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BILIARY EPITHELIAL CELLS, THE IMMUNE SYSTEM AND PSC PATHOGENESIS

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Biliary epithelial cells, the immune system and PSC pathogenesis

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ABSTRACT

Primary sclerosing cholangitis (PSC) is a chronic, inflammatory liver disease that leads to destruction of the bile duct system. PSC is strongly associated with inflammatory bowel disease, and PSC patients have an increased risk of developing malignancies, especially cholangiocarcinoma (CCA). The pathogenesis of PSC is still insufficiently understood, although T cells have been suggested to play a major role. Little is known about the involvement of other immune cell populations. In addition to this, a better understanding is needed about the capacity of liver resident immune cells to prevent tumor development. In this thesis we have investigated cellular and humoral components of the immune system, in PSC and the PSC-associated malignancy CCA.

In the first study, we examined the role of autoreactive antibodies in PSC patients. Flow cytometry was used to investigate the presence of IgA and IgG antibodies in PSC patient sera, and its reactivity against isolated human biliary epithelial cells. A majority of the patients had antibodies that bound to the cells, while only low levels could be detected in serum of healthy individuals. Moreover, IgA autoantibodies in PSC patients were associated with a reduced survival, and therefore their presence may be of importance in the pathogenesis of PSC.

In the second and third study, immunohistochemistry and image analysis was used, to explore the cell compositions in PSC and CCA livers. Specific phenotypic patterns, associated with severity of disease, were revealed in PSC livers. T cells were enriched, mainly localizing to fibrotic fields, whereas MAIT cells were not equally increased. Furthermore, one group of PSC patients, characterized by a potential loss of smooth muscle cell function, was found to have increased numbers of T cells and a more extensive bile duct proliferation. The tumor microenvironment in CCA was characterized by a selective loss of Kupffer cells and MAIT cells, and contained high numbers of regulatory T cells. Moreover, the expression of IL-33 was significantly lower in tumors. This distinct intratumoral phenotype was unaffected by tumor location, tumor differentiation, or an underlying PSC.

Altogether, our studies provide insights into the pathogenesis of PSC and CCA and opens up for further studies of disease mechanisms.

LIST OF SCIENTIFIC PAPERS

I. L Berglin, NK Björkström, A Bergquist.
Primary sclerosing cholangitis is associated with autoreactive IgA antibodies against biliary epithelial cells.
Scandinavian Journal of Gastroenterology, 2013, 48, 719-728

II. L Berglin, A Bergquist, H Johansson, H Glaumann, C Jorns, S Lunemann, H Wedemeyer, EC Ellis, NK Björkström.
In situ characterization of intrahepatic non-parenchymal cells in PSC reveals phenotypic patterns associated with disease severity.
PLoS One, 2014, 9, e105375

III. L Berglin, E von Seth, H Glaumann, A Bergquist, NK Björkström.
Immunohistochemistry assessment of intratumoral immune cells in cholangiocarcinoma.
Manuscript

TABLE OF CONTENT

LIST OF ABBREVIATIONS

α-SMA	alpha-smooth muscle actin
ACIA	acquired computerized image analysis
AIH	autoimmune hepatitis
ALP	alkaline phosphatase
ANA	anti-nuclear antibody
ANCA	anti-neutrophil cytoplasmic antibody
BEC	biliary epithelial cell
CCA	cholangiocarcinoma
CCL	CC chemokine ligand
CD	cluster of differentiation
cDNA	complementary DNA
CK7	cytokeratin 7
CK19	cytokeratin 19
Ct	cycle threshold
CTL	cytotoxic T lymphocyte
CY	cyanine
DC	dendritic cell
ECM	extracellular matrix
EpCAM	epithelial cell adhesion molecule

ERCP	endoscopic retrograde cholangiopancreatography
FITC	fluorescein isothiocyanate
HCC	hepatocellular carcinoma
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LSEC	liver sinusoidal endothelial cell
M-CSF	macrophage colony-stimulating factor
MADCAM-1	mucosal addressin cellular adhesion molecule 1
MAIT	mucosal-associated invariant T cell
MCP-1	monocyte chemoattractant protein-1
MDR	multidrug resistance protein
MFI	mean fluoresence intensity
MHC	major histocompatibility complex
MRCP	magnetic resonance cholangiopancreatography
mRNA	messenger RNA
NK	natural killer
PAMPS	pathogen-associated molecular patterns

PBC	primary biliary cirrhosis
PCA	principal component analysis
PCR	polymerase chain reaction
PSC	primary sclerosing cholangitis
ROS	reactive oxygen species
SMA	smooth muscle antibody
TBB-5	beta-tubulin isotype 5
TGF-β	transforming growth factor beta
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
UC	ulcerative colitis
UDCA	ursodeoxycholic acid

1 INTRODUCTION

1.1 IMMUNOLOGY

The immune system is under constant selection, to provide us protection against pathogenic microorganisms in the environment. The ability to distinguish self from non-self is one of the most critical challenges for the immune system to overcome, in order to mobilize a proper response against invading pathogens. The initial response is generated by cellular components of the innate immune system, such as neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins. This is a highly conserved system between species, and crucial for the ability of the individual to survive. Innate immune cells recognize and bind to microbial structures, termed pathogen-associated molecular patterns (PAMPS). The binding initiates effector functions of the cells, such as phagocytosis, opsonization, and complement-mediated lysis, leading to the elimination of the pathogen. In addition to the innate immune system, higher animals have evolved an adaptive immune system. This system is highly specific, but often require days or weeks before it is fully activated (1). B and T cells, which are the main actors of the adaptive system, develop from progenitor cells in the bone marrow. At an early stage during development, T cell precursors migrate to the thymus for further development, whereas B cells remain in the bone marrow. B and T lymphocytes acquire their antigen-specific receptors through complex gene rearrangements, which leads to a large pool of lymphocytes, estimated to contain over 10^8 unique T-cell receptors and 10^{10} antibody specificities. B and T lymphocytes continuously circulate through secondary lymphoid organs, and upon antigen delivery by antigen presenting cells, they get activated and start to differentiate. Thereafter, T cells are able to home to the infected site, where they exert effector functions, and B cells release antibodies into blood and tissue fluids (1). For T cells to combat intracellular pathogens, it is not enough with the ability to recognize structures of the microbe. They must also recognize the cells that have been infected. For this reason, T cells have developed unique properties of recognition of antigens

in combination with self-molecules. They only recognize antigenic peptides bound to major histocompatibility complex (MHC) molecules. In humans, this complex is commonly referred to as HLA (human leukocyte antigen). MHC class I molecules bind to peptide fragments produced by the cell´s own machinery, which occurs when the cells are virus or tumor transformed. MHC class II molecules bind to antigenic peptides derived from the extracellular environment, taken up by phagocytosis by the cell. MHC class II is particularly expressed by professional antigen-presenting cells, whereas all nucleated cells express MHC class I. However, some pathogens have developed strategies to escape presentation by MHC molecules (2), and genes within the MHC region are under a constant selective pressure. Therefore, genes within the MHC locus are highly polymorphic, i.e., there are multiple variants of each gene. This can be both favorable and unfavorable for the individual, because some gene variants may contribute to susceptibility to autoimmune, infectious, or inflammatory diseases (3).

1.1.1 Liver immunology

The liver has unique immune properties, largely due to its anatomical position in the body. It has a dual blood supply, both from the systemic blood and the gastrointestinal tract. From an immunological perspective this is important, since it does not only receive oxygen-rich blood from the systemic circulation, but also nutrient and antigen-rich blood from the gut. The blood enters the liver mainly through the portal vein, and drains through a highly branched vascular system, called sinusoidal endothelium, before leaving the organ via the central veins. Liver sinusoids are composed of a layer of fenestrated liver sinusoidal endothelial cells (LSEC), which makes them permeable. A large amount of lymphocytes from the peripheral blood passes the LSECs every minute (4). Due to the small diameter of the vessels, the lymphocytes are in close contact with LSECs, and are easily able to extravasate through the fenestrated cell layer (5). The liver harbors approximately ten billion lymphocytes (6). These

include cells of both the innate and the adaptive immune system. There are also several different cell types in the liver with the capability to present antigens to circulating lymphocytes. These include LSECs, Kupffer cells, hepatic stellate cells, and dendritic cells (DCs).

1.1.1.1 Liver sinusoidal endothelial cells

Up to half of the non-parenchymal cells in the liver are comprised of LSECs. They differ from other vascular endothelial cells in the body, as they express receptors for both antigen uptake and antigen presentation, such as scavenger receptors and MHC class I and II (7, 8). These properties give them a unique role in liver immunology and tolerance, as will be discussed in more detail.

1.1.1.2 Kupffer cells

Kupffer cells are liver resident macrophages and constitute 1/5 of the non-parenchymal cells of the liver (7). They are strategically positioned in the sinusoids and constitute a first line of defense against invading microorganisms. One of the effector functions of Kupffer cells upon bacterial stimulation is the production of cytokines (8). Secreted cytokines influence and shape the further immune response. As an example, they can activate and induce the differentiation of natural killer (NK) cells by the release of interleukins (IL), such as IL-12 and IL-18 (9).

1.1.1.3 Natural killer cells

NK cells are innate immune cells with cytotoxic activity against virus or tumor-transformed cells. They constitute about 30% of the total lymphocyte population in the liver, and are regulated by a balance of signals between activating and inhibitory receptors. The ligands for the inhibitory receptors on NK cells are MHC class I molecules, and as long as these are

expressed on the surface of nearby cells, NK cells are kept in an inactive state (10). Both the absence of MHC class I, as well as presence of activating signals, is required for the induction of NK cell cytotoxicity. Upon activation they release cytokines, such as interferon (IFN)-γ, which leads to the expression of chemokines by LSECs and recruitment of T cells (11).

1.1.1.4 T cells

The majority of lymphocytes in the liver consist of T cells. Cytotoxic T cells are characterized by the expression of the glycoprotein CD8 on the cell surface and thus, commonly referred to as $CD8^+$ T cells. They are restricted to recognize peptide fragments in conjunction with MHC class I molecules, and are therefore key immune cells in the defense against virus and intracellular pathogens (12). $CD8^+$ T cells are known to be more abundant in the liver than the $CD4^+$ T cells (6), which constitute the second population of conventional T cells. They are characterized by the expression of CD4 on the cell surface and recognize antigens presented by MHC class II molecules. One of their main role is to help cytotoxic T cells to become fully activated (13).

1.1.1.5 Mucosal-associated-invariant T cells

Other T cell populations, with a more innate-like phenotype, such as mucosal-associated-invariant T (MAIT) cells, are found at higher frequencies in the liver than in the peripheral blood (14). MAIT cells are characterized by the expression of a semi-invariant T cell receptor (TCR-Vα7.2) and respond to bacterial antigens, presented by the MHC-like molecule MR1, with immediate effector functions (15).

