

several protective mechanisms have been developed to protect cholangiocytes from injury (139). Bile acids that would induce apoptosis and necrosis to cholangiocytes normally form mixed micelles with phosphatidylcholine and cholesterol to prevent bile acid toxicity (6). Impairment of transporters that are responsible for maintaining the bile acid/phospholipid ratio (MDR3 or BSEP) or bicarbonate excretion and hydration of bile (CFTR or AE2) can potentially lead to toxic bile formation and damage of cholangiocytes (140) (141) (84) (142). Variants and functional mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) have been described in patients with PSC (142). Bile stasis, a frequent phenomenon in PSC, may also lead to toxic bile formation leading to exacerbation of bile duct injury (6).

In PBC, reduced expression of the chloride-bicarbonate anion exchanger AE3/SLC4A2, the sodium-hydrogen exchanger NHE/SLC9A3, and the inositol 1,4,5-triphosphate receptor has been demonstrated (143-145). The expression of MRP4, molecule involved in basolateral export, is induced 3-fold in PBC. As the condition progresses, sodium-taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP) (key transporters for hepatic bile acid uptake and excretion) are increased and expression of organic anion transporting polypeptide 2 (OATP2) is decreased (146, 147). These adaptive mechanisms may be an effort to prevent accumulation of toxic bile acids in chronic cholestasis, and a possible target for future therapeutic strategies e.g. Farnesoid X receptor agonists.

Mechanisms of injury

The mechanisms underlying the breakdown of self-tolerance in autoimmune diseases have not been fully elucidated, though there is mounting evidence that a defect in homeostatic processes, that normally keep the response to self-antigens under control, is involved. PBC is a disease of small bile ducts, affecting the lining epithelial cells, the cholangiocytes. In PBC, these cholangiocytes show irregular shape and arrangement with infiltration of mononuclear cells. Bile ducts are eventually lost and cholestasis becomes chronic. It still remains unclear what are the exact factors that lead to the development of biliary specificity, albeit a

combination of cholangiocyte apoptosis, cellular senescence and autophagy might be involved (148).

Apoptosis of cholangiocytes has been described as a potential contributing factor in the pathogenesis of cholangiopathies (149). In PBC, the small rather than the large bile duct cholangiocytes are more susceptible to apoptosis. In most cells in the human body that undergo apoptosis, the mitochondrial PDC-E2-autoantigen undergoes covalent modification by glutathione. However, in PBC, cholangiocytes undergoing apoptosis fail to bind glutathione to the lysine-lipoyl residue of the dehydrogenase complex, thus preserve the antigenic epitope as it is (150), and subsequently translocate intact, immunologically active PDC-E2 to apoptotic bodies, creating an “apotope” (151). In PBC, this apotope is recognized by circulating AMA autoantibodies and the resulting AMA-autoantigen complex may then stimulate the innate immune system in genetically susceptible individuals. Interactions between apotopes, macrophages, and serum can lead to a potent production of injurious cytokines such as TNF α , which can exacerbate apoptosis of neighbouring cells (90). The intact PDC-E2 in apoptotic fragments can be also taken up by local antigen-presenting cells and transferred to regional lymph nodes for the priming of the cognate T cells. Recent reports have shown that apoptotic cholangiocytes can be phagocytosed by neighbouring cholangiocytes in PBC, consequently providing an additional source of autoantigens (152). Cholangiocytes express intact PDC-E2 and MHC and respond to cytokines that induce their apoptosis, thus they are vulnerable to ongoing attack (Figure 2). Therefore, cholangiocytes have been implicated as effector cells in the loss of immune tolerance via the impaired phagocytic clearance of apotopes (153). This may explain the efficacy of UDCA (ursodeoxycholic acid), which acts by decreasing biliary apoptotic rate, sustaining bile flow, and enhancing apotope clearance (154). Transgenic mice expressing PDC-E2 on the surface of cholangiocytes do not develop spontaneous hepatobiliary lesions, thus is unlikely that aberrant PDC-E2 alone is pathogenic (155).

