

REVIEW

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Immunobiology of primary sclerosing cholangitis

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic inflammatory progressive cholestatic liver disease. Genetic risk factors, the presence of autoantibodies, the strong clinical link with inflammatory bowel disease, and associations with other autoimmune disorders all suggest a pivotal role for the immune system in PSC pathogenesis. In this review, we provide a comprehensive overview of recent immunobiology insights in PSC. A particular emphasis is given to immunological concepts such as tissue residency and knowledge gained from novel technologies, including single-cell RNA sequencing and spatial transcriptomics. This review of the immunobiological landscape of PSC covers major immune cell types known to be enriched in PSC-diseased livers as well as recently described cell types whose biliary localization and contribution to PSC immunopathogenesis remain incompletely described. Finally, we emphasize the importance of time and space in relation to PSC heterogeneity as a key consideration for future studies interrogating the role of the immune system in PSC.

Keywords: cholestatic liver diseases, liver immunology, fibrosis, single-cell methods, autoimmunity

KEY POINTS

- Using time and space axes, we position the current immune knowledge in PSC and highlight areas to be further explored.
- Most insights originate from the liver of patients after diagnosis and/or fibrotic end-stage disease, where a number of single-cell and spatial immune characterizations have been performed.
- Mononuclear phagocytes, Th17 T cells, and neutrophils close to the bile duct and within the fibrotic areas are some of the most documented cell types where modification of their numbers, location, and function is observed in PSC.
- Studies on intercellular and inter-organ crosstalk, such as the immune-cholangiocyte and gut-liver axes, reveal important propagators of disease activity and warrant further research.

Abbreviations: CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; cDC, conventional DC; DC, dendritic cell; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FoxP3, forkhead Box P3; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; IFN, interferon; ILC, innate lymphoid cell; MAIT, mucosal-associated invariant T; MR1, major histocompatibility complex, class I-related; NK, natural killer; NKT, natural killer T; PSC, primary sclerosing cholangitis; scRNAseq, single-cell RNA sequencing; ST, spatial transcriptomics; STAT, signal transducer and activator of transcription; Tregs, T regulatory cells; TRM, tissue-resident memory T cells; UDCA, ursodeoxycholic acid.

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GENERAL INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory progressive cholestatic liver disease affecting intrahepatic and/or extrahepatic bile ducts, eventually leading to cirrhosis.^[1] Patients with PSC also stand at high risk of developing biliary tract malignancies.^[1] Many patients with PSC have concomitant inflammatory bowel disease (IBD), and overlap with autoimmune hepatitis occurs.^[1,2] Several genetic risk factors, including a strong human leukocyte antigen (HLA) association, implicate the immune system in PSC.^[3] Beyond this, patients with PSC frequently have autoantibodies, although their role in diagnosis is minor.^[4] All of this together suggests a pivotal role for the immune system, both in the development and progression of PSC. However, it is in this context intriguing that immunomodulatory treatments have limited to no effect on PSC, which contrasts with many other immune-mediated disorders.

Immune cells are abundant in the human liver, which is notably enriched for innate immune cells when compared with peripheral blood.^[5,6] This includes specialized liver macrophages (KCs), natural killer (NK) cells, and unconventional T cells such as CD1d-restricted natural killer T (NKT) cells in mice and mucosal-associated invariant T (MAIT) cells in humans.^[5,6] Beyond this, it is now clear that both fractions of myeloid cells and lymphocytes represent tissue-resident cells adapted to the hepatic microenvironment.^[5,6] Recent technological advancements in single-cell technologies, such as single-cell RNA sequencing (scRNAseq) have improved our understanding of liver immunology by enabling highly granular identification of many discrete subpopulations of immune cells. Indeed, several such single-cell resources now exist for late-stage PSC.^[7,8] Alongside this, the revolution in spatial biology has illustrated that numerous gradients of immune cells exist at the microscopic level in liver lobules^[5] and that the biliary niche represents yet another distinct immune compartment containing specialized myeloid cells and intraepithelial lymphocytes.^[9,10] Also here, spatial resources are now available on end-stage disease PSC livers.^[7,11] These resources and others hold promise for guiding future research regarding the role of the immune system in PSC pathogenesis.

Moving forward, in this review, we will discuss recent immunological insights into PSC pathogenesis, spanning from findings in murine experimental models to translational human studies and implications for immunomodulation in treatment. Recent immunological concepts such as tissue residency and the importance of the spatial microenvironment for cells will be covered in relation to PSC in the upcoming sections that relate to major innate and adaptive immune cell types. As an outlook, unresolved issues will be brought up and open questions for the future will be outlined, with a particular emphasis on time and space in contextualizing PSC disease heterogeneity.

IMMUNE CELLS IN PSC

In the following sections, current knowledge and possible roles for different innate and adaptive immune cells in PSC pathogenesis will be discussed, incorporating findings from both murine experimental systems and human translational studies (Figure 1).

Neutrophils

Neutrophils, the most abundant immune cell type in the human blood, are essential mediators of inflammatory innate immune responses. Most notably known for their role in resolving acute infection through phagocytosis and degranulation, neutrophils also shape the adaptive immune responses through chemokine and inflammatory cytokine production.^[12] While neutrophil accumulation in tissues is essential for combating bacterial and viral infections, neutrophils have been shown to contribute to the immunopathology of many chronic inflammatory conditions.^[13,14] Indeed, elevated levels of neutrophils in the bile ducts of patients with PSC, both at earlier and later disease stages, represent an important immunological hallmark of disease pathology.^[9] In the *Mdr2*^{-/-} mouse model, a commonly used mouse model for PSC pathogenesis studies where bile secretion is affected, neutrophils have been shown to be important propagators of biliary inflammation.^[15] Immunofluorescence staining of liver biopsies from *Mdr2*^{-/-} mice revealed increased presence of activated neutrophils and formation of neutrophil extracellular traps (NETosis) compared with wild-type mice.^[15] In this model, chemical inhibition of neutrophil elastase proved hepatoprotective, as indicated by decreased serum levels of ALP and ALT.^[15] Regarding neutrophil trafficking, elevated C-C motif chemokine ligand (CCL)24 expression from cholangiocytes and liver macrophages in *Mdr2*^{-/-} mice has been shown to promote neutrophil recruitment to the inflamed bile ducts.^[16] Subsequently, the use of a CCL24-neutralizing antibody abrogated neutrophil recruitment to the bile ducts and mitigated liver fibrosis.^[16] In the human setting, CCL24 was found to be enriched in liver biopsies of patients with PSC.^[16] In addition, CCL24 serum levels correlated with Enhanced Liver Fibrosis score.^[16,17] IL-8, a chemokine that attracts neutrophils through CXCR1/2, was also found to be elevated in the bile of patients with PSC and to correlate with the number of neutrophils in bile ducts of patients with PSC.^[9,18] Together, these cytokines and chemokines represent important modulators of neutrophilic infiltration in PSC, and blocking the accumulation of neutrophils in the bile ducts of patients with PSC represents a promising therapeutic avenue for mitigating biliary inflammation.^[19]