1.1.1.6 B cells

B cells are known to be scarce in the liver, and little is known about their immunological activities here (6). According to the classical model, activation and maturation of B cells

occur in secondary lymphoid organs (16) and at present, it is not known whether B cells can be primed in the liver. However, plasma cells (antibody-producing B cells) line the biliary epithelium in the liver, and by producing and secreting immunoglobulin (Ig) A antibodies into bile, they likely have a role in the protection of bile ducts against invading pathogen (17). Antibodies are components of the humoral immune response and offer protection against extracellular pathogens. They develop as membrane-bound receptors on B cells in the bone marrow, each one unique for its particular antigen. B cells migrate to secondary lymphoid organs, where they meet their specific antigen and start to mature with the help of costimulatory signals from CD4+ T helper cells. Upon activation, B cells start to differentiate and proliferate, and become either antibody-producing effector cells or memory B cells. Effector cell-released antibodies have the same antigen specificity as the initially produced membrane-bound antibodies (18).

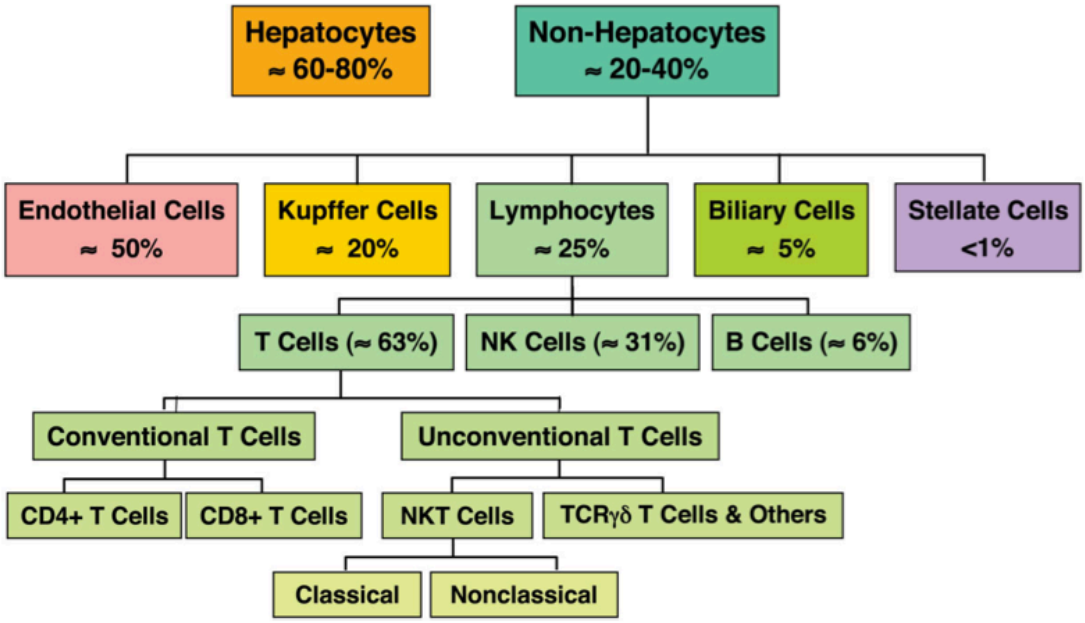

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Figure 1.1: The distribution of cells in the healthy liver. Percentages refer to estimated frequencies of the different cellular subsets in relation to the total number of liver cells.

1.2 LIVER STRUCTURE AND FUNCTION

The liver is the largest gland in the body (19). It has many important roles in glucose, protein and lipid metabolism as well as functions in detoxification and removal of waste products (20). The microanatomical structure of the liver is divided into functional units, called liver lobules. Each liver lobule has a central vein in the middle, from which the functional liver cells, the hepatocytes, radiate to the portal tracts, also known as portal triads, in the periphery. Each portal triad consists of one hepatic artery, one hepatic portal vein and one common bile duct (21).

Bile, which is produced by the hepatocytes, is transported via a complex network of bile conduits, lined by biliary epithelial cells (BECs), and finally converges into the common bile duct. From there it empties into the gall bladder before further secretion to the intestine. The major function of the BECs is to modify the bile before its secretion. These cells also constitute the main target cells in several cholestatic diseases, commonly referred to as cholangiopathies.

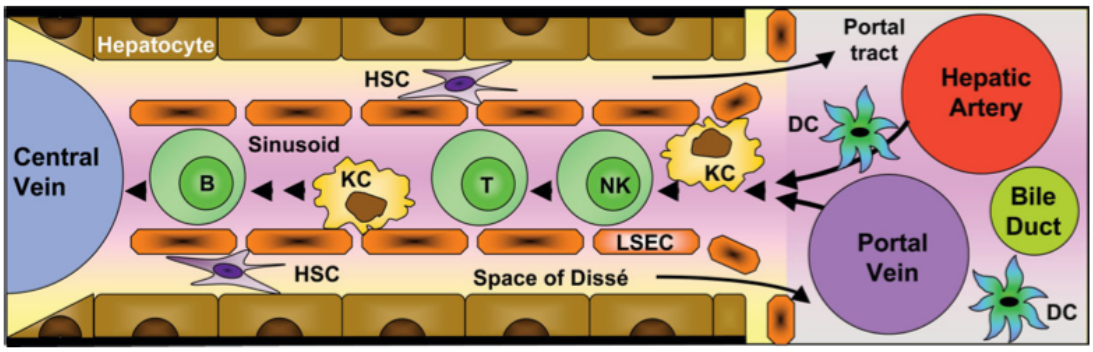

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Figure 1.2: Schematic drawing of a portion of a liver lobule. To the left is the central vein, from which the hepatocytes radiate towards the periphery (to the right), where the portal triads are located. Each portal triad contains one hepartic artery, one portal vein, and one bile duct. Within the lumen of the sinusoidal endothelium, resident Kupffer cells patrol the area, whereas DCs mainly locate near the portal zones. Immune cells, such as T cells and NK cells

arrive from the peripheral blood and flow within the sinusoids, in close contact with the LSECs.

1.3 LIVER TOLERANCE

The liver is constantly exposed to microbial products from the portal blood. For this reason, the liver has evolved unique immunosuppressive mechanisms, which together cooperate to keep the environment tolerogenic. LSECs have efficient scavenger functions, that keep the vessels clean from antigens and bacterial compounds (22), and they also have the capacity to present antigens to $CD4^+$ and $CD8^+$ T cells (23). However, under non-inflammatory conditions, these interactions fail to activate an immune response (24). Instead, tolerance is induced, as T cells start to secrete suppressive cytokines, such as IL-4 and IL-10 (25). Other resident liver cells, such as Kupffer cells, contribute to the tolerance as well. Under normal conditions, they do not get triggered by soluble antigens, since they do not express MHC class II or co-stimulatory molecules (26). Instead they interact with regulatory T cells (Tregs), which leads to more IL-10 production and, thus, an immunosuppressive environment (27). During inflammation in the liver, tolerance can be broken and Kupffer cells can get activated to induce T cell proliferation (28).

1.3.1 Breaking of tolerance

Immune tolerance can be broken if the liver is exposed to live bacteria, viruses, or increasing levels of PAMPs. This induces a robust inflammatory response, with recruitment of large numbers of lymphocytes from the peripheral blood to the liver (29).

1.3.1.1 Liver inflammation

Inflammation in the liver begins when a primary injury, caused by viral or bacterial attacks for example, initiates cell death of hepatocytes and /or BECs. The leakage of cellular contents into the local environment, and increased levels of reactive oxygen species (ROS), attracts

and activate Kupffer cells and hepatic stellate cells. These cells start to remove dying cells by phagocytosis. By doing so, they also secrete pro-inflammatory mediators, such as tumor necrosis factor (TNF), IL-6 and IL-1β, which in turn attract T cells and neutrophils to the site of injury. The recruitment of different cellular subsets further amplifies the inflammatory response, and secreted cytokines, in particular transforming growth factor beta (TGF-β), causes the transdifferentiation of hepatic stellate cells into myofibroblasts. When these cells mature and become fully activated, they start to produce extracellular matrix (ECM) proteins, which initiate the fibrotic process (30).

1.3.1.2 Liver fibrosis

Hepatic stellate cells normally reside as lipid and vitamin A-storing, quiescent cells, situated in the space of Disse, between the hepatocytes and sinusoidal endothelium. Upon liver injury, they change phenotype and start to proliferate, migrate and acquire contractile properties. Their full activation leads to the synthesis and secretion of extracellular matrix (ECM) proteins, especially type I collagen, as well as secretion of pro-fibrogenic factors, such as TGF-β (30). Macrophages are observed to localize in the vicinity of myofibroblasts in fibrotic livers and soluble mediators in the environment, such as monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF), have been shown to prolong the activation state of myofibroblasts, suggesting an active cross-talk between these two cell types (31).

Furthermore, the recruitment of T cells, and the balance between the differentiated subtypes of Th cells, Th1 and Th2 cells, has also been shown to be of importance for the fibrotic outcome. Th1 cytokines have been shown to be more prone to reduce fibrosis (32), whereas Th2 cytokines, such as IL-13 and IL-33, have pro-fibrogenic effects (33). Fibrosis is part of the healing process, and type I collagen has been shown to protect hepatocytes against toxic compounds (34). However, if tissue injury persists and the fibrotic process continues in a

dysregulated manner, clinical complications can arise (Figure 1.3). If the primary injury is eliminated, the liver tissue can regain its normal structure, since the fibrosis, in its early stages, is reversible. However, once cirrhosis is established, the process is in most cases irreversible. In advanced stages, when liver failure occurs, the only curative option is liver transplantation (30).

Figure 1.3: Stages of chronic liver disease. Inflammation, caused by an initial injury leads to fibrosis development with deposition of scar matrix. This process is reversible, if the injury is healed. Continued inflammation leads to further increased fibrosis and eventually cirrhosis. Once cirrhosis is established, the possibility to reverse the process is decreased (30).

1.4 BILIARY EPITHELIAL CELLS

1.4.1 Physiological functions

BECs constitute only about 4-5% of the total number of cells in the liver, but have many important physiologic and immunologic functions. Modification of bile is one of their major functions, and occurs by the secretion of ions, solutes and water into the bile duct lumen (35). This is a process tightly regulated by hormones, peptides and neurotransmitters. Another function is to secrete IgA into bile (36). This occurs via a mechanism where serum IgA first bind to the secretory component on the basolateral side of the biliary epithelium and thereafter get endocytosed and further transported via vesicles to the apical membrane. At this site, proteolytic cleavage of the secretory component takes place, which results in the release of IgA into bile (37). Since the distal part of the biliary system, the common bile duct, drains into the duodenum, secreted IgA has a crucial role in protecting the biliary tree against

gastrointestinal-derived pathogens. Main effector functions of IgA is to aggregate bacteria and prevent them from attaching to the mucous membranes, as well as protecting against intracellular pathogens during the transcytosis pathway, through the biliary epithelium (38).