Similar to apoptosis, permanent withdrawal from the cell cycle (i.e. senescence) functions as a protective mechanism to remove damaged cells from the

population. The accumulation of senescent cholangiocytes has been identified in both PBC and PSC (156, 157). In PBC, cholangiocytes show increased expression of the markers of cell senescence such as senescence-associated beta-galactosidase (SA- β gal), p16^{INK4A}, p21^{Waf1/Cip1}. Furthermore, a significant decrease in telomere length has been observed in cholangiocytes lining damaged bile ducts and bile ductules in PBC (158). The exact mechanism of how cellular senescence contributes to duct loss in PBC is incompletely understood. After cellular senescence occurs in injured cholangiocytes they are not replaced by normal cells (158) but they are transitioning to a “senescence-associated secretory phenotype” (SASP), characterized by the robust secretion of chemokines (CX₃CL1, CXCL8, CCL2), cytokines (IL-6, IL-1), growth factors and matrix metalloproteinases (MMPs) that function in repair/remodelling and recruiting of immune cells (159) (Figure 2). Cellular senescence is also seen in ductular reaction, which is thought to harbour hepatic stem cells (HSCs) in PBC (156). Therefore the inability of senescent HSCs to proliferate may thus fail to replace damaged cholangiocytes, thereby exacerbating bile duct loss. Interestingly, chronic liver allograft rejection, which is characterized by bile duct loss akin to PBC, also shows similar biliary epithelial senescence (160). An increase in the number of senescent cells in PSC patients compared to the IBD and control groups has been also observed (161).

Abnormal autophagy may also result in autoimmune disease, whereby autophagy-related processing of self-proteins provides a source of immunostimulatory molecules and autoantigens. Upregulated autophagy has been reported in the damaged bile ducts in PBC, the initiation of autophagy being associated with reduced stress-induced cellular senescence (162).

In AIH patients there is significant evidence for innate immunity as being the initial trigger that precipitates the immunopathology, much like the Con A mouse models. This is critical as it suggests a very rapid onset and perhaps a short latency time between an environmental exposure and induction of pathology. Monocytes/macrophages represent a major component of the portal/periportal cellular infiltrate in AIH. Studies have reported that monocytes in peripheral

circulation of AIH patients have a vigorous spontaneous migration, which cannot be further augmented by migration-inducing stimuli (136). Moreover, monocytes in AIH show a higher TNF α over IL-10 production and an elevated TLR4 expression, which suggest a more pro-inflammatory phenotype. Interestingly, the finding of a marked monocyte activation during active disease, a time when also CD4 and CD8 T cell autoimmune responses are at their highest in terms of proliferation and IFN γ production, suggests a monocyte participation in the pathogenesis of liver damage in AIH, possibly promoted by autoreactive cells belonging to the adaptive arm of the immune system (136). An increase in $\gamma\delta^+$ T cells is also detected in AIH, which show an inverted V δ 1/V δ 2 ratio and a higher IFN γ and granzyme B production, the latter being correlated to biochemical indices of liver damage, suggesting a prevailing effector over regulatory function for these cells in this condition (109). It should be noted that in the case of PBC while innate immunity may be critical in the early phases, there appears to be a significant delay between the appearance of autoantibodies and the development of clinical symptoms. Therefore, in AIH it is believed that innate immune cells play a role in the loss of immune tolerance and further perpetuation of the autoimmune attack. Of note, a successful adaptive response, whether a normal immune response or an autoimmune response requires innate immunity. Break of tolerance in AIH can terminate a previously unresponsive state of liver-related autoantigens and further presentation of such self-antigenic peptides to uncommitted T helper (Th0) lymphocytes by HLA class II molecules on APCs, such as macrophages, DCs and B lymphocytes. This renders Th0 lymphocytes to become activated; in the presence of IL-12 they differentiate into Th1 cells, or in the presence of IL-4 into Th2 cells, further initiating a series of immune reactions determined by the cytokines they produce. Th1 cells predominantly secrete IL-2 and IFN γ , the latter being the main orchestrator of tissue damage because of its ability to stimulate cytotoxic lymphocytes (CTL), enhance HLA class I molecule expression on APCs and of HLA class II molecules on hepatocytes (163) and activate monocytes/macrophages, which in turn release IL-1 and TNF α . The induction of HLA class II on hepatocytes, enables them to present the autoantigen to Th1 cells and hence further perpetuate the autoimmune process. The function of Th1 cells is counterbalanced by the Th2

cells, which arise in the presence of IL-4 and mainly produce IL-4, IL-10 and IL-13. These cytokines induce also the maturation of B cells into plasma cells, with consequent production of autoantibodies. If regulatory cells are numerically deficient and/or impaired to perform their suppressor function, these effector responses are perpetuated with ensuing persistent liver cell destruction by the direct action of CTL, cytokine release by Th1 cells and monocytes/macrophages, complement activation and engagement of NK cells by the autoantibodies bound to the hepatocyte surface (Figure 3) (164).