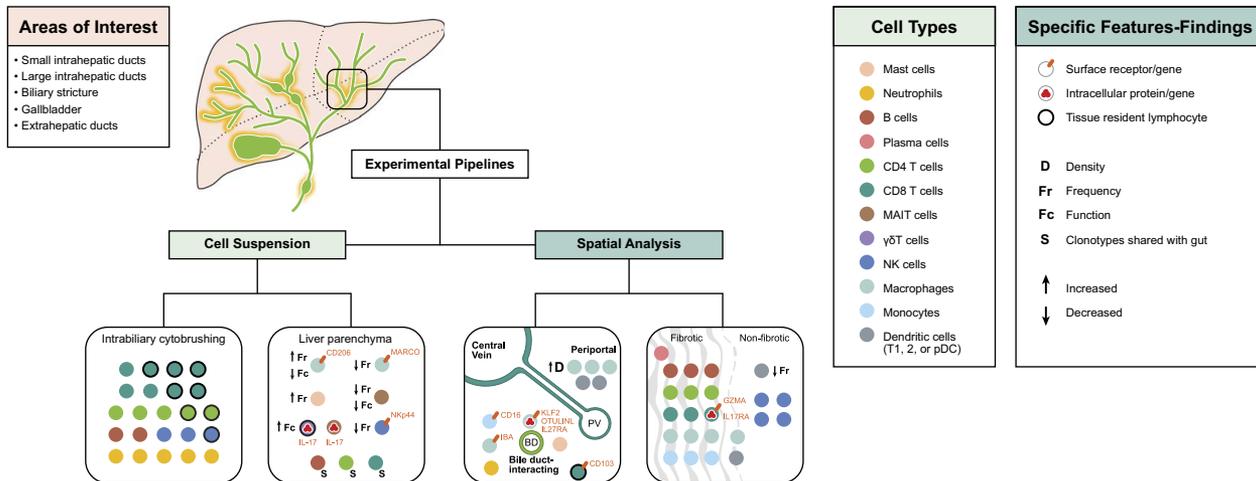


FIGURE 1 Overview of PSC immunobiology. Summary of the immune features from PSC livers is presented in extended circles depending on the techniques used. These mainly arise from studies on parenchyma of advanced PSC livers, whereas less is known regarding extrahepatic bile duct, intrahepatic and extrahepatic strictures, gallbladder, or intrahepatic small duct areas as indicated by the areas of interest box. Immune cells and specific features/findings are depicted by colored circles, symbols, and letters. Analysis of cell suspensions shows the immune composition of the biliary tree (cytobrushing) and the global modifications of the immune system in the liver parenchyma. Spatial analysis highlights differences within the liver based on a central to portal (PV) axis, bile-duct interacting (BD) cells, as well as in fibrotic versus nonfibrotic areas. Abbreviations: BD, bile duct; PSC, primary sclerosing cholangitis; PV, portal vein.

Pathogenic IL-17 signaling has been implicated as a propagator of biliary inflammation in PSC (Th17 responses are discussed more in subsequent sections covering T cells),^[8,20,21] yet the specific role of neutrophils in driving IL-17 production in PSC remains to be elucidated. Neutrophils are capable of IL-17 production and have been shown to produce IL-17 in different contexts.^[22,23] More importantly, neutrophils have been shown to skew T-cell differentiation toward a Th17 phenotype through alarmins and histones contained within NETs,^[12] as well as through IL-23 production.^[24] Alongside this, studies in mice leveraging intravital microscopy have demonstrated that IL-17 (synergistically with TNF) mediates neutrophil rolling and endothelial transmigration through selective upregulation of E-selectin and expression of neutrophilic chemokines CXCL1, CXCL2, and CXCL5 by endothelial cells.^[25] Taken together, these findings suggest a positive feedback loop in which neutrophils are recruited to inflamed bile ducts, thereby skewing resident T cells toward a Th17 phenotype, which further propagates neutrophil recruitment to the inflamed bile duct. Therefore, the extent to which this occurs in PSC patients represents an important direction for research regarding the immunopathogenesis of PSC.

Although neutrophils are short-lived, their heterogeneity has recently been explored in the context of infection and cancer. Indeed, these studies have uncovered vast cellular heterogeneity upon emergency granulopoiesis or when neutrophils enter tissues.^[26–28] Immature neutrophils can display immunosuppressive effector functions relative to their mature counterparts, which can display a more proinflammatory phenotype.^[29,30] While *Mdr2*^{-/-} mice primarily contained mature and activated

CXCR2^{high}*CXCR4*^{low}*CD62L*^{low} neutrophils,^[15] limited studies have been conducted to assess biliary neutrophil heterogeneity in humans. One recent study investigating neutrophil heterogeneity in chronic liver diseases uncovered a subset of *CXCR4*^{hi} *CD62L*^{low} aged neutrophils termed ductular reaction-associated neutrophils or “DRANs.”^[31] These cells accumulate at the site of ductular reaction in patients and display a proinflammatory transcriptomic profile in 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-fed mice.^[31] Lastly, depletion of these neutrophils (with either an anti-Ly6G antibody or a *CXCR1/2* antagonist) in the DDC-fed mouse liver mitigated ductular reaction, fibrosis, and angiogenesis, demonstrating that these neutrophils are important propagators of ductular reaction in chronic liver diseases.^[31] Thus, further research into the phenotypic and functional heterogeneity of biliary neutrophils in patients with PSC is warranted. Beyond directly studying neutrophils in relation to PSC, it will also be important to determine the impact bacterial cholangitis, a feature of patients with PSC, has as a secondary late phenomenon on neutrophil infiltration and function.