1.4.2 Activation of BECs

In the healthy liver, BECs do not divide. As a response to biliary injury, however, they can start to proliferate and become immunologically active, a phenomenon known as ductular reaction. BECs express toll-like receptors (TLRs), which upon binding to pathogen-derived ligands start to synthesize IL-6, IL-1β and IL-23 (38). These cytokines mediate recruitment and differentiation of T cells. Recently, the differentiation of naive T cells into Th17 cells have been proposed to be of importance in the pathogenesis of chronic inflammatory diseases. Reactive BECs are also important players in fibrosis, since they produce pro-inflammatory and profibrogenic cytokines, whose interactions lead to an exaggeration of the mesenchymal response, with further deposition of ECM proteins into the liver parenchyma. Furthermore, the release of IL-6 stimulates BECs in an autocrine fashion, thereby leading to a continued release of inflammatory mediators (38).

1.5 PRIMARY SCLEROSING CHOLANGITIS

PSC is a chronic, cholestatic liver disease that is characterized by inflammation, fibrosis and destruction of the intra- and/or extrahepatic bile ducts. This leads to development of cirrhosis and eventually liver failure. PSC is strongly associated with inflammatory bowel disease (IBD), especially ulcerative colitis (UC), which is present in up to 80% of the PSC patients in northern Europe (39). Based on population studies from Norway, Great Britain and United States, the prevalence of PSC is estimated to be 8.5-13.6/100 000, which is 10-100 times higher than in the southern parts of Europe and Asia (40). Two thirds of the patients are male and the median age at onset is 30-40 years (39). There are no effective medical treatments for

PSC, and more than half of the patients need a liver transplant within 10-15 years after the first symptoms. Although survival is increased after liver transplantation, one fifth of the patients are at risk of developing recurrent disease in the liver graft (40). There is also an increased risk for PSC patients to develop malignancies, particularly cholangiocarcinoma (CCA), which often has a poor outcome.

1.5.1 Diagnosis

PSC is often asymptomatic in its initial stage, and is usually detected first when the patient's liver enzymes increase. As the disease progresses, symptoms such as jaundice, pruritus and abdominal pain arise, accompanied by episodes of bacterial cholangitis, with fever and worsening of liver biochemistries. The diagnosis of PSC is based on a typical cholangiogram with multiple strictures and dilatations. Serum liver tests usually show increased liver enzymes, in particular alkaline phosphatase (ALP). The presence of autoantibodies is also common. A majority of the patients have a history of IBD and show signs of cholestasis. The use of magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP) is used to visualize the bile ducts (Figure 1.4). ERCP is reserved for patients with dominant strictures and who are in a need for biliary intervention (41).

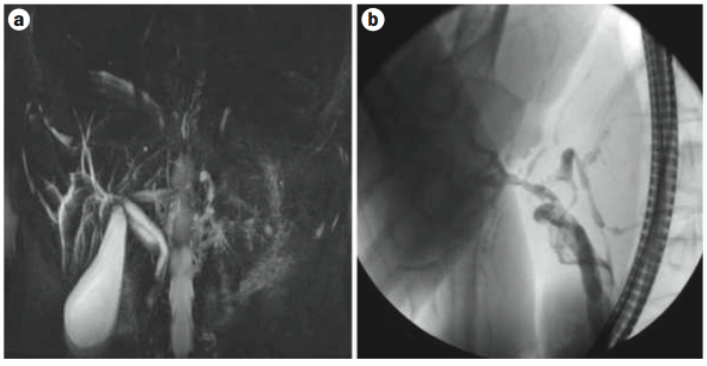

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Figure 1.4: Left: MRCP (left) and ERCP (right) showing typical, multiple strictures and dilatations of the biliary tree (41).

1.5.2 Pathogenesis

The etiology and pathogenesis of PSC is incompletely understood, but probably involves both genetic susceptibility and environmental factors. There are different theories on whether the original injury to the bile ducts is caused by immune-related mechanisms or biochemical factors associated to bile toxicity (42).

1.5.2.1 Autoantibodies

The observation that PSC patients have many circulating autoantibodies has led to the hypothesis that PSC is caused by immune reactions against self-antigens (43). Further support for this is the strong risk factors correlated to certain HLA haplotypes (44), but also the fact that 25% of PSC patients are reported to have other, coexisting autoimmune diseases (45). However, the specificity of many of the detected autoantibodies is relatively low, and it is often argued that they arise due to secondary responses, following the sustained inflammation, rather than as a consequence of autoimmunity against a specific target autoantigen (43).

Examples of such non-specific antibodies detected in PSC and in other autoimmune liver diseases are anti-nuclear (ANA), anti-cardiolipin, and anti-smooth muscle (SMA) antibodies (43), as well as antibodies against neutrophil components (ANCAs) (46). However, the identification of β-tubulin isotype 5 (TBB-5) as a candidate autoantigen for ANCAs, suggests that PSC patients might have a dysregulated immune response to intestinal microbes, since TBB-5 shares high structural homology with the bacterial cell division protein FtsZ (47).

1.5.2.2 T cells

Several experimental studies suggests T cells to be highly involved in the pathogenesis of PSC (42). The strong association with IBD indicate that the immune system may be triggered by bacterial compounds from the gut (48). Experimental data further support this. Bile from PSC patients have been shown to contain increased levels of bacterial species, with the capability to induce expansion of Th17 and Th1/Th17 cells in peripheral blood (49). Moreover, mechanistic studies suggest that the biliary injury is caused by cytotoxic $CD8^+$ T cells, initially activated in the gut (50). Such observations led to the hypothesis of "aberrant homing of lymphocytes", presented by Adams et al. 2006, which is further explained in the next section.

1.5.2.3 Lymphocyte recruitment and homing

The fact that PSC can occur in IBD patients after colectomy, and IBD arise in PSC patients after liver transplantation, has led to the hypothesis of aberrant lymphocyte homing. According to this theory, activated T cells in the gut are proposed to persist as memory cells and may be recruited to the liver by aberrant expression of mucosal addressin cellular adhesion molecule 1 (MADCAM-1) and CC chemokine ligand (CCL) 25 on the liver endothelium. The up-regulation of these molecules leads to the recruitment of gut-resident $CCR9^+$ $\alpha4\beta7$-$integrin^+$ lymphocytes. Epithelial produced chemokines, such as CXCL12, CXCL16, and CX_3CL1, place them around bile ducts, and upon encountering of antigen(s), they become activated and promote inflammation (51, 52).

1.5.2.4 Exposure to endotoxins

Bile fluids of PSC patients have been shown to be frequently colonized with bacteria (49). Alterations in the epithelial barrier, due to overexposure to microbial compounds, might be an explanation to the biliary injury. Studies in animal models have shown that the disruption of

epithelial tight junctions leads to a leakage of bile into the portal tracts, followed by an inflammatory response. This response includes influx of T cells, activation of myofibroblasts and development of fibrosis (51). Furthermore, BECs from PSC patients have been observed to express higher levels of TLRs, TNF-α, IFN-γ and IL-8, than in patients without PSC, suggesting an exaggerated innate immune response (52).

1.5.2.5 Toxic bile

There is evidence that toxic bile directly or indirectly might be involved in the pathogenesis of PSC. Cholestasis in itself can lead to damage of the liver cells, since it naturally leads to an accumulation of bile acids. This is a common characteristic of all cholestatic diseases (53). However, genetic findings suggest that the toxic effect of bile acids in PSC may be more specific and caused by defects in the secretion functions of biliary epithelial cells (54). Dysfunction in the multidrug resistance protein (MDR) 3 gene leads to bile-duct injury, as a consequence of an abnormal composition of bile acids. MDR3 encodes a transporter protein, whose function is to secrete phospholipids, that together with cholesterol encloses the bile acids in micelles, which protects the biliary epithelium from toxicity (53). Mice, that are devoid of this gene, have been observed to have PSC-like intrahepatic changes with disrupted tight junctions, leakage of bile acids into the portal tracts and infiltrating inflammatory cells (51). However, no direct association between PSC and defects in *MDR3* have been found (55).

Variations found in the gene encoding the bile-salt-sensing receptor TGR5 may be of more importance in PSC pathogenesis (54). It is involved in the complex regulation of HCO3- secretion and defects in this system has led to the hypothesis of a protective HCO3- umbrella (56). According to this theory, damage to the BECs is caused by reduced concentrations of secreted chloride and bicarbonate into the bile ducts. This makes BECs more vulnerable to bile acids, as the protective glycocalyx layer (termed glycocalyx umbrella) disappears.

Irrespective of whether toxic bile acids are the direct cause of inflammation in PSC or not, their leakage into the parenchyma likely contributes to the acceleration of disease, with worsened fibrosis as a result.

1.6 GENETICS

First-degree relatives of PSC patients have a 9- to 39-fold increased risk of developing the disease (57) and the strongest risk factors are located within the MHC complex, at chromosome 6p21 (58). Most of the genes identified as being risk associated in PSC are related to inflammation. Classic examples include the HLA class I and II genes (59), whose encoded proteins direct the T cell response. Other risk-associated genes that have been found include *IL2/IL21, REL* and *CARD9* (60). This further suggests that inflammation plays an important role in PSC, since all of these genes encode proteins that are implicated in the activation and function of immune cells (61). Several PSC-associated susceptibility genes have also been found outside the MHC region, such as *CD28* and *CD226* (58). These genes encode receptors that are involved in co-stimulation of T and NK cells respectively (62), suggesting components of both the innate and adaptive immune system to be involved in the pathogenesis of PSC. Gene variants have also been identified in *MST1*, whose protein product is implicated in the suppression of macrophages (61). Taken together, even though no PSC-specific susceptibility loci have been identified so far, several genetic findings suggest that PSC might be caused by immune-mediated mechanisms.

Despite several proposed hypotheses and the recent identification of new genetic susceptibility loci in PSC, the pathogenesis still remains incompletely understood. The main reason for this is that when PSC is diagnosed, the disease has often progressed to a late stage. By that time it is impossible to know whether the molecular changes observed are the direct causing factors, or secondary effects due to inflammation. However, heterogeneity within the PSC population suggests that several pathogenic mechanisms may be involved at different

stages of the disease.

1.7 CHOLANGIOCARCINOMA

CCA is the malignant transformation of the biliary epithelium. Although it is a rare form of cancer, it is the second most common primary liver cancer after hepatocellular carcinoma (HCC), and accounts for approximately 3% of all gastrointestinal tumors (63). The difficulty of early diagnosis is a contributing factor to the low survival of the patients. The median 5-year survival is as low as 10% (64) and due to severity of fibrosis or a poor health, only a third of the patients can undergo curative liver resection (65). The anatomical location of the tumor along the biliary tree determines the classification of CCA (66). If it originates from small intrahepatic ductules or large intrahepatic ducts, close to the right and left hepatic ducts, it is classified as intrahepatic CCA. Extrahepatic CCA is the collection name for hilar cholangiocarcinomas (also called Klatskin tumors) and distal tumors. These two subtypes are anatomically separated from each other by the cystic duct. Perihilar CCA is the most common form of CCA and accounts for 50-67% of all cases (67). The surgical options for patients diagnosed with perihilar CCA are limited (68). The different subtypes of CCA differ in epidemiology, etiology, pathogenesis and treatment possibilities (63).

The pathogenesis of CCA is insufficiently known, and, there are no early, reliable detection markers. Early diagnosis is difficult, especially in PSC where the biliary tree has strictures. CA19-9 is the most commonly used serum biomarker for CCA. This marker is unfortunately unspecific and can also be increased in bacterial cholangitis, cholestasis and other malignancies (69).