Animal Models

There are several murine models that simulate features of human PBC, including spontaneous models such as the NOD.c3c4 mouse, mice depleted of dnTGF β on a CD4 promoter, and mice with knocked out gene for IL-2 R (IL-2R α KO mice) (165-168).

Research on the pathogenesis of AIH has been hampered by the lack of animal models reproducing faithfully the human condition. Recent studies have focused on animal models of AIH type 2, since the autoantigen is well defined (169). C57BL/6 female mice are immunised with a plasmid containing the antigenic region of human CYP2D6, the target of anti-LKM1, and formiminotransferase cyclodeaminase, the target of anti-liver cytosol-1, an additional marker for AIH type 2 (107). Another model of AIH type 2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an adenovirus-CYP2D6 vector (170). However, a model mimicking closely AIH in humans is still missing.

A reliable and reproducible single animal model for PSC is still needed. A classification scheme for animal models has been described by Pollheimer et al (65). In the existent PSC animal models, cholangitis is induced by enteric bacteria cell wall components, infectious agents (such as *Cryptosporidium parvum*), biliary obstruction, chemicals (such as lithocholic acid), knock out genes such as *Mdr2* or *Cftr* (in mice) or primary biliary and endothelial cell injury (65). Mice injected with death receptor 5 agonists have also provided insight into apoptosis in cholestatic liver disease (90).

Concluding Remarks

The mechanisms responsible for the pathogenesis of autoimmune liver diseases are still poorly understood. Evidence suggests that all three autoimmune diseases are the result of a complex interaction between genetic and environmental factors. Given the plethora of genetic susceptibility loci that have been identified in PBC and PSC in particular, efforts now are needed to translate genetic risk into true biology. This when combined with understanding environmental triggers/microbiome influences, and patterns of immune homeostasis, should provide better insights into the mechanisms of disease such that new rational therapies can be applied in the future.

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Figure Legends

Figure 1. Mechanisms of tissue injury in PSC. (a) Enterohepatic circulation brings potential mediators of inflammation, such as microbes or metabolites of enteric microbiota from the gut to the hepatic sinusoids, where sinusoidal endothelial cells (SECs), Kupffer cells (KC), hepatocytes, hepatic stellate cells (HSCs), dendritic cells and monocytes (M) through their pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), can recognize and respond to pattern associated molecular patterns (PAMPs). Biliary epithelial cells (BECs) express multiple TLRs, which (b) upon pathogen recognition produce $\text{TNF}\alpha$, IL-6 and IL-8, in order to (c) promote recruitment and activation of T cells, monocytes/macrophages (M), neutrophils (N) and natural killer cells (NK) that will initiate biliary repair responses. (d) Endogenous molecules released from injured or dying cells (damage-associated molecular patterns, DAMPs, heat shock proteins) can activate TLRs and/or DAMP receptors (DAMP R). Components of bile such as oxysterols, can activate cholangiocytes to express IL-6 and IL-8. (e) Exposure of cholangiocytes to LPS may cause disruption of tight junctions exposing cholangiocytes to a variety of substances, such as bile acids, that could eventually promote injury and inflammation. (f) Autoantibodies against biliary epithelial cells can activate the immune system by upregulating TLRs, increasing expression of IL-6 and adhesion molecules such as CD44 that could thereby promote lymphocyte proliferation, Ig production and cell adhesion. (g) $\text{TNF}\alpha$ -rich microenvironment can induce the loss of CD28 expression from T cells, leading to accumulation of CD28-ve T cells, which are able to release $\text{TNF}\alpha$ and $\text{IFN}\gamma$, perforin and granzyme B, that act upon biliary epithelial cells inducing activation and death. (h) PSC hepatic NK cells have decreased cytolytic activity, likely because of the high levels of local $\text{TNF}\alpha$ production. (i) Activation of vascular adhesion protein 1 (VAP-1) in the liver, in the presence of pro-inflammatory $\text{TNF}\alpha$, can induce expression of MAdCAM-1, thereby promoting the recruitment of $\alpha 4\beta 7^+$ mucosal effector cells to the liver. (j) Bacterial RNA has been detected within the portal tracts of PSC patients; activation of PSC PBMCs with heat-inactivated bacteria has led to a marked induction of Th17 responses. (k) Th17 cells have been found around bile ducts in PSC patients. In humans, CCL20 is expressed on inflamed bile ducts, thus able to