Mononuclear phagocytes

The mononuclear phagocyte system mainly comprises monocytes (blood circulating), macrophages/KCs (tissue-resident), and dendritic cells (DCs), all of which display phenotypic and functional heterogeneity.^[32] Collectively, these cells contribute to tissue repair and resolution of inflammation, especially through the clearance of pathogens and cell debris involving phagocytosis, but can also cause inflammation.^[32] They

further play a pivotal role due to their specific secretome, interactome, and capacity to perform antigen presentation that can modulate both the innate and adaptive compartments of the broader immune system.^[32] Monocytes from the blood of patients with PSC have been reported to display an increased capacity to secrete IL-1 β and IL-6 upon microbial stimulation as compared with healthy individuals with consequences for T-cell responses (discussed below).^[33] Circulating monocytes patrol and infiltrate the inflamed liver and have the capacity to differentiate and become tissue macrophages/KCs.^[34] In this context, CD16⁺ monocytes (receptor binding to the Fc portion of IgG antibodies) were seen preferentially accumulated in the liver of patients with PSC^[35] and located around bile ducts.^[33] Spatial transcriptomics (ST) and scRNAseq studies have recently provided further details on the transcriptional profiles and locations of monocyte and macrophage subsets in PSC. A first ST study reported an increased number of monocytes in fibrotic regions associated with a modification of the tissue monocyte subsets, suggesting a preferential infiltration of fibrotic areas with higher frequencies of tissue monocytes (*IL17RA+S100A8+FCER1G+*) and KCs (*VCAM1+SDC3+*).^[11] A second ST study further described the infiltrate around the biliary tract, showing an increased density of inflammatory macrophages (expressing *CD209*, *CCL4*, *IL1B*, and *FCGR3A*).^[36] A third study combining ST and scRNAseq reported a general increase in macrophage diversity as well as several PSC-specific clusters predominantly of monocyte-like macrophages. Upregulation of *INFR2*, *CXCL16*, and *CCR1* was noticed in PSC. More specifically, macrophages had increased expression of inhibitory molecules *KLF2*, *OTULINL*, and *IL27RA*, whereas monocyte-like macrophages were described to display a “fibrosis-associated phenotype” with the upregulation of *LGALS3*, *SPP13*, and *ADA2*. ST indicated an enrichment of these cells around cholangiocytes in fibrotic regions, whereas KCs were localized outside further completing the picture of cell subsets associated and possibly contributing to the fibrotic processes. Beyond phenotyping, macrophages were further described in the same study as hypofunctional with a decreased response to lipopolysaccharide (assessed by CD45⁺CD68⁺CD206⁺TNF⁺ cells using flow cytometry).^[7] Besides ST and scRNAseq studies, immunohistochemistry using the pan-macrophage marker CD68 showed an accumulation of such cells in both parenchymal and peribiliary areas of PSC livers as compared with control livers.^[37] Finally, an increased percentage of CD206⁺ macrophages was found in PSC livers in comparison with controls^[7,35] fewer MARCO⁺ macrophages (macrophage receptor with collagenous structure, a scavenger receptor), although the total number of macrophages was comparable.^[38,39] Altogether, these data suggest that spatial diversity of

mononuclear phagocytes within the PSC livers might be globally affected rather than particular types of cells being excluded from specific areas. Along those lines, an increased macrophage diversity was reported in PSC together with the identification of several PSC-specific clusters of monocyte-like cells.^[7] These studies suggest that monocytes and macrophages from fibrotic areas have the potential to both secrete and respond differently to the microenvironment and that this might contribute to the propagation of the immunofibrogenic process.

Several animal models have described the importance of macrophages in shaping cholestatic and fibrotic processes. Of note, the importance of IBA⁺ macrophages seen in humans was also confirmed in the *Mdr2*^{-/-} model as they were described to originate from infiltrating monocytes, increased in number as compared with wild type, and located close to the bile duct.^[40] Furthermore, inhibition of the C-C motif chemokine receptor (CCR)2/CCL2 axis (using CCR2 agonist and *Ccr2*^{-/-} mice), a major monocytic recruitment pathway, attenuated biliary injury in the BV6 (acute sclerosing cholangitis following intrabiliary instillation of BV6 which sensitizes for apoptosis) and *Mdr2*^{-/-} models^[37] and deletion of farnesoid X receptor in myeloid cells (*Mrd2*^{-/-} mice) led to the identification of a macrophage-Th1/17 axis associated with a sclerosing cholangitis phenotype. Finally, the Trem2-expressing monocytes/macrophages subset,^[41,42] as well as the expression of *Arid3*^[43] and *CCL24*^[16] in macrophages, were identified as important in driving the severity of cholestatic models. However, conditional depletion of KCs (using *Clec4f*^{DTR} transgenic mice) before cholestasis induction by DDC diet and common bile duct ligation had no effect on disease outcome, thus contesting these cells as major drivers of early pathogenesis.^[44] Overall, data from mouse models mostly suggest a causative role of these cells in driving fibrosis development in the context of cholestasis, although considerable differences exist in the composition of the mononuclear phagocyte system across species.

Beyond monocytes and macrophages, DCs have been studied to some extent in PSC. They comprised several subsets, such as plasmacytoid DC, and 3 main conventional DC subsets with distinct phenotypes and functions.^[45,46] DC density has been reported as increased in the portal area of patients with PSC as compared with liver disease controls, and mouse models of cholangitis reported an increase of intrahepatic conventional DC2 (cDC2) but not cDC1 or plasmacytoid DC.^[47] In humans, deconvolution of ST data from livers of patients with PSC indicated a decreased proportion of cDC1 (not cDC2) in fibrotic regions as opposed to the parenchyma.^[11] In contrast, the deconvolution of DCs from RNAseq of PSC livers suggested an increase in conventional DCs with PSC disease stages.^[40] Finally, the assessment of DCs in PSC livers using scRNAseq

identified these cells as one of the disease-associated cell types (cell subset frequency increased in PSC, both plasmacytoid and conventional) as compared with normal livers.^[7] However, compared with our knowledge of monocytes and macrophages, much less is known about DCs in PSC. If and how they crosstalk with conventional and unconventional T cells or B cells warrants further studies. Similarly, priming events taking place either in the gut or in liver/bile duct-draining lymph nodes remain relevant to assess.

In summary, the last decade has provided insights into the immune infiltrate of fibrotic PSC livers, and mononuclear phagocytes have repeatedly been identified as key cell types in shaping liver fibrosis. The most recent studies have further revealed mononuclear phagocyte transcriptional profiles possibly imperative to inflammation and fibrosis observed in PSC. As these detailed phenotypes should be further confirmed and their functional implication better explored, whether these are a consequence of the liver damage or driver of the disease is currently unknown. Comparisons of PSC myeloid cell transcriptomic signatures to those of fibrotic controls from other liver diseases, alongside mechanistic studies exploring the role of such signatures will help us better understand the involvement of mononuclear phagocytes in PSC pathogenesis.

Mast cells

The role of mast cells in the progression of liver diseases, including cholestatic diseases, has recently been reviewed and involves these cells possessing the capacity to release a broad range of molecules acting on immune and parenchymal cells such as cytokines (TNF, IL-1 β , IL-6, IL-8, and TGF- β), peptidases, and other mediators such as histamine, leukotriene, and prostaglandins.^[48,49] Mast cell infiltration has been reported in the livers of patients with PSC by immunohistochemistry near bile ducts^[50] and portal tracts.^[51] Mast cells have further been identified as one of the disease-associated cell types using scRNAseq of cirrhotic PSC livers compared with normal livers.^[7] However, most of the current knowledge on these cells comes from animal models where their role in cholangitis and fibrosis has been described. Using the *Mdr2*^{-/-} model, inhibition of mast cell-derived histamine,^[52] antagonism of histamine receptors,^[53] inhibition of the apical sodium BA transporter (expressed on mast cells),^[54] treatment with ursodeoxycholic acid (UDCA),^[55] or cromolyn sodium^[50] all support a role for mast cells in cholangitis and fibrosis progression.