Chronic inflammation is an established risk factor for CCA and PSC patients have 160 times higher risk than the general population of developing CCA (70). PSC patients develop CCA much earlier than the patients without PSC. The age of onset among individuals with PSC is about 40 years (74), while it is over 70 years for the non-CCA patients (75). Furthermore, a

PSC patient often receives the diagnosis of CCA within one year from the determination of PSC (71). Thus, the duration time of PSC does not seem to be linked to the malignant transformation, even though inflammatory factors are assumed to be probable causes for the progression to cancer. Evidence for this is the association of CCA with other inflammatory diseases in the liver, such as parasitic infections and hepatitis B and C infections (72).

A commonly observed phenomenon in various cancers is the infiltration of suppressive immune cell subsets, such as regulatory T cells (Tregs) (73). This infiltration is often beneficial for the tumor cells, since the functions of Tregs commonly lead to a decreased capacity of cytotoxic immune cells to kill tumor cells. Studies in CCA have led to different observations. Positive associations have been made between infiltrating Tregs and the pro-inflammatory subset of T cells, known as $IL17^+$ cells (74). This was further correlated with a poorer survival for the patients. In another study of CCA, infiltrating Tregs were reported to associate with increased numbers of alternatively activated macrophages (M2 cells) (75). These cells are known to have anti-inflammatory properties (76). Moreover, no unifying results have been obtained from studies of cytotoxic T lymphocytes (CTLs) and their functions in CCA. Some studies found CCA tumors to contain lower numbers of CTLs than the surrounding tissue (74), whereas another study observed similar CTL levels inside as outside the tumor (77).

In addition to risk factors such as inflammation, the genetic profile of the patients probably also has a significant role. Variations in the gene encoding the activating natural killer receptor G2D (*NKG2D*) have been associated with risk for CCA in PSC patients (78). NKG2D has an important role in tumor surveillance (79) and variants in the gene encoding its ligand, *MICA*, have also been identified in CCA, as well as in HCC. This suggests that the MICA-NKG2D axis might have an overall role in the development of hepatobiliary cancer (80).

In conclusion, inflammation is strongly linked to CCA and other cancer forms. Some cancers are preceded by an inflammatory condition, whereas in others, inflammation develops due to the malignant transformations (81). Regardless of what comes first, the tumor cells are often favored by the immune suppressive nature of the cancer-related inflammation (73). A better understanding of the inflammatory cells involved in the cancer-related inflammation can lead to the development of targeted therapies against these cellular factors.

2 AIMS

The general aim of this thesis was to improve the understanding about pathogenic mechanisms in PSC and the PSC-associated malignancy CCA, by exploring both humoral and cellular aspects of the immune response.

Aim of study I:

To evaluate the presence of autoreactive IgA antibodies in PSC patients.

Aim of study II:

To characterize non-parenchymal intrahepatic cells in PSC livers and to investigate associations between immune phenotypes and PSC disease severity.

Aim of study III:

To enumerate innate and adaptive immune cell subsets in CCA in livers from PSC and non-PSC patients.

3 MATERIAL AND METHODS

3.1 ETHICAL CONSIDERATIONS

Informed written consent was obtained from all patients and the Regional Ethics Committee, Stockholm, Sweden, approved the studies.

Material from deceased liver donors were included in the studies according to the regulations of the organ transplantation law of Sweden (1995:831), that is, the donors prior written declaration was followed as well as the written informed consent from next of kin.

3.2 PAPER I

3.2.1 Clinical material

Serum samples from 81 PSC patients were used in this study. The samples had been collected while the patients underwent routine controls, at our Unit at Karolinska University Hospital in Huddinge, during a total time period of 16 years. The diagnoses of PSC and IBD were based on biochemical, clinical, and cholangiographic features, typical history, and characteristic endoscopic and histologic findings (41). Serum was collected from 42 healthy volunteers, and all samples were cryopreserved until later usage. For isolation of liver cells, tissue from donor livers, not used for transplantation because of technical reasons, were collected.

3.2.2 Establishing cultures of BECs

To avoid the risk that a possible autoantigen had been destroyed by earlier immune attack following inflammation, we chose to isolate human BECs from livers of deceased individuals with no known liver diseases. The tissue was disintegrated into small pieces, thereafter enzymatically digested with 0.05% Collagenase V and 0.002% DNAse to release the cells.

The liver digest was filtered and washed, thereafter the mixed cell population was resuspended in epithelial cell medium (ECM) and grown until confluence in gelatin-coated tissue culture flasks. Selection for epithelial cells was performed using immunomagnetic isolation with EpCAM-conjugated (clone HEA-125) microbeads. Enriched cells were further cultured and used freshly before reaching passage 6, or cryopreserved in liquid nitrogen for later usage. The EpCAM-selected cells were observed for the characteristic cuboidal morphology of epithelial cells with a phase contrast microscope.

3.2.3 Phenotypic characterization of isolated cells by flow cytometry

EpCAM-enriched cells were characterized by flow cytometry for epithelial markers such as EpCAM, Cytokeratin 19 (CK19) and Cytokeratin 7 (CK7), and for non-epithelial markers such as CD90, CD31, CD45, α-SMA, Calponin and Caldesmon. Antibody staining was performed with monoclonal antibodies, either on primary liver cells or isolated, passaged cells. Levels of background staining were assessed with corresponding isotype control. Samples were acquired using a FACS Calibur flow cytometer and analyzed using FlowJo version 9.3.

3.2.4 Detection of autoreactive IgA and IgG antibodies in patient sera

EpCAM-enriched cells were stimulated overnight with 20 ng/ml TNF-α and 200 ng/ml IFN-γ, according to a previously described method (82). Before staining, the single-cell suspension was incubated with patient or control sera, diluted 1:4 in PBS, at room temperature for one hour. Unbound antibodies were washed away and thereafter the cells were incubated with secondary fluorescein isothiocyanate (FITC)- conjugated goat anti-human IgA antibody or cyanine (CY) 3- conjugated rabbit-anti IgG. Samples were acquired using a FACS Calibur flow cytometer and analyzed using FlowJo version 9.3.

3.2.5 Data Analysis

Statistical analyses were carried out using Graphad Prism software version 5.0. The parametric two-tailed Student´s t-test was used, since the groups were large enough (containing more than 15 observations) and normally distributed. Linear regressions were performed to investigate the degree of correlation between two variables. Deviations between the variables were visualized using r^2 values. Kaplan Meier survival analyses were performed, with comparison of survival curves using the Gehan–Breslow–Wilcoxon test. Hazard ratios were calculated using the Mantel–Haenszel method, for assessment of the rate of deaths in the two groups (a hazard ratio of 2.0 means that the rate of deaths is twice the rate in the other group) (www.graphpad.com). Follow-up time was calculated from PSC diagnosis to the date of liver transplantation or death.

3.3 PAPER II

3.3.1 Patients and liver tissue

Liver tissue from 17 patients with PSC and 17 non-PSC controls were used in this study. Sixteen of the PSC patient samples were received at the time of liver transplantation, and one was obtained from a patient undergoing liver resection due to suspicion of CCA. As control samples, tissues from four donor livers (not used for liver transplantation because of technical reasons) and 13 patients undergoing liver resection due to suspicion of cancer, were included. All the obtained samples were from non-tumor-affected areas of the resected livers, as far away from the resection margin as possible. Patients with cholestatic disease or primary liver cancer were excluded from the non-PSC cohort. Upon collection, all liver specimens were embedded in Tissue-Tek OCT, snap-frozen, and stored at −80°C until sectioning.

3.3.2 Gene expression analysis

For the measurement of gene expression, total messenger (mRNA) was extracted from liver tissues with Trizol, based on the protocol by the manufacturer. Complementary (c)DNA was produced by the use of Applied Biosystem´s High capacity cDNA reverse transcription kit. The DNA was amplified and quantified using TaqMan real time polymerase chain reaction (PCR), employing the Applied Biosystems 7500 Fast Real-Time PCR System. Expression levels of *CK19*, *Caldesmon*, *NKp46*, and *HLA-A* and *HLA-B* were calculated from the cycle threshold (Ct) values against the endogenous housekeeping gene *Cyclophilin A*, using the comparative delta-Ct method.

The remaining methods; histological grading, immunohistochemistry, automated image analyses and statistical analyses, are the same as for paper III and therefore described further below.

3.4 PAPER III

3.4.1 Patients and liver tissue

Twenty CCA-patients and five non-CCA controls were enrolled in this study. Liver tissue specimens were obtained from patients admitted for liver resection at Karolinska University Hospital, for reason of a primary malignancy in the liver. Inclusion criteria were a diagnosis of CCA, which was confirmed by routine pathology. Control samples were obtained from five patients undergoing liver resection due to suspicion of gall bladder cancer, but where no cancer was found in the surgical specimen and the liver histology was evaluated to be normal.

3.5 PAPER II & III

3.5.1 Histological grading

Liver tissues were sectioned into a thickness of 5 µm and placed onto SuperFrost Ultra Plus slides. To visualize the histology, the samples were stained with hematoxylin and eosin. Thereafter a liver pathologist, who had no information about the included patients, evaluated the samples with regard to inflammation (grade) and fibrosis (stage), based on a non-continous, 0–4 graded scale. Levels of bile duct proliferation were assessed using a scoring method with; 0 = 1–2 bile ducts per portal zone, 1 = 3–4 ducts per portal zone, evaluated as minimal proliferation, 2 = 5–7 ducts per portal zone, corresponding to a moderate proliferation, 3 = 8 or more bile ducts per portal zone, corresponding to intensive proliferation (83).

3.5.2 Immunohistochemistry

Immunostainings were performed using the ImmPRESS system (Figure 3). Briefly, sections were air-dried for 10 minutes and thereafter fixed in 4% paraformaldehyde for 20 minutes on ice. For intracellular staining, fixation and permeabilization was performed with Foxp3/Transcription Factor Staining Buffer Set (study III). Sections were incubated with Bloxall (10 minutes), followed by Innovex background Buster (20 minutes) to reduce unspecific background stainings. Samples were incubated with primary antibodies over night at +4˚C. Secondary ImmPRESS antibodies were added to the sections for 30 minutes at room temperature, and specific staining was detected by incubation with ImmPACT DAB. Finally, tissue sections were counterstained with Hematoxylin and mounted with Kaisers´s glycerol gelatine.