recruit CCR6⁺ Th17 cells. Cholangiocytes express the IL-17R and upon stimulation with IL-17A cholangiocytes produce IL-1 β , IL-6 and IL-23 pro-inflammatory cytokines, which (l) promote the survival of Th17 cells, but also stimulate fibroblasts to induce periductular fibrosis. (m) Increased senescence has been reported in PSC patients compared to IBD and control groups.

Figure 2. Mechanisms of tissue injury in PBC. (a) The portal tracts in PBC are rich in chemokines CXCL10, CXCL9 and CX₃CL1, which are responsible for recruiting CD4 and CD8 T cells, as well as NK cells, that bear their cognate receptors CXCR3 (for CXCL9 and CXCL10) and CX₃CR1, respectively. (b-e) Autoantigenic stimuli provided by bacterial mimics of the PDC-E2 autoepitope, xenobiotically modified PDC-E2 or “spillage” of native mitochondrial autoantigens derived from biliary epithelial apoptotic cells, can be presented by APCs via MHC class II to autoreactive CD4⁺ T cells. (f) CD4⁺ T cells in turn can activate (g) CD8 cytotoxic T lymphocytes able to damage biliary epithelial cells. (h) Tregs that normally restrain the activated autoreactive T cells are reduced in PBC. (i) Activation of PDC-E2 specific B cells leads to their differentiation into plasma cells and production of anti-mitochondrial antibodies (AMA). (j) Interactions between BEC apoptoses, macrophages, and serum AMA can lead to potent production of injurious cytokines such as TNF α , which can exacerbate apoptosis of neighboring cells and recruitment of immune cells. (k) In PBC, high frequencies of NK cells compared to healthy controls have been detected. Hepatic macrophages previously primed with TLR3,-4 ligands, can induce activation of NK cells and thus enhance their cytotoxicity against BECs. (l) It has been suggested that the NK cell-mediated lysis of BECs is associated with the TRAIL-R5-TRAIL pathway. (m) In PBC, cholangiocytes show increased expression of the markers of cell senescence such as senescence-associated beta-galactosidase (SA- β gal), p16^{INK4A}, p21^{Waf1/cip1}. After cellular senescence occurs in injured cholangiocytes they are not replaced by normal cells but they are transitioning to a “senescence-associated secretory phenotype” (SASP), characterized by the robust secretion of chemokines (CX₃CL1, CXCL8, CCL2), cytokines (IL-6, IL-1), growth factors and matrix metalloproteinases (MMPs) that function in repair/remodelling and recruiting of immune cells.

Figure 3. Mechanisms of tissue injury in AIH. (a) Significant evidence suggests innate immunity as being the initial trigger that precipitates the immunopathology in AIH patients. Monocytes from peripheral circulation of AIH patients show a vigorous spontaneous migration, with high TNF α over IL-10 production and elevated TLR4 expression, suggesting a highly pro-inflammatory phenotype. NK cells have been also detected in the interface hepatitis, and are responsible for antibody-mediated cellular toxicity. $\gamma\delta$ T cells are also elevated in AIH; cells that pursue antigenic targets on the hepatocyte without prior sensitization or after presentation by nonclassical MHC, and show high IFN γ and granzyme B production, further suggesting a prevailing effector over regulatory function. (b) Loss of tolerance in AIH can terminate a previously unresponsive state of liver-related autoantigens and further presentation of such self-antigenic peptides to uncommitted T helper lymphocytes (Th0) by antigen presenting cells (APCs). The Th0 lymphocytes exposed to the antigen presented by an HLA class II molecule on APC, becomes activated; in the presence of IL-12 Th0 differentiate into a Th1 cells, whereas in the presence of IL-4 into Th2 cells. (c) Th1 cells predominantly secrete IL-2 and IFN γ , the latter being able to stimulate cytotoxic lymphocytes (CTL), which are antigen-specific and release cytokines within the liver that promote hepatocyte death. Th1 cells also enhance HLA class I molecule expression on APCs and HLA class II molecule on hepatocytes, and activate monocytes/macrophages, which in turn release IL-1 and TNF α . The induction of HLA class II on hepatocytes enables them to present the autoantigen to Th1 cells, and further perpetuate the autoimmune response. (d) Th2 cells, which arise in the presence of IL-4, mainly produce IL-4, IL-10 and IL-13 cytokines, (e) which induce the maturation of B cells into plasma cells. The clonal expansion of plasma cells results in excess production of immunoglobulins, which then bind to normal membrane constituents of the hepatocytes, and induce complement activation, engagement of NK cells and hepatocyte death. (f) Intrahepatic Th17 cells are numerically expanded in AIH, where they release IL-17, IL-23, IL-6 and IL-1 β . IL-17 induces IL-6 expression in hepatocytes, which in turn may further stimulate Th17 cells, thus triggering a positive feedback loop. (g) Studies have reported that Tregs are numerically decreased and functionally defective in AIH,