Innate lymphoid cells

Innate lymphoid cells (ILCs) are immune cells enriched in mucosal barriers defined by their rapid production of

effector cytokines in response to epithelial stress signals and other myeloid-derived cytokines.^[56–59] From an effector standpoint, ILCs mirror conventional T helper cell subsets in their cytokine production yet notably lack T-cell receptors and, therefore, antigen specificity.^[56–59] Canonical ILC subsets include NK cells, ILC1s, intraepithelial ILC1s, ILC2s, natural cytotoxicity receptor-negative ILC3s, and natural cytotoxicity receptor-positive ILC3s.^[56–59] In contrast to peripheral blood, where terminally differentiated CD56^{dim} NK cells are abundant, CD56^{bright} NK cells and other ILC subsets predominate in tissues.^[6,60] In humans, CD56^{bright} NK cells expressing CD69, CXCR6, and the transcription factor Eomes, termed liver resident NK cells, represent the most abundant liver ILC subset, constituting up to 35% of all lymphocytes in the healthy human liver.^[61–63] Work investigating peripheral blood NK cells in patients with PSC found that terminally differentiated CD57⁺ CD56^{dim} NK cells were decreased relative to healthy controls.^[64] In addition, the reduction of CD57 on peripheral CD56^{dim} NK cells correlated with serum bilirubin and ALP levels.^[64] However, whether these cells traffic to bile ducts remains to be determined. Recently, an HLA-DP risk haplotype has been described in PSC directly related to NK-cell functionality.^[65] After confirming HLA-DPA1*02:01-DPB1*01:01 binding to NKp44 (an activating NK-cell receptor) with a bead-based assay, it was shown that plate-bound HLA-DPA1*02:01-DPB1*01:01 induced significantly higher degranulation of primary NKp44⁺ NK cells *in vitro*.^[65] This activation was specifically inhibited through an anti-NKp44 blocking antibody, uncovering a possible novel inflammatory axis in PSC.^[65] Of note, while HLA-DP expression was increased in PSC liver explants, the frequency of NKp44⁺ NK cells was decreased in these livers relative to nonautoimmune liver disease controls.^[65] Therefore, the extent to which NK cells localize to inflamed bile ducts in PSC, and whether they contribute to immunopathogenesis, remains to be elucidated. Along similar lines, little is known about non-NK ILCs in PSC, both in peripheral blood and in the inflamed liver and biliary tract system.

Unconventional T cells

While granulocytes likely represent transiently infiltrating effector cells responding to inflammation, human bile ducts are populated by tissue-resident lymphocytes that likely reside *in situ* for many years at a time, similar to other organ systems.^[5,63] Conventional tissue-resident memory T cells (TRMs) (discussed more in detail in the upcoming section) have been shown to be the most abundant lymphocyte population in human bile ducts.^[9] However, a plethora of other innate lymphocytes and unconventional T cells have been described

in human bile ducts, with their abundance and phenotype altered in the context of PSC.

MAIT cells, NKT cells, and gamma delta T ($\gamma\delta$ T) cells represent 3 classes of unconventional T cells identified in the human bile ducts, with each of these subsets responding to different classes of antigens. MAIT cells, prevalent in the human liver, recognize microbially derived vitamin metabolites presented by MR1, an MHC class I-like molecule, and exhibit potent Th1 and Th17 responses.^[66] NKT cells, prevalent in the murine liver, are restricted by lipid antigens presented by CD1d and are also capable of Th1 and Th17 responses.^[67] Finally, $\gamma\delta$ T cells can recognize a host of different antigens, including phosphoantigens and stress-induced ligands such as MICA/MICB.^[68,69] In the context of PSC, circulating MAIT cells were found to be substantially reduced in abundance relative to control patients.^[70] While they displayed a highly activated phenotype *ex vivo*, *in vitro* analysis revealed diminished interferon (IFN)- γ and TNF responses compared with MAIT cells from healthy controls.^[70] Indeed, subsequent *in vitro* experiments coculturing bile from patients with PSC with peripheral blood mononuclear cells, with and without MR1-blockade, demonstrated that MAIT cell activating antigens are present in the bile of patients with PSC.^[71] In this context, recent work has suggested that sulfated bile acids represent a host-ligand for MAIT cells.^[72] However, it remains to be determined if those bile acids are dysregulated in PSC. Furthermore, recent work outside of the liver and PSC suggests that MAIT cells not only contribute to proinflammatory processes but can also have an active role in wound healing.^[73] Future studies on such reparative (or fibrosis-causing) processes are warranted in PSC.

Similar to that of MAIT cells, bile from patients with PSC has also been shown to contain lipid antigens activating NKT cells.^[74] Diluted bile samples from patients with PSC added to murine NKT cells induced IL-2 production, and this activation was inhibited by blocking CD1d.^[74] To further investigate the role of NKT cells in PSC immunopathology, researchers used the NKT cell activating agent oxazolone in a murine model.^[75] Oxazolone injection into the bile duct increased liver fibrosis in a CD1d-dependent manner since CD1d^{-/-} mice were protected against weight loss, increased ALT, and inflammation.^[75] While the described studies concerning NKT cells have implicated their importance in PSC immunopathology, their abundance and possible deregulation in the human liver remain to be detailed. Regarding $\gamma\delta$ T cells, Mdr2^{-/-} mice have been shown to have increased levels of IL-17+ $\gamma\delta$ T cells in the liver compared with controls.^[76] Alongside this, *i.v.* injection with an antibody directed against the $\gamma\delta$ T-cell receptor reduced fibrosis in Mdr2^{-/-} mice with a corresponding decrease of IL-17 detected in the serum.^[76] In humans, intrahepatic $\gamma\delta$ T cells from

patients with PSC could produce IL-17 upon stimulation, in contrast to $\gamma\delta$ T cells from patients with other liver diseases.^[76] This suggests that $\gamma\delta$ T cells might be part of the dysregulated Th17 axis in PSC.

In summary, the liver is enriched in unconventional T cells, and they could represent important contributors to inflammation and fibrosis progression. Species differences exist between mice and humans (high numbers of NKT in mice but low MAIT and vice versa in humans and more potent Th17 responses in mice), and consolidation of findings from murine models in translational settings is therefore of importance.