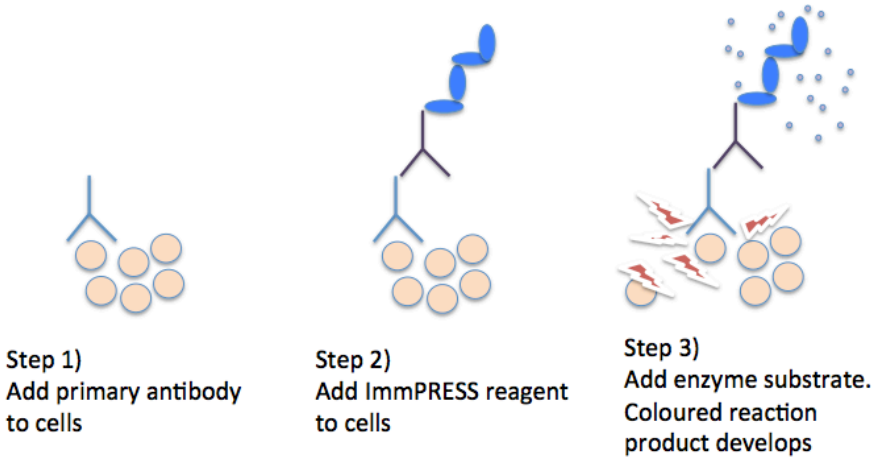

Figure 3. Brief description of the general concept behind immunohistochemistry, and the ImmPRESS method used. In a first step, primary antibody, targeted against the specific protein of interest, is added. In step two, the bound primary antibodies are captured with a peroxidase-conjugated secondary antibody. In the last step, the enzyme substrate for peroxidase, 3,3´-diaminobenzidine (DAB), is added. This will lead to the development of a brown reaction product, which makes the detection of the protein possible and further measurable for image analysis.

3.5.3 Automated image analysis

IHC-stained specimens were analyzed by acquired computerized image analysis (ACIA) in a blinded fashion, that is, without knowledge of which samples that were controls and which ones that were patients. Fifteen to twenty consecutive photomicrographs were taken from each tissue section, which covered the majority of the sample area. ACIA-values were calculated as the percentage of area stained positively by a phenotypic marker, multiplied by the mean intensity of the positive staining.

3.5.4 Data Analysis

Statistical analyzes were performed using Prism version 6.0 (84). Kolmogorov-Smirnov or A D´Agostino-Pearson omnibus test were performed to probe if values were normally distributed. The Students t test was used when the investigated population assumed a normal distribution. If not, the nonparametric statistical test, Mann Whitney *U* test, was used. For comparison of paired samples, a paired t test was used, when the values had a Gaussian distribution. Otherwise, the Wilcoxon matched pairs test was performed. One-way ANOVA (parametric) or Kruskal Wallis (nonparametric) tests were applied for multiple groups. To analyze the pairwise differences between any of two groups, a Bonferroni or a Dunn´s post-analysis was performed, after a parametric and nonparametric test, respectively. For correlation analyzes, linear regressions were performed. Principal component analysis (PCA) was performed using Qlucore OmicsExplorer v2.2, to identify the largest variances between included variables

4 RESULTS

4.1 PAPER I

4.1.1 Patients

The clinical characteristics of the patients and controls included in this study are presented in Table 1. The median age of the PSC patients was 58 years (range 25-94). The distribution between males and females was 63% and 37% respectively. The median age of the controls was 48 years (range 28-71), with 24 males (57%) and 18 females (43%). Eighty percent of the PSC patients suffered from IBD, and 53% of the total numbers of patients reached clinical endpoint during the follow-up period.

Table 1.

Clinical characteristics of patients and controls in paper I.

	Controls (n=42)	PSC patients (n=81)
Age, median (range)	48 years (28-71)	58 years (25-94)
Male	24 (57%)	51 (63%)
CCA	NA	10 (12%)[1]
IBD	NA	65 (80%)
Colectomy	NA	15 (19%)[2]
Duration of PSC disease	NA	89 months (4 – 408)
Ltx or death	NA	43 (53%)
Follow-up time	NA	65 months (1 – 164)

Abbreviations: CCA = cholangiocarcinoma; IBD = inflammatory bowel disease; UC = ulcerative colitis; CD = Crohn's disease; Ltx = liver transplantation.

[1]Information not available in four patients; [2]Information not available in five patients.

4.1.2 Establishment of biliary epithelial cell-selected cultures

After mechanic and enzymatic digestion of healthy human liver tissues, a population of cells that expressed the biliary epithelial-specific markers epithelial cell adhesion molecule (EpCAM), cytokeratin (CK) 7 and CK19 was identified with flow cytometry (Figure 4.1).

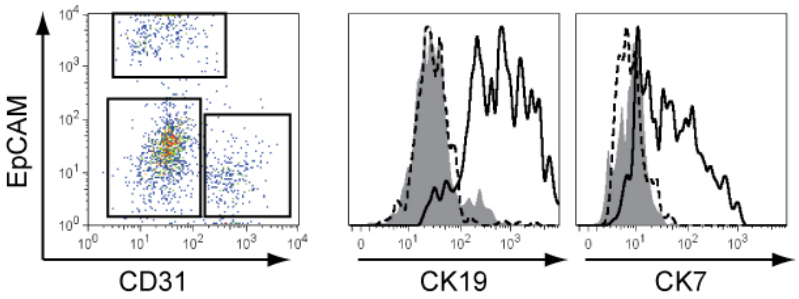

Figure 4.1: Characterization of primary and *in vitro* expanded biliary epithelial cells. Representative stainings for EpCAM and CD31 on freshly isolated liver cells and for CK19 and CK7 after gating for EpCAM-positive (solid black line), CD31-positive (dashed black line), and EpCAM-negative CD31-negative (solid gray histogram) cells.

After four to six passages, the cells started to express molecules often associated with fibrotic processes, such as α-SMA, calponin, caldesmon, and CD90 and had lost their expression of EpCAM and BerEp (another antibody clone for epithelial adhesion molecule). However, as a control for purity of the cultures, cells also stained negative for the endothelial marker CD31 (Figure 4.2).

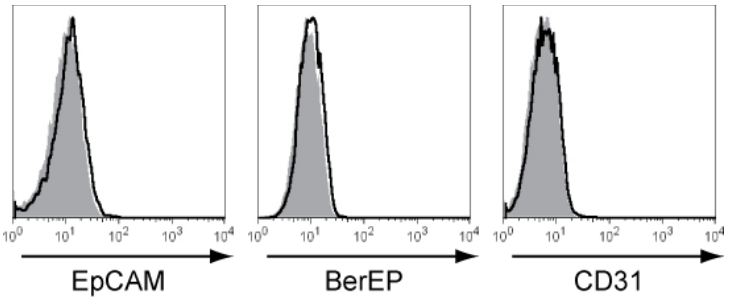

Figure 4.2: Representative stainings for EpCAM, BerEP, and CD31 (all solid black lines) on

biliary epithelial-enriched cells after six passages. Solid gray histograms represent isotype controls.

4.1.3 PSC patients have autoreactive IgA and IgG antibodies

Serum from a total of 81 PSC patients and 42 healthy controls was investigated for the presence of serum IgA and IgG with reactivity against the EpCAM-selected cells. Using MFI measurements, with a MFI cut-off set to the highest MFI value of the healthy control group, 65% of the investigated PSC patients were shown to have IgA that reacted against the cells and 68% had reactive IgG antibodies (Figure 4.3). Only low or no binding could be detected when applying serum from healthy controls (Figure 4.4).

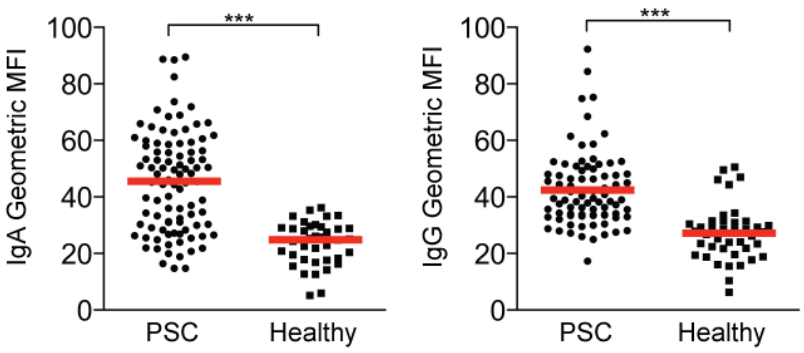

Figure 4.3: Geometric MFI for IgA (left) and IgG (right) binding to epithelial-enriched liver cells summarized for the patients and controls.

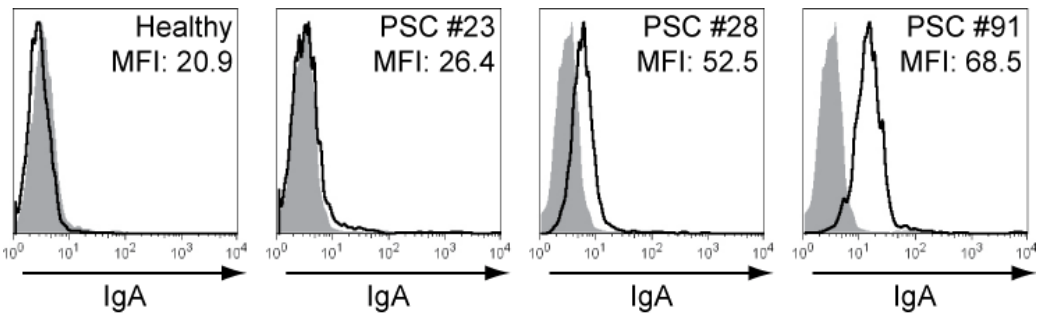

Figure 4.4: Representative flow cytometry staining for IgA (black solid line) and isotype

control (gray solid histogram) on epithelial-enriched liver cells using serum from one healthy control and three PSC patients. The figure shows that PSC patients have antibodies that react against biliary epithelial-enriched cells.

4.1.4 Levels of autoreactive IgA is associated with higher total levels of circulating IgA antibodies

Since a majority of the patients were found to have reactive antibodies against the selected cells, we next investigated possible underlying factors that could explain their appearance in the sera. For this purpose, levels of autoreactive IgA and IgG were correlated with total serum concentrations of IgA and IgG in the patients. We found that patients who were autoreactive IgA-positive had higher levels of total serum IgA than the patients that were autoreactive IgA-negative. No such correlation was found between serum IgG and autoreactive IgG levels. Another possible explanation for the presence of autoreactive antibodies in PSC patient sera could be secondary effects, following long disease duration with chronic inflammatory and fibrotic processes in the liver. However, when levels of autoreactive IgA and IgG antibodies in PSC patient sera were correlated with the median duration of PSC disease, no such association was found.

4.1.5 Autoreactive IgA is associated with more rapid PSC disease progression

Next, we wanted to investigate if the presence of autoreactive antibodies in PSC patient sera could be connected to disease characteristics. When investigating this, we found that the levels of autoreactive IgA or IgG in the patients were neither correlated with age, gender or later development of CCA. No correlation was either found between the presence of autoantibodies and a coexisting IBD in the patients.

Finally, we wanted to investigate if disease progression was affected by the presence of

circulating autoantibodies in PSC patient sera. Survival analysis studies were performed to evaluate this, using death or liver transplantation as the clinical endpoints. Autoreactive IgA-positive PSC patients were found to progress more rapidly to a clinical endpoint than autoreactive IgA-negative patients, with a hazard ratio of 1.78 ($p = 0.03$) (Figure 4.5). A similar association could not be found for autoreactive IgG-positive patients.