although others have shown that the frequency and function of AIH Tregs is not impaired. (h) Reduced frequencies of NKT cells in AIH patients have been reported, which produce less immunoregulatory IL-4, a potent inhibitor of Th17 cell development.

Figure 1
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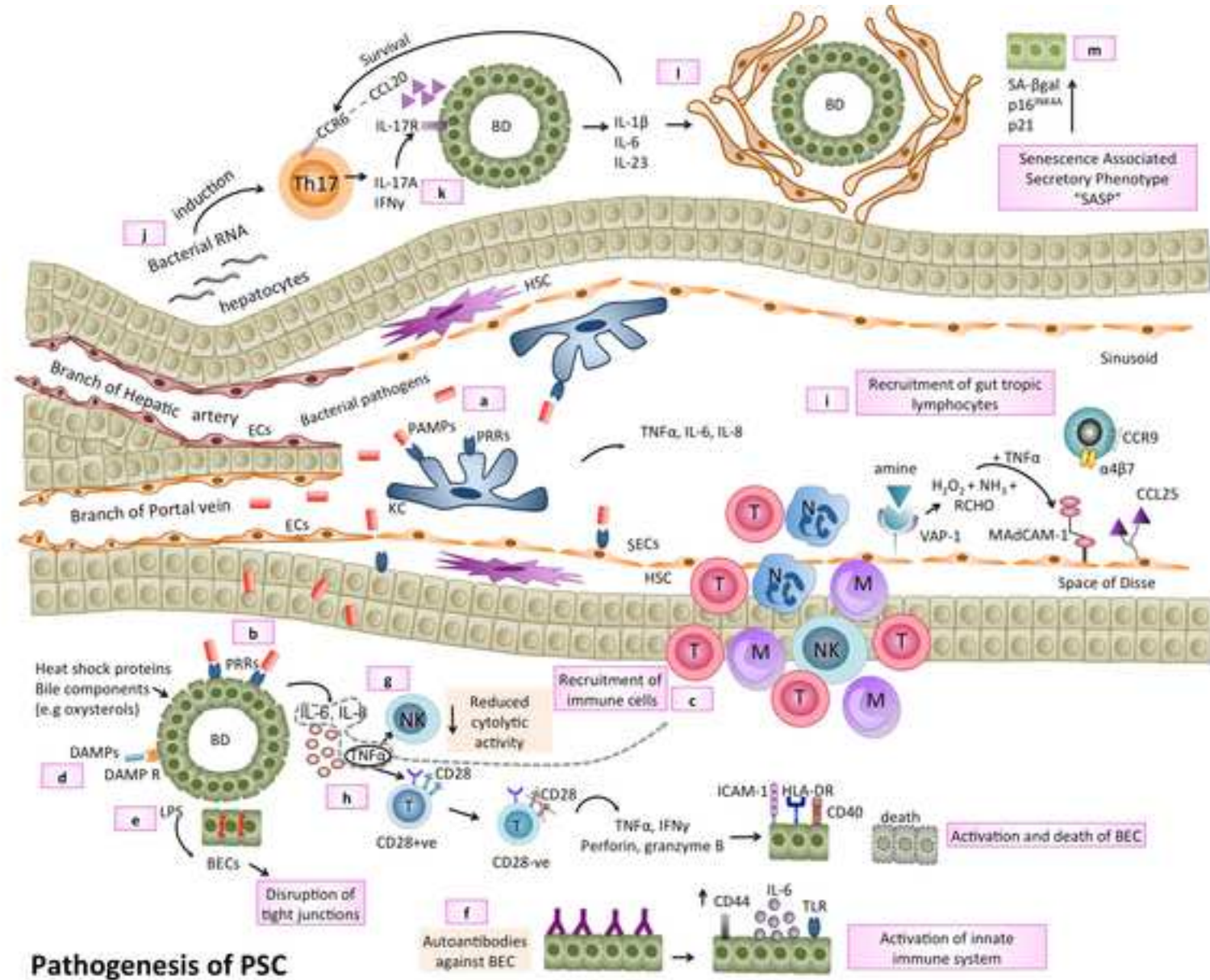
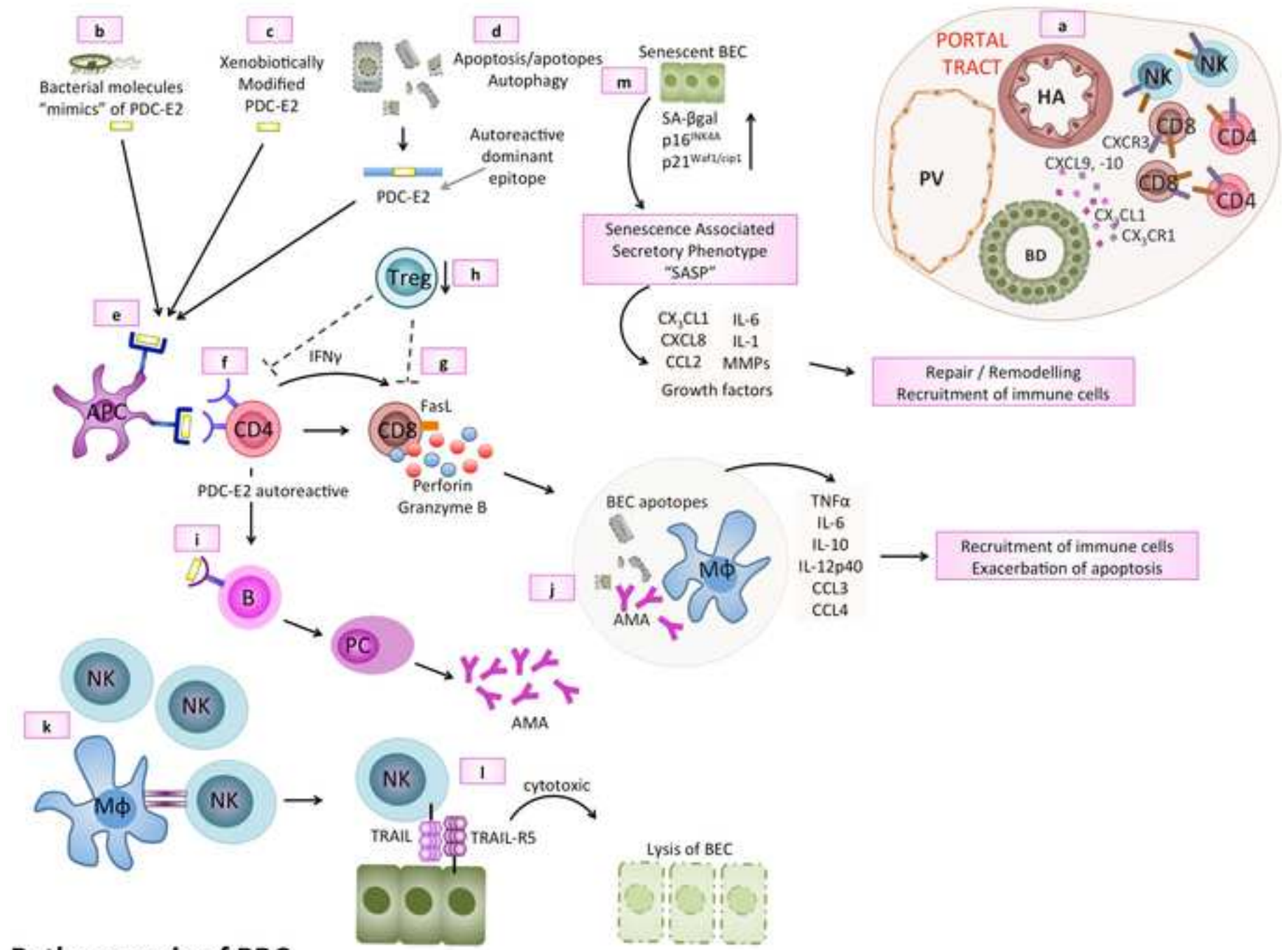