Conventional T cells

Many of the identified PSC susceptibility genes can be related to the function of adaptive lymphocytes and T cells in particular (eg, the strong HLA association as well as *CTLA4/CD28*, *IL2/IL21*, *IL2RA*, *SIK2*, *PTPN2*, *BACH2/MIR4464*, *HDAC7*, *SH2B3*, *MMEL1/TNFRSF14*, *CCL20*, *FOXP1*, *CD226*, *PRKD2*, and *CCDC88B*).^[1,77,78] Yet many of the PSC risk-variants reside in noncoding regions of the genome, making it harder to link them directly to pathogenesis. To address this, a recent study made use of cell-type-specific epigenomic data and assessed if the identified SNPs could contribute to epigenetic dysregulation and found an enrichment of risk-variants within immune-cell-specific enhancers involved in T-cell responses.^[79] An additional link between genetics, T cells, and PSC came from the identification of a family with autosomal dominant PSC having a missense-mutation in *SEMA4D* (semaphorin 4D) encoding CD100.^[80] Biliary-infiltrating T cells were further shown to be the main CD100-expressing cells, and the mutation boosted Th17 differentiation.^[20] More support for a dysregulated Th17-axis in PSC comes from both human translational studies and murine models. A large study relying on brush samples from endoscopic retrograde cholangiopancreatography revealed an enrichment of TRMs with the capacity to produce IL-17 in large bile ducts of both patients with early and end-stage PSC.^[9] The increase of biliary TRMs in PSC has been confirmed by subsequent work as well as positively associated with disease severity.^[81] A role for the transcription factor Runx3 in driving this phenotype has also been suggested.^[81] Furthermore, scRNAseq of cirrhotic PSC compared with control livers have identified an enrichment for naïve-like CD4 T cells epigenetically primed for Th17 differentiation in advanced PSC.^[8] The PSC risk variant associated with the *BACH2/MIR4464* loci was also recently shown to increase the likelihood of naïve T cells polarizing toward a proinflammatory phenotype.^[82] Spatial assessment of cirrhotic PSC livers revealed enrichment of both Th17 cells and cytotoxic T cells in fibrotic compared with parenchymal areas.^[11] Beyond Th17, studies of Mdr2^{-/-} mice have also

suggested a role for Th1-responses with enrichment of intrahepatic cytotoxic T cells and NK cells as well as exaggerated IFN- γ production and amelioration of disease upon depletion of T cells or cytotoxic molecules.^[83–85]

The dysregulated Th17-axis has been linked to proinflammatory cytokines and diminished T regulatory cell (Treg) function. As discussed above, monocytes respond to microbial dysbiosis in PSC with enhanced production of IL-1 β and IL-6 that subsequently promote Th17 cells and IFN- γ responses from T cells.^[33,86] IL-6 signaling in CD4 T cells occurs through STAT3 and increased phosphorylated STAT3 can be found around bile ducts in PSC livers compared with controls.^[86] However, while JAK1/2 inhibitors experimentally blocked STAT3 and diminished the elevated IFN- γ responses, no effect on IL-17 was seen.^[86] Reduced and dysfunctional Tregs in PSC might be one factor allowing for elevated proinflammatory signaling. In more detail, the risk SNP in the *IL2RA* gene is associated with reduced circulating Tregs in PSC. One mechanism could be “less stable” Tregs because of weaker IL-2 signaling, reduced forkhead Box P3 (FoxP3) induction, and a lower level of FoxP3 demethylation.^[87,88] Similar results have been obtained in *Mdr2*^{-/-} mice with ensuing increased proinflammatory signals.^[89]

What is then the origin of the pathogenic Th17 cells and where are they primed? Early work suggested gut priming, possibly then connected to dysbiosis, followed by liver-homing of T cells.^[90] However, more recent studies have shown that such a homing mechanism might not be specific to PSC.^[91] Nevertheless, TRMs in the bile ducts of patients with PSC express a combination of gut and liver-homing chemokine receptors.^[9] Break of tolerance in *Mdr2*^{-/-} mice by genetic removal of the inhibitory ectoenzyme CD39 promoted influx of T cells expressing the gut-homing integrin $\alpha 4\beta 7$ to the liver.^[92] Intriguingly, a proportion of T cells in the gut and liver also share the same T-cell receptor clonotypes suggestive of a common origin.^[93]

Altogether, this suggests that the balance between tolerance and reactivity with respect to T-cell responses is offset in PSC, leading to exaggerated Th17 and Th1 responses. However, most work until today has been performed in end-stage disease, not fully taking the distinct niches existing within the liver into account.

B cells

Compared with T cells, fewer studies have focused on the possible pathogenic role of B cells, despite the clinical overlap between PSC and many autoimmune disorders. Nevertheless, work deconvoluting B cells from bulk RNAseq revealed an increased B-cell signature in advanced PSC livers.^[40] These B cells could, in ST analysis of cirrhotic PSC livers, be localized to fibrotic

regions as opposed to the parenchyma.^[11] Furthermore, a similar clonal overlap as has been described for T cells is also evident for B cells in the gut and liver.^[94] These clones had relatively shorter CDR3 lengths and a high degree of somatic hypermutations as compared with non-overlapping clonotypes, suggesting antigen-driven activation.^[94] Similar findings have been reported in another study focusing on the intestinal mucosa where PSC colitis was characterized by an increased proportion of IgG-secreting plasma cells, not seen to the same degree in non-PSC-IBD, where both IgG and IgA clones showed signs of affinity maturation.^[95] In *Mdr2*^{-/-} mice, circulating IgG and hepatic B cells increased upon fibrosis progression, and these hepatic B cells produced IgG in the absence of any stimulation.^[96] Moreover, in these mice, a much higher number of hepatic antibody-secreting cells was observed as compared with wild type, and this was associated with fibrosis progression.^[96] Dominant clonotypes were uniquely present in intrahepatic B cells from fibrotic as compared with nonfibrotic areas of the liver or splenic compartments, and analysis of somatic hypermutation rates in hepatic B cells suggested a liver-specific selection of the BCR repertoire occurring during hepatic fibrosis.^[96] Finally, *Mdr2*^{-/-} mice also had aberrant antibody concentration, elevated autoantibodies (ANA), and immune complexes, mimicking some of the features of patients with PSC. The depletion of B cells by anti-CD20 strongly reduced fibrosis, levels of IgG, ANA, immune complexes, and hepatic immune infiltrate,^[96] a therapeutic strategy that might have some bearing in humans.^[97] Furthermore, in vitro cultures of PSC explants also identified antibody-secreting cells secreting autoantibodies, and flow cytometry analysis revealed increased plasmablasts but fewer plasma cells as compared with primary biliary cholangitis.^[98–100] Finally, IgG4+ lymphoplasmacytic infiltration has been reported in PSC and seems to be associated with the presence of more severe disease.^[101] Indeed, more than 20% of explanted PSC livers contain increased IgG4+ periductal plasma cells.^[102] This is coupled with increased levels of antibodies against food and animal antigens in patients with PSC with high IgG4 levels, similar to IgG4-related disease.^[103] Taken together, although B-cell dysregulation is evident in PSC and elevated IgG in the blood commonly is seen in patients, mechanistic insights into such dysregulation remain to be elucidated. Furthermore, we are still lacking knowledge of the possible functional pathogenic roles of autoantibodies.