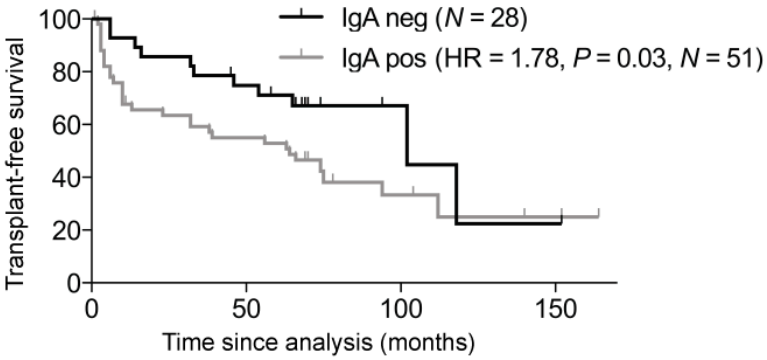

Figure 4.5: Autoreactive IgA is associated with more rapid PSC disease progression. Kaplan Meier survival analysis comparing the effects of having IgA autoantibodies on progression of disease defined as liver-transplantation-free survival. PSC patients were subdivided into autoantibody-positive and -negative patients. As a cut-off for IgA in the patients, the highest measured geometric MFI of the healthy controls was used.

4.2 PAPER II

4.2.1 Patients

The clinical characteristics of patients and their evaluated liver histology are presented in Table 2. Ten of the patients (59%) were male and 7 (41%) were female. Four patients were histologically assessed as having a mild disease and 13 patients as having a severe disease.

Table 2.

Clinical characteristics of patients and controls in paper II.

	Controls (n = 17)	PSC patients (n = 17)
Age, median (range)	58 (16-86)	36 (19-68)
Male	9 (53%)	10 (59%)
IBD	0[1]	14 (82%)
Ltx	0	16 (94%)
MELD score, mean ± SD	NA	13 ± 8
Serum IgG (g/L), mean ± SD	NA	15.5 ± 4.6
ALP (μkat/L), mean ± SD	1.8 ± 1.0	5.4 ± 4.4
Bilirubin (μmol/L), mean ± SD	7.9 ± 4.9	89.2 ± 166.1
ALT (μkat/L), mean ± SD	0.6 ± 0.2	1.3 ± 0.7
CRP (mg/L), mean ± SD	5.3 ± 6.6	24.4 ± 32.9
UDCA dose (g/day), mean ± SD	NA	0.8 ± 0.6
Fibrosis stage 0/1/2/3/4	12/5/0/0/0	0/4/0/0/13
Inflammation grade, 0/1/2/3/4	14/3/0/0/0	4/4/9/0/0
Bile duct proliferation stage, 0/1/2/3	3/14/0/0	3/2/7/5

Abbreviations: IBD = inflammatory bowel disease; UC = ulcerative colitis; CD = Crohn´s disease; NA = not applicable, Ltx = liver transplantation, MELD = Model for End-Stage Liver Disease, SD = standard deviation, ALP = alkaline phosphatase, ALT = alanine aminotransferase, CRP = C-reactive protein, UDCA = ursodeoxycholic acid

[1]Information not available for four controls

4.2.2 IHC characterization of non-parenchymal non-immune cells in PSC-patient livers

After IHC staining for several different cellular subsets of non-immune origin, we could conclude that our results support previous findings regarding increased numbers of BECs in

PSC patients. All the BEC-specific markers (CK7, CK19 and EpCAM), were significantly increased (visualized for CK19 in Figure 4.6), whereas numbers of endothelial cells were at the same levels in patients and controls.

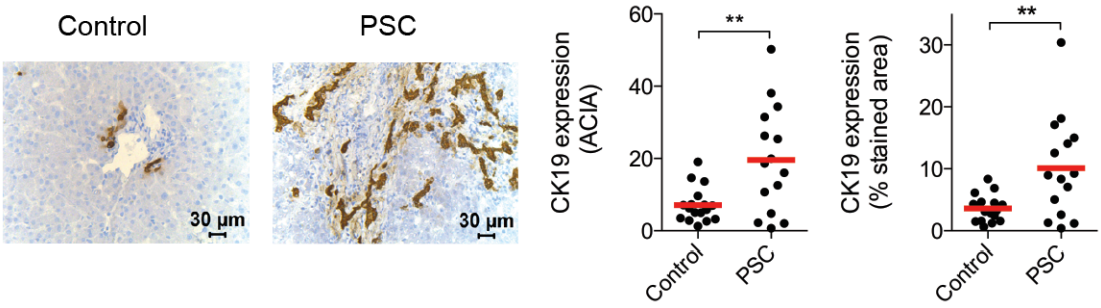

Figure 4.6: Representative immunostaining and quantification analysis of CK19 expression in controls and PSC patients. Samples were counterstained with hematoxylin and DAB chromogen provided the brown reaction product, identifying the biliary epithelial cells. Quantification of protein expression was performed by calculating both the intensity of the IHC staining (ACIA) and the frequency of cells that expressed the protein (percentage of stained area of the total area).

4.2.3 Gene expression analysis

When investigating the gene expression of CK19, only a trend toward elevated levels of *CK19* mRNA was detected in the patients compared to the controls. No significant difference could either be observed for *NKp46*, *HLA-A* and *HLA-B* mRNA, although the total protein expression of HLA class I was significantly reduced in patients compared to controls, as revealed by the IHC stainings.

4.2.4 T cells infiltrate PSC-patient livers and localize to fibrotic fields

Next, we wanted to locate and enumerate different immune cell populations in patients and control livers. IHC staining and image analysis was performed to quantify the expression of CD3, TCR-Vα7.2, NKp46 and CD163. Protein levels of MHC class I were examined (HLA-A, B, C) as well. Our data reveal that the total numbers of $CD3^+$ T cells in PSC patients were

significantly higher than in controls, and that these cells specifically localized to fibrotic areas (Figure 4.7). MAIT cells exhibited a similar pattern, but were not more numerous in the patients than in the controls (Fig 4.7). CD163 was more strongly stained in the patients, whereas no difference was seen for NKp46 expression. Both Kupffer cells and NK cells were evenly distributed throughout the parenchyma with no specific accumulation in fibrotic areas.

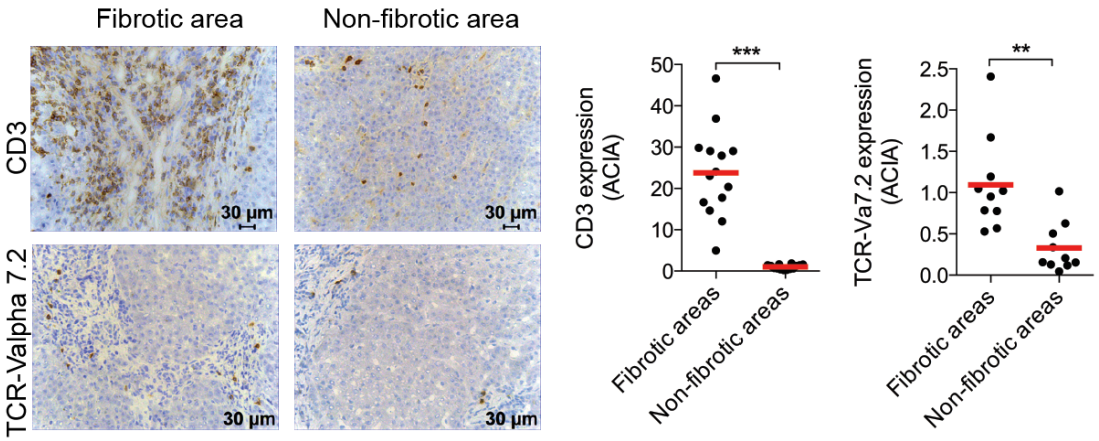

Figure 4.7: Representative immunostaining and quantitative determination of intensity of staining (ACIA) of CD3 and TCR-Vα7.2 in fibrotic and non-fibrotic areas in PSC patient livers.

4.2.5 Low expression of Caldesmon in PSC-patients correlates with higher numbers of T cells and more extensive bile duct proliferation

Immunohistochemistry and enumeration for Caldesmon expression was performed, since smooth muscle cells might participate in processes leading to development of fibrosis. No significant differences in protein expression could be detected between patients and controls, however, some patients were distinguished by being almost completely negative for Caldesmon expression (Caldesmon^{neg}), while the other patients had a similar expression level as the controls (Caldesmon^{pos}) (Figure 4.8). The two groups were compared regarding expression of phenotypic markers and histological parameters. Caldesmon^{neg} patients had significantly higher expression of CD3 and a more extensive bile duct proliferation than

Caldesmon^{pos} patients (Figure 4.9).

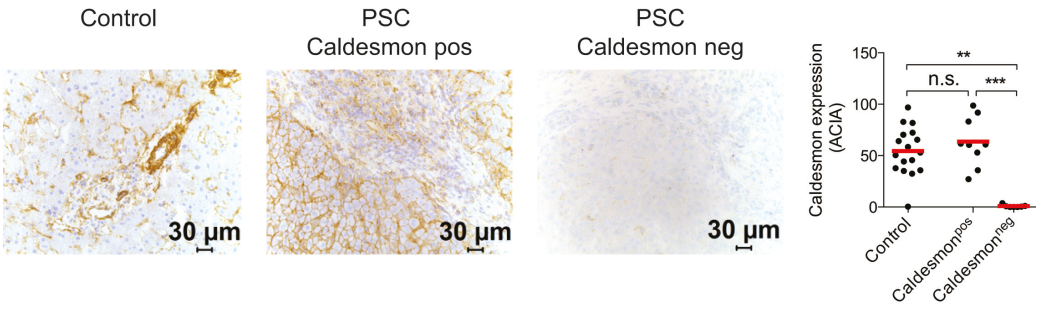

Figure 4.8: IHC and quantitative image analysis of Caldesmon, showing one control (left), one PSC patient with a negative expression (right) (Caldesmon^{neg}), and one PSC patient with similar level of expression as the control (middle) (Caldesmon^{pos}).

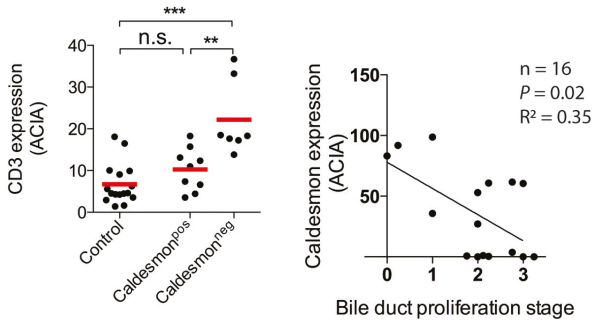

Figure 4.9: Quantification with image analysis of CD3 expression in controls and PSC patients with positive (pos) or negative (neg) expression of Caldesmon (left). Linear regression analysis of Caldesmon expression versus bile duct proliferation in the PSC-patients (right).