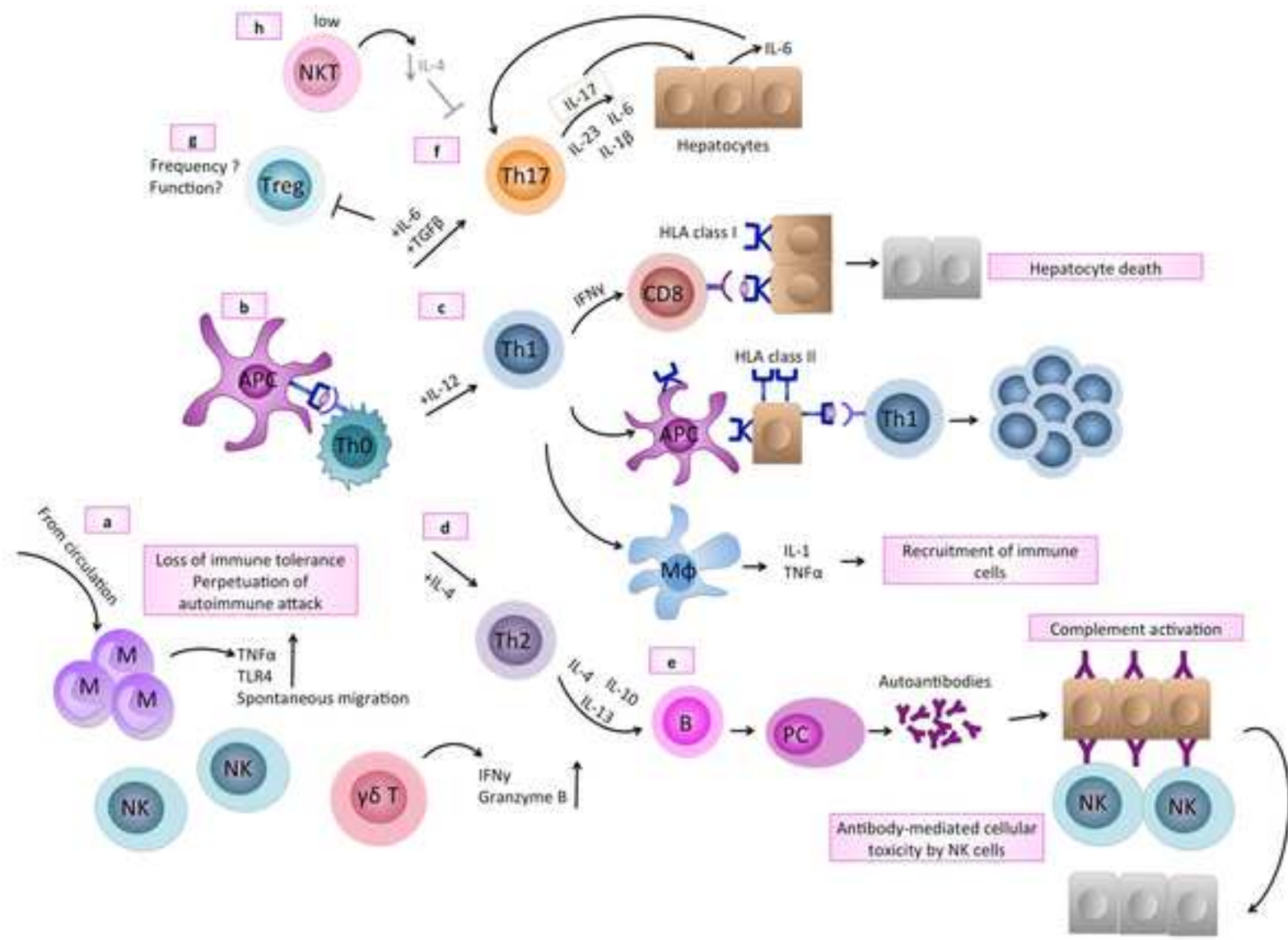


Figure 2
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Pathogenesis of PBC

Figure 3
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Pathogenesis of AIH