CROSSTALK BETWEEN PARENCHYMA AND IMMUNE CELLS

While immune cells certainly contribute to PSC immunopathology, hepatocytes and cholangiocytes undergo phenotypic and functional changes in PSC that likely drive biliary inflammation and re-structuring and, therefore, disease trajectories. In addition to essential physiological functions, hepatocytes and cholangiocytes can sense

pathogens through pattern recognition receptors and secrete antimicrobial peptides and inflammatory cytokines to initiate immunologic defenses.^[104,105] These immune functions are, therefore, critical for containing and controlling infection in the liver but may also contribute to sustained biliary damage upon persistent and chronic inflammation.^[104] Hepatocytes and cholangiocytes can interact with immune cells directly via antigen presentation and indirectly through cytokine secretion. Hepatocytes and cholangiocytes can express MHC class I molecules and are therefore capable of antigen presentation to CD8+ TRMs, the most abundant lymphocyte subset in human bile ducts.^[9,106,107] In mouse models, activated hepatocytes are capable of MHC class II upregulation for antigen presentation to CD4+ TRMs.^[108] MHC class II expression by cholangiocytes has also been observed in patients with PSC.^[109] As previously discussed, bile from PSC livers contains MAIT and NKT cell antigens, and cholangiocytes have been shown to present antigens to NKT cells through CD1d and to MAIT cells through MR1.^[71,74,107] Nevertheless, the exact role of hepatocyte and cholangiocyte antigen presentation in coordinating immune responses through classical and nonclassical MHC molecules remains unclear in humans and requires further study. As we understand today, other cell types, such as KCs and DCs, represent the professional antigen-presenting cells that initiate adaptive immune responses in the liver and biliary tract.^[106]

Single-cell and spatial technologies have, in recent years, shed light upon nonimmune and immune phenotypes and interactions in PSC. Notably, a single-cell atlas has been generated using technologies including scRNA-seq, single nuclear RNA sequencing, and ST on cirrhotic PSC livers to uncover novel cellular subsets associated with inflammation.^[7] One major finding was that CK7+HNF4A+ cholangiocyte-like hepatocytes were enriched in PSC livers relative to control livers.^[7] These trans-differentiating hepatocytes were marked by high levels of TNF and IFN- γ signaling, emphasizing their role in orchestrating immune-mediated inflammation in PSC.^[7] Additional novel findings include endothelial-mediated recruitment of monocyte-derived macrophages, which displayed an immunosuppressive phenotype in contrast to recruited lymphoid subsets in PSC livers.^[7] This research highlights the immunological complexity of PSC and provides a key data set for researchers to explore specific pathways of inflammation in PSC in more detail.

In recent years, cholangiocyte organoid models have been employed to study cholangiocyte-immune interactions *ex vivo*. scRNAseq of extrahepatic cholangiocyte organoids identified 8 cholangiocyte clusters *ex vivo*, including an inflammatory cluster marked by the expression of chemokines and TNF superfamily proteins.^[21] Of note, all 8 of these clusters were shared between PSC and non-PSC-derived organoids.^[21] However, PSC-derived cholangiocyte organoids were marked by an increased number of differentially expressed genes in response to IL-17

treatment relative to non-PSC-derived cholangiocyte organoids.^[21] In addition, supernatants from PSC cholangiocyte organoids were enriched for inflammatory proteins such as IL-6 and CXCL9, demonstrating their potential inflammatory phenotype *in vivo*.^[21] Beyond phenotypic assessment of cholangiocyte organoids *ex vivo*, cholangiocyte organoid-immune cell cocultures have been used to uncover potential pathogenic interactions between these cell types driving inflammation in PSC.^[20] Specifically, cholangiocytes were shown to mediate cleavage of CD100 expression on T cells, which resulted in a phenotypic skewing of these resident T cells toward a Th17 phenotype.^[20] Taken together, cholangiocyte organoids—and systems leveraging cholangiocyte-immune cell coculture—hold promise for discovering how interactions between cholangiocytes and immune cells propagate biliary inflammation and fibrosis in PSC.

Immune cells also have important effector functions targeting cholangiocytes. scRNAseq of PSC liver explants and *Mdr2*^{-/-} mice uncovered the role of CD8+ T cell and NK-cell-derived granzyme B (cytotoxic effector molecule) and TRAIL (apoptosis-inducing molecule) in PSC immunopathogenesis, both of which were elevated in PSC livers.^[83] *Mdr2*^{-/-} mice genetically lacking granzyme B demonstrated reduced cholangiocyte apoptosis, fibrosis, and liver injury relative to *Mdr2*^{-/-} control mice.^[83] Conversely, *Mdr2*^{-/-} mice genetically lacking TRAIL displayed elevated IFN- γ and granzyme B expression alongside elevated ALT levels and cholangiocyte apoptosis relative to *Mdr2*^{-/-} control mice.^[83] Further research into how inflammatory cytokines from recruited or resident immune cells modify cholangiocytes is therefore warranted.

Bi-directional cholangiocyte-immune cell crosstalk is, therefore, an important feature of PSC immunopathogenesis. Inflammatory cues from both cholangiocytes and immune cells likely contribute to a positive feedback loop of inflammation. Interrupting the cascade of inflammatory signaling at this axis holds promise in ameliorating structuring, fibrosis, and liver failure in PSC. Future studies should, therefore, explore this axis further. More sophisticated methods, such as “organ-on-a-chip” models incorporating multiple immune cell types and other supportive parenchymal cells, hold promise in elucidating these interactions between immune and nonimmune cells in more detail.

IMPACT OF DYSBIOSIS, A LEAKY GUT, AND INTESTINAL INFLAMMATION ON BILIARY IMMUNE CELLS

The intricate relationship between PSC and IBD is difficult to dissect. Current studies estimate that 2%–7% of patients with IBD could have a concomitant PSC, often diagnosed later in life. In addition, 60%–90% of patients with PSC have IBD with specific features and represent a

relatively milder progressive phenotype.^[110,111] In this context, the relationship between the gut and the liver, including its immunobiology, has been studied, mainly focusing on the intestinal microbiota, the mucosal inflammation, the associated leaky gut, and bile acid homeostasis. In this context, the shared clonal overlap of both T cells and B cells (both discussed above) between gut and liver is intriguing.^[93,94]