4.2.6 Principal component analysis of non-parenchymal liver cells in PSC

Having analyzed a total of 23 unique parameters, including both phenotypic markers and histological scores, we next wanted to identify factors that distinguished patients from controls, and further, within the PSC group, identify patterns associated with disease severity. PCA was first performed to compare the expression pattern in patients and controls. Histological scores (fibrosis, inflammation, and bile duct proliferation) were in this first set of

analysis excluded (Figure 4.10). Thereafter, a second analysis was performed, with inclusion of histological scores, to identify phenotypic markers that differentiated patients with respect to severity of fibrosis or bile duct proliferation (Figure 4.10). The results revealed that numbers of bile duct cells, T cells, Kupffer cells, and MHC class I expression, were all phenotypic factors that significantly distinguished PSC-patients from controls. In the next analysis,

EpCAM and NKp46 were identified as phenotypic factors that distinguished patients with mild from severe fibrosis, whereas Caldesmon and CK19 separated patients according to stage of bile duct proliferation (Figure 4.10).

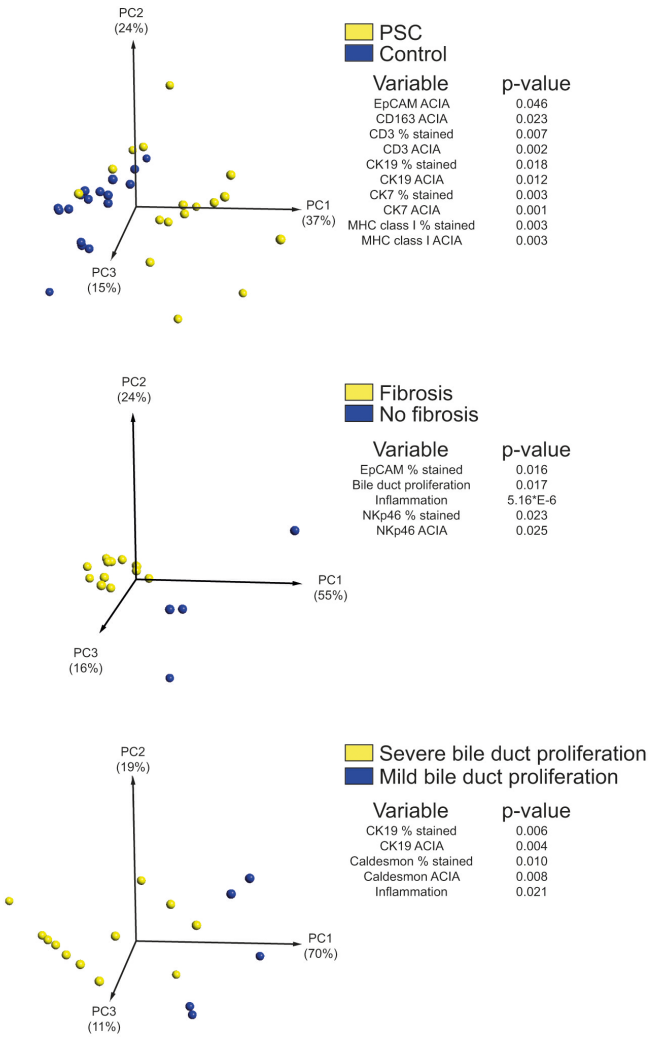

Figure 4.10: PCA plot showing phenotypic factors that separate PSC patients from controls (upper). In the next PCA, PSC patients were compared according to severity of disease, with inclusion of scores of fibrosis (middle) and bile duct proliferation (lower).

4.3 PAPER III

4.3.1 Patients

The clinical characteristics and histological scores of the patients included in this study are presented in Table 3. In total, 20 CCA patients (11 males and 9 females) and 5 non-tumor controls (2 males and 3 females) were included in the study. The median age was 63 years (range 30-81) for the CCA-patients and 67 years (range 53-70) for the controls.

Table 3.
Clinical characteristics of patients and controls in paper III.

	Controls (n=5)	CCA patients (n=20)
Age, median (range)	67 (53-70)	63 (30-81)
Male	2/5	11/20
PSC	NA	5/20
Intrahepatic tumor	NA	14/20
Tumor differentiation (low/moderate)	NA	13/6[1]
ALP (μkat/L), mean ± SD	1.3 ± 0.5	4.4 ± 2.6
Bilirubin (μmol/L), mean ± SD	12.4 ± 15.6	16.2 ± 36
CRP[2] (mg/L), mean ± SD	12.4 ± 22.2	38.5 ± 44.2
Fibrosis score[3] 0/1/2/3/4	2/3/0/0/0	6/12/2/0/0
Inflammation grade[3] 0/1/2/3/4	4/1/0/0/0	10/9/0/1/0
Bile duct proliferation stage[3] 0/1/2/3	1/3/1/0	2/8/7/3

Abbreviations: ALP = alkaline phosphatase; CRP = C-reactive protein;

PSC = primary sclerosing cholangitis; SD = standard deviation.

[1]Information not available from one patient; [2]CRP was measured the day before surgery;

[3]Non-tumorous tissue

4.3.2 IHC characterization and quantitative image analysis of non-parenchymal non-immune cells of CCA-patient livers

We characterized non-parenchymal intrahepatic cells of immune as well as of non-immune cell origin in CCA-patients and controls. Expression of CK19, CD31 and Caldesmon were assessed with IHC and quantitative determination. As expected, CK19 was more dramatically increased in the tumorous areas of the CCA patients than in the non-tumorous areas, and also

in comparison to the non-CCA controls, whereas no change in presence of endothelial cells or smooth muscle cells were detected in the tumor areas.

4.3.3 MAIT cells and Kupffer cells are selectively lost in the CCA microenvironment

The establishment and growth of tumors are tightly linked to the numbers and composition different immune cell subsets in the tumor microenvironment (85).

Immunostainings were performed to enumerate $CD3^+$ T cells, $CD8^+$ cytotoxic T lymphocytes, TCR-Vα7.2$^+$ MAIT cells and $CD163^+$ Kupffer cells in controls and in non-tumorous and tumorous areas of CCA-patients. Few, if any, differences were present when comparing the non-tumorous areas of CCA-patients with the control tissues. Furthermore, the tumor areas contained similar levels of $CD3^+$ and $CD8^+$ cells as in the surrounding non-affected areas. Instead, a selective two-fold loss of TCR-Vα7.2 and CD163 expression was noted in the tumorous areas (Fig. 4.11). In summary, these results indicate that the intratumoral compartment in CCA has preserved numbers of T cells and CTLs but a selective loss of innate MAIT cells and Kupffer cells.

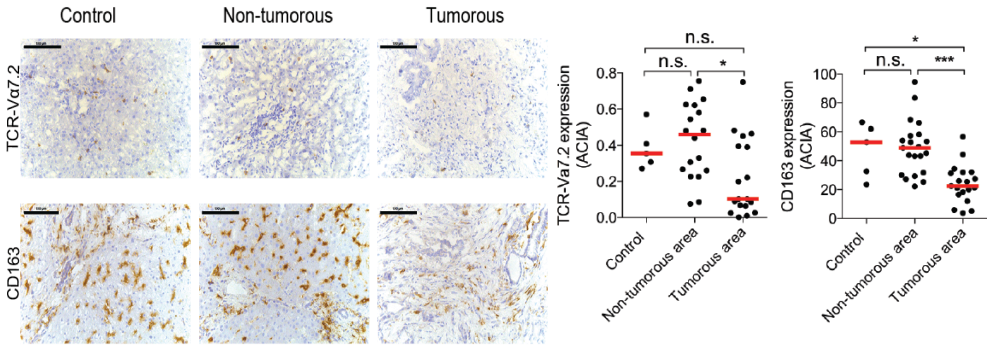

Figure 4.11: Representative immunostainings (left) of TCR-Vα7.2 (upper) and CD163 (lower) expression in controls, non-affected and affected areas of CCA patient livers. Samples were counterstained with hematoxylin and dark brown indicate immunoreaction

product. Quantitative analysis (right) of the mean intensity of TCR Vα7.2 and CD163 IHC stainings (ACIA) in controls, and non-affected and affected areas in CCA patient livers.

4.3.4 Retained Th1 but diminished Th2 responses in the tumor microenvironment

Having observed that the tumor areas of CCA-patients contained less innate immune cells than the surrounding non-tumorous areas, we next wanted to investigate the shape of the adaptive immune response within the tumor. Expression levels of perforin, IL-33, and Foxp3 were measured, reflecting Th1, Th2, and regulatory immune responses respectively. In summary, CCA tumors were characterized as having conserved Th1, but clearly reduced Th2 responses, and high numbers of regulatory T cells. This was evident by the sustained expression of perforin, significantly lower protein levels of IL-33 and higher levels of Foxp3 (Figure 4.12).

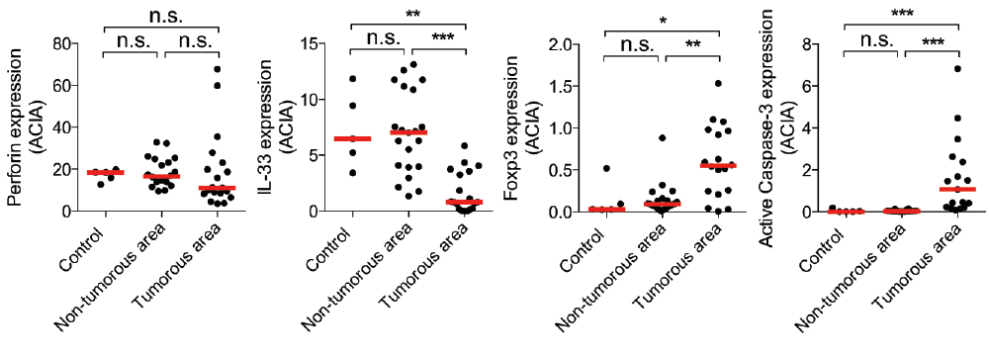

Figure 4.12: Quantification of the mean intensity of staining (ACIA) of Foxp3, IL-33, Perforin and Caspase-3, in non-affected and affected areas of CCA patients and in non-affected and affected areas of CCA-PSC patients.

4.3.5 Underlying PSC, tumor location, and degree of tumor differentiation are not associated with the revealed immune cell composition

PSC is known to be one of the strongest risk factors for CCA, but there is limited knowledge about immunological differences between CCA livers with and without PSC. Thus, we evaluated the immune cell composition in the five PSC-CCA patient livers included in the study and compared it to the non-PSC CCA-patient livers. We also investigated if tumor location or the degree of tumor differentiation had an impact on the presence and numbers of cells. All the eleven included IHC-parameters previously studied were compared between the groups. We found that neither PSC nor tumor location nor tumor differentiation seemed to affect the immune cell pattern in CCA.

5 GENERAL DISCUSSION

The general goal of this thesis was to improve the knowledge about pathogenic mechanisms in PSC. So far, no unifying explanation exists for how the chronic injury of the bile ducts is initiated in PSC. However, genetics as well as the observations of inflammatory infiltrates around portal tracts, other coexisting autoimmune diseases and many different autoantibodies in patient sera, suggests that specific immune reactions are of importance. With this knowledge as a basis, we aimed for investigating the presence and effect of autoantibodies in PSC patient sera, specifically targeted against biliary epithelial cells. Other aims were to study the intrahepatic localization and composition of various immune cell subsets in PSC patient livers, and in a subgroup of patients, diagnosed with cholangiocarcinoma.

In paper I, we investigated the presence and effect of specifically targeted autoantibodies against BECs in PSC. We examined serum samples from 81 PSC patients and 42 controls, for IgG and IgA antibodies. The reactivity against isolated and cultured human BECs was explored and we found that most PSC patients had IgA antibodies that were reactive against the isolated cells, while few of the control samples showed any reactivity at all.

The identification of autoantibodies, targeting a specific antigen in PSC, would be valuable from both diagnostic and prognostic purposes. However, studies to date suggest that autoantibodies in PSC arise as a result of secondary effects due to inflammation, rather than by a specific immune response towards the biliary epithelium. An early study (86) reported that sera from PSC and UC patients have autoantibodies that react with human tropomyosin isoform 5 (hTM5), a protein expressed in both intestinal and biliary epithelial cells. However, the specificity of this autoantibody is insufficient, since this protein is also found in the eyes, skin and cartilage, and furthermore, the presence of anti-hTM5 autoantibodies have been reported in sera from UC patients without PSC.

Why would autoreactive IgA antibodies form in PSC patients? We observed that PSC patients who were seropositive for autoreactive IgA generally had higher levels of total serum IgA. This observation led us to two different theories. One was that the inflammation itself causes an increased production of IgA antibodies, and that some of these antibodies react with endogenous proteins, exposed as a result of cell death. Another possibility is that the autoantibodies themselves contribute to the inflammatory process, and thereby accelerate the disease, with an increased total IgA production as a consequence. To investigate this, we correlated the presence of autoantibodies with disease duration. Since no association was found, we speculate that the appearance of autoantibodies might be related to factors other than prolonged inflammation.

We found that PSC patients that were positive for autoreactive IgA antibodies at follow-up, reached clinical endpoint (death or liver transplantation) faster than patients that were negative for autoreactive IgA. The reason for this is unclear. The knowledge about the function of IgA antibodies in liver diseases is scarce although several studies suggest that elevated serum IgA levels are associated with reduced liver function and fibrosis (87). We did not have access to assessments of liver histology for the included patients, but since approximately half of them reached clinical endpoint within the follow-up period, it seems reasonable to believe that many of them were cirrhotic.

The possibility that autoreactive IgA antibodies are produced in response to intestinal inflammation could not be excluded. However, no correlation could be found between the presence of IBD and autoreactive IgA antibodies in our patient cohort, suggesting that IBD, as a single parameter, has no effect on the formation of autoantibodies against the biliary epithelium. The specificity of these antibodies for PSC can also be questioned. Other

autoimmune or inflammatory liver diseases such as primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH) may also produce autoreactive IgA antibodies. Unfortunately, this question must be left open for further investigation, since we did not have access to such patient samples in this study.

Sera from PSC patients and controls were reacted against EpCAM-selected, *in vitro* cultured liver cells in this study. After a few passages, the cells lost their expression of epithelial markers and started to express myofibroblast-like surface proteins, possibly indicating an epithelial to mesenchymal transition (EMT). Interestingly, previous studies have shown that the development of fibrosis in PSC patient livers is accompanied by an upregulation of EMT-associated markers on the biliary epithelium (88), which possibly suggests that the cultured cells might have reflected a PSC-like *in vivo* situation. Although the precise cellular phenotype could not be identified, a majority of the patients had IgA antibodies targeted against the selected cells, which was further associated with a lower survival rate for the patients. The exact phenotype of the selected cells and the antigen(s) to which the antibodies bind, need further investigations.

In summary, we found that the presence of IgA antibodies, targeted against biliary enriched cells, were associated with a lower survival for the PSC patients. Regardless of whether these autoantibodies are contributing factors to the inflammation in PSC, or if they arise as secondary effects due to it, their presence is evidence of an dysregulated immune response.

A characterization and quantification of non-parenchymal cells in PSC livers has previously not been systematically and comprehensively performed. Such knowledge is of importance for better understanding of the inflammatory process in the liver and why it occurs. The biliary epithelium appear to be the main target for the immune attack in PSC, but less is

known about other non-parenchymal cells and how they are affected during the course of PSC disease (89). Many experimental studies have focused on investigating the role of T cells in PSC pathogenesis (42), and the proposed theory that PSC is driven by long-lived memory T lymphocytes, initially activated in the gut, represents an attractive link between PSC and IBD (90). The enterohepatic circulation of lymphocytes constitutes a possible explanation of how inflammation of the liver can occur long after an IBD patient has undergone colectomy. The purpose of the study in paper II was to characterize and quantify non-parenchymal cells in PSC livers and explore if specific phenotypic patterns were associated with severity of disease. In addition to the immunohistochemical characterization, followed by computer-based quantifications, a PCA was performed, to identify the phenotypic factors that most strongly separated PSC patients from healthy controls.

Major findings were, as observed by the immunostainings, that $CD3^+$ T cells were significantly more abundant in PSC patient livers than in controls and almost exclusively localized to areas of fibrosis. TCR $V\alpha7.2^+$ MAIT cells, a recently discovered T cell population with an innate-like phenotype and the capacity to respond to bacterial stimulation (91), were not increased in numbers to the same extent. However, as the rest of the $CD3^+$ T cell population, MAIT cells were preferentially localized to fields of fibrosis. Previous studies have shown that PSC patients have higher amounts of bacterial and fungal species in bile than controls and PBC patients have. Moreover, isolated bacterial compounds from PSC bile fluid were shown to initiate an increased number of Th17 and Th1/Th17 cells in peripheral blood (49). Since MAIT cells are competent producers of IL-17 (92), a cytokine that is described to be involved in fibrosis and pro-inflammatory interactions in the liver, it is tempting to speculate on the possibility that the expanding Th17 cells in the peripheral blood actually were MAIT cells. Although we did not see higher amounts of MAIT cells in PSC

patient livers than in controls, future investigations of the functions of MAIT cells in PSC will be of importance to determine a possible involvement in PSC disease.

Experimental and genetic studies have shown that macrophages may be involved in the pathogenesis of PSC. Significantly higher numbers of Kupffer cells have been observed in PSC patients than in patients with primary biliary cirrhosis (PBC) and normal controls (93). The group behind this study proposed that Kupffer cells might be involved in early stages of PSC disease, since they were found in similar numbers both in cirrhotic and non-cirrhotic livers. In our study we saw that Kupffer cells were more intensely stained in the patients than in controls, while the frequencies of them were similar. In contrast to T cells, Kupffer cells localized in a scattered manner throughout the parenchyma and not only to fibrotic fields.

Monocytes that migrate to sites of injury differentiate into macrophages with different phenotypes, as a response to local cytokines in the environment. Immunosuppressive interleukins (ILs), such as IL-10, IL-4 and IL-13 are known to induce the alternative activation of macrophages into the M2 phenotype, while pro-inflammatory mediators, such as IFN-γ, lead to the classical activation into M1 macrophages (76). M2 macrophages are associated with suppression of adaptive immune responses, and therefore, they have been the focus of several cancer studies (94). We used CD163 as a phenotypic marker for Kupffer cells. It should be mentioned that CD163 positive cells might represent the population of M2 macrophages, reported to be involved in wound healing and anti-inflammatory activities (95). Future investigations should include CD68 as a phenotypic marker for characterization and localization of the pro-inflammatory M1 subset of macrophages in PSC.

NK cells were observed to localize throughout the parenchyma, but were at similar levels in patients and controls. Except for a study conducted in the 1990ies (96), little is known about

NK cells and a possible involvement in PSC. Experimental studies using murine models, have suggested them to participate in anti-fibrotic activities (97) and therefore it is not unlikely to suspect them to be involved in the pathogenesis of PSC. Interestingly, although the quantification showed similar levels of NK cells in patients and controls, the PCA analysis revealed NKp46 as a phenotypic factor that distinguished PSC patients with mild from severe fibrosis.

PCA is a mathematical method, with which multivariate datasets can be visualized as completely as possible, with as few graphable dimensions as possible. More specifically, a PCA analysis detects new variables (principal components), which account for the vast variability in the data set. By doing so, hidden structures in a complex data set can be revealed, while noise is filtered out (98). In a first PCA analysis, we aimed to identify phenotypic markers that most strongly separated all PSC patients from controls. Therefore, histological scores were excluded. Not unexpectedly, the biliary epithelial markers CK7, CK19 and EpCAM were all identified as phenotypic markers that separated PSC patients from controls.

In a second analysis, the purpose was to find phenotypic factors that separated PSC patients according to inflammation, fibrosis and bile duct proliferation. Therefore, the histological assessments were included in this analysis. In addition to the identification of NKp46 as a factor that distinguished patients according to mild and severe fibrosis, Caldesmon was revealed as a marker that separated patients according to mild or severe bile duct proliferation.

Caldesmon is a protein involved in contractile functions of smooth muscle cells in the liver and in other organs, but it is also reported to participate in cellular processes, such as division,

migration, apoptosis etc. (99). Interestingly, some of the PSC patients were observed to have a lost expression of Caldesmon, and this was further associated with an increased bile duct proliferation and higher numbers of T cells. It is currently unclear why some PSC patients lose Caldesmon expression and the eventual effects of it in terms of liver physiology. The reduced expression might be a response to inflammation, wound healing or cellular reactions that leads to bile duct proliferation. However, the increased T cell infiltration observed in patients with lost Caldesmon expression, indicate that their liver disease is more active than in patients with normal levels of Caldesmon expression.

Genetic susceptibility within the HLA-region has been reported many times in PSC, but few studies have investigated expression at the protein level. In the present study we stained and measured the surface expression of MHC class I in PSC patients and controls and found that the patients had significantly lower expression. This was further supported by the PCA analysis, which revealed MHC class I as a factor that separated the patients from controls. Conflicting results have been reported regarding the usefulness of ursodeoxycholic acid (UDCA) in the treatment of PSC. Interestingly, in a study where PBC-patients were treated with UDCA, the MHC class I expression was observed to be reduced in hepatocytes (100). More detailed investigations showed that IFN-γ, which under normal circumstances up-regulate MHC class I expression, was decreased upon treatment with UDCA (101). In the present study, all the PSC patients received UDCA treatment, and consistent with the reported finding in PBC, the PSC patients in our study showed a lower MHC class I expression than controls.

A limitation of paper II is that the majority of the samples were from patients who underwent liver transplantation, that is, patients that had reached end-stage liver disease. Therefore, our results probably represent such cellular events that occur during the late stages of the disease,

rather than those that occur early. Correlations with clinical parameters should be considered with caution, as the number of patients with mild disease was small. Yet another limitation is the lack of control samples from individuals with other chronic liver diseases, such as PBC or autoimmune hepatitis (AIH). The objective of this study, however, was primarily to characterize and localize immune and non-immune cells in PSC livers. The design of it was mainly descriptive, and our findings can be viewed as hypothesis generating. Future studies of pathogenic mechanisms behind the lost expression of Caldesmon and MHC class I in PSC patients, as well as the biology behind specific T cell recruitment to fibrotic