Intestinal inflammation and associated gut dysbiosis are thought to disrupt bile acid homeostasis,^[112,113] a feature described in patients with PSC.^[114,115] Moreover, these bile acids have been shown to modulate the balance of Th17 cells and Tregs in experimental models.^[112,116] Recently, right-sided colonic biopsies from patients with PSC-IBD and IBD analyzed by bulk RNAseq, scRNAseq, and flow cytometry identified distinct features of intestinal inflammation in PSC-IBD compared with IBD where PSC-IBD inflammation was characterized by the presence of IL-17+FoxP3+ CD4 T cells as well as IgG-secreting plasma cells.^[95] In addition, even in the absence of IBD, an increased expression of *IL17A* and *IFNG* in intestinal biopsies and a dysbiotic microbiota was reported in the vast majority of patients with PSC.^[117] However, a study analyzing biliary brush samples from patients with PSC revealed no difference in biliary neutrophil or T-cell abundance in relation to IBD status.^[9] Gut inflammation increased mucosal permeability and translocation of bacterial product could potentially cause biliary inflammation. In such a context, an enhanced response of cholangiocytes to endotoxins has been reported in patients with PSC.^[118] In addition, human cholangiocytes exposed to bile from patients with PSC, which often contained microbial DNA, were able to in some cases activate MAIT cells in an MR1-dependent manner.^[71]

PSC microbiota studies have revealed a global decrease in diversity associated with an overrepresentation of specific strains^[119–122] and some functional specificities.^[123] Results from germ-free mouse models suggested both protective and aggravated effects on cholestasis progression due to the microbiota.^[124,125] However, fecal microbiota from patients with PSC-IBD transferred to gnotobiotic mice led to, in some cases, activation of Th17 cells in the liver.^[126] These mice had increased susceptibility to DDC diet-induced hepatobiliary injury with higher serum bilirubin, ALP, and Th17 response in the liver as compared with germ-free mice.^[126] 16s rRNA sequencing of mesenteric lymph nodes identified *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus gallinarum* in those mice as well as in metagenomic analysis of PSC patient microbiota as compared with controls.^[126] In DDC-fed gnotobiotic mice colonized with these bacteria, inhibition of Th17 differentiation ameliorated hepatobiliary injury and fibrosis.^[126] Interestingly, *K. pneumoniae* derived from mesenteric lymph nodes was found to induce pores in colonic epithelial cells in vitro, and the use of a *K. pneumoniae*

non-pore-forming strain in the 3-bacterial mix inoculated into germ-free mice led to decreased Th17 response in the liver.^[126]

Although most patients with PSC also have IBD, the shared and specific immunobiological features of these entities remain poorly characterized. In recent years, studies have provided data on the microbiota composition of patients with PSC as well as their inflammatory landscape of the gut. Still, the mechanistic involvement of the liver and gut immune systems in this context remains poorly understood.

IMMUNE-FOCUSED THERAPIES IN PSC—PAST, PRESENT, AND FUTURE

To date, no medical therapy has proven to have a significant impact on clinical outcomes in PSC.^[1] There are critical obstacles to the development of new therapies, such as a lack of statistical power and the slow and variable progression of the disease. Clinical trials are also hindered by the lack of disease activity markers associated with prognosis that can be used as endpoints. The window of treatment opportunity in PSC is unclear, and the point at which medical treatment may no longer be beneficial has not been identified.^[127] ALP, along with fibrosis progression markers (histology, liver stiffness measurement, and serum fibrosis markers), is the current gold standard endpoint but is insufficient due to a silent inflammatory process leading to disease progression that is difficult to detect.^[128] In addition, the patchy distribution of fibrosis makes histology and liver stiffness measurement variable and unreliable. Early-stage disease with low ALP levels, where immune-focused therapies are most likely to play a role, progresses very slowly.^[129,130] Consequently, drugs tested in short-term trials always risk being shown as ineffective, and patients with early-stage PSC with normal ALP levels are excluded from clinical trials. Conducting small, randomized studies over a short exposure time may result in potentially important drugs being dismissed despite their effectiveness.

The first randomized study in PSC was published in 1988, using D-penicillamine which was known to be effective in rheumatoid arthritis.^[131] Studies on corticosteroids followed, both orally and intrabiliary, and they showed no convincing beneficial effects but led to adverse metabolic and systemic effects.^[132–137] Therefore, the use of steroids is not recommended in the recent clinical guidelines to treat PSC.^[138,139] Very limited data exist on other immunosuppressive drugs. There are small, non-randomized, trials or pilot studies of azathioprine,^[136] methotrexate,^[140] cyclosporine,^[141] tacrolimus,^[142] mycophenolate mofetil,^[143] and etanercept^[144] usage. While tacrolimus, mycophenolate mofetil, and cyclosporine showed modest biochemical improvements, these drugs

demonstrated no significant treatment benefits to justify their use, especially given their substantial adverse effect profiles.^[141]

The most used drug today is UDCA.^[139,145] UDCA improves ALP levels, but no clear survival benefit has been shown.^[146] Its mechanisms of action on the immune system are somewhat unclear, but UDCA is known to exert a direct effect on adaptive immunity by inhibiting DCs.^[147] The development of new immune therapies or biologics in IBD is rapidly advancing. Targeting gut inflammation through drugs or colectomy or the gut microbiome itself is hypothesized to play an important role in PSC progression as well. Unfortunately, immunotherapies effective for IBD have not demonstrated clear efficacy for PSC. Neither anti-TNF therapies (infliximab and adalimumab)^[148–150] nor vedolizumab (integrin $\alpha_4\beta_7$ blockade)^[151–153] have been associated with improvements in cholestatic markers despite their effectiveness in treating IBD.^[154] Results from a retrospective study on the effect of the JAK inhibitor tofacitinib showed some promising results with improvement of cholestasis in PSC-IBD.^[155] There is one interesting ongoing phase II trial using CM-101 that targets the soluble protein CCL24, promoting fibrotic and inflammatory activities in the liver through its receptor, CCR3 (ClinicalTrials ID: NCT04595825). Finally, more experimental approaches, such as autologous hematopoietic stem cell transplantation^[156] and autologous Treg transfer^[157], have also been proposed.

Effective treatment is urgently needed for PSC. There are ongoing trials, including some experimental efforts. So far, immune-focused treatments, unfortunately have failed, but the rapidly growing field in IBD is also promising for PSC. However, further understanding of the immunopathology of PSC is required to choose the most promising immune treatment targets.

SUMMARY AND OUTLOOK—SPACE AND TIME OF PSC

We propose that the current immunobiological knowledge of PSC, to a greater extent, is placed into the context of time, space, and clinical presentation of the disease for improved interpretation (Figure 2). The time of study/sampling regarding the clinical course of the disease is an important aspect as PSC progression is observed over the course of several decades and can lead to a variable degree of fibrosis and multifocal biliary strictures. To date, most of the immunobiological knowledge, out of necessity, is from the time window of “post-diagnosis” to “end-stage disease” (Figure 2). Studying preclinical onset as well as posttransplant recurrent disease will likely provide additional key insights to better understand disease etiology and progression. While posttransplant recurrent disease will

be easier to target in translational study designs, identifying cohorts of patients before the disease presents poses a challenge. However, clever usage of historical biobanks (serum and biopsies), patients with IBD who later present with PSC-IBD, as well as ongoing (or future) large population-based studies (UK Biobank and similar efforts), should likely yield translational opportunities, especially since novel technologies (proteomics, scRNAseq, and spatial methods) now permit detailed and broad analysis on for instance formal-fixed tissue.

Furthermore, although PSC is mostly studied and managed as a single disease entity, its clinical presentation and progression are heterogeneous, which should be looked upon when placing immunobiological knowledge into context. Indeed, PSC can affect both sexes, and the age at disease onset can span from childhood to late adulthood. These aspects might be of importance as both age and sex are associated with differences in the composition and function of the immune system.^[158,159] To date, limited immunological knowledge is available on these aspects to understand the predominance of PSC diagnosis in males in their 30s.

Regarding the spatial component of the disease, a large proportion of patients also have IBD with specific characteristics, as well as a high risk of developing cancer both in the biliary tract and the colon. As we know it today, the composition and function of the immune system vary in each tissue and evolve with age.^[160] Knowledge provided from the liver and the gut has revealed complementary immunological views of PSC. This highlights the need for looking at various tissue niches to better understand the complex interplay between tissues driving PSC pathogenesis. Beyond space at the organ level, technological development now also allows for spatial assessment of RNA, proteins, and metabolites at the single-cell level in situ.^[5] As discussed above, such efforts have already yielded novel insights into PSC pathogenesis, and it is clear that the biliary niche is distinct from that of the liver parenchyma.^[5,9] Future work will likely benefit from an even greater consideration of events occurring within micro-niches.

Finally, the heterogeneity of PSC is driven by additional features such as the levels of severity of disease, secondary effects of the presence of high-grade strictures, recurrent bacterial cholangitis, circulating IgG4, the involvement of small or large bile duct, or the presence of autoimmune hepatitis features. Immunological investigations are needed to better dissect these PSC subtypes. Specifically, as the portfolio of immunotherapeutic agents is expanding, immunobiological knowledge placed in the context of time, space, and heterogeneity of PSC might provide future options for precision medicine.

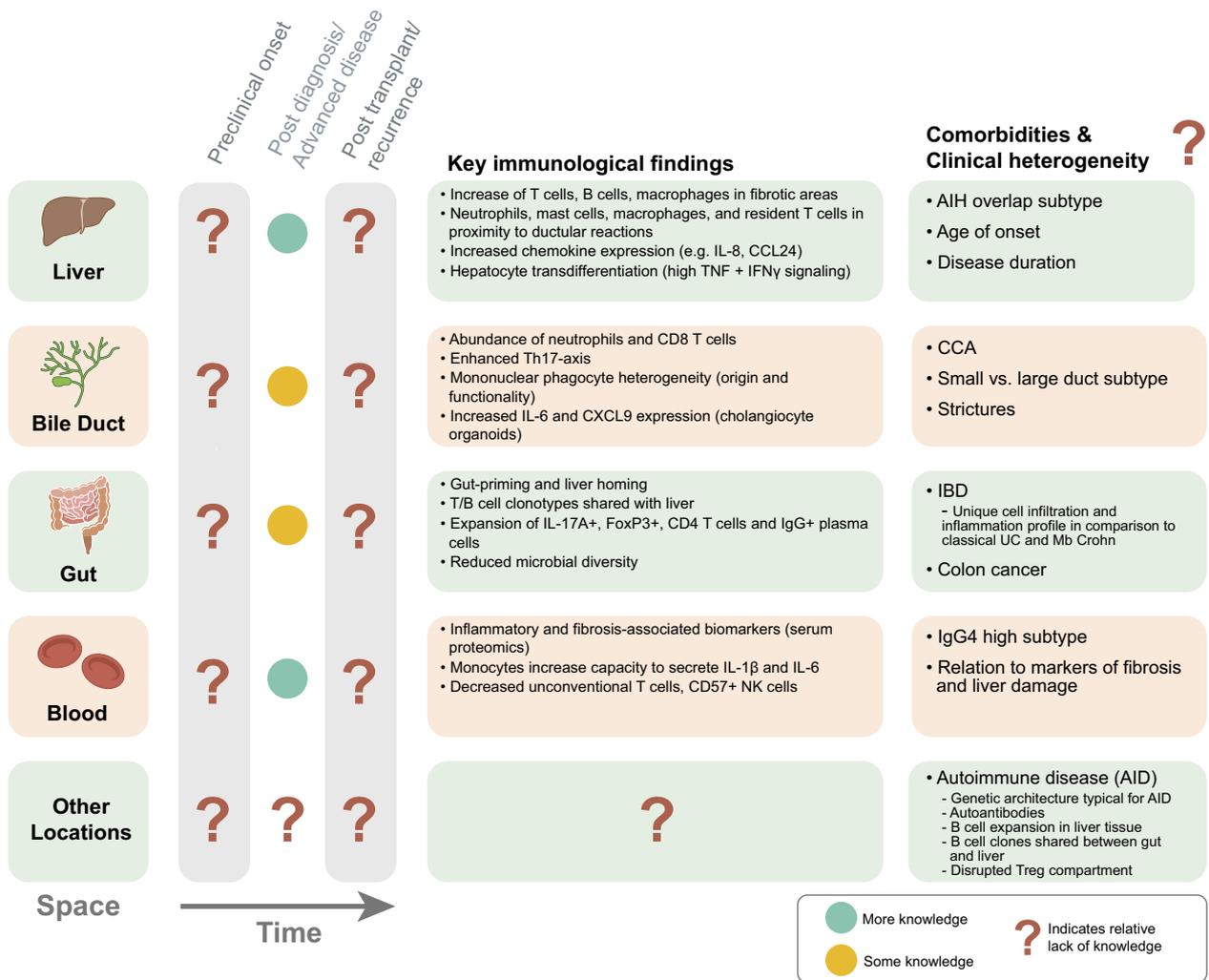


FIGURE 2 PSC pathogenesis through time, space, comorbidities, and clinical heterogeneity. Locations (space) of the immune studies are indicated in the left part of the figure. The extent of immunological knowledge in relation to disease stage (time) is thereafter depicted (preclinical, postdiagnosis/advanced disease, and posttransplant recurrent PSC). Most of the current immunobiological knowledge originates from a narrow spatiotemporal window, mostly from liver tissue postdiagnosis or in end-stage disease. The main immune features per location are detailed in bullet points. On the right side, related comorbidities and clinical heterogeneity–related aspects for each location are brought up. In most instances, it remains unclear how these aspects affect immunology. Abbreviation: PSC, primary sclerosing cholangitis.

AUTHOR CONTRIBUTIONS

The authors contributed equally to all aspects of the article.

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CONFLICTS OF INTEREST

Annika Bergquist consults for Calliditas. She advises Ipsen. The remaining authors have no conflicts to report.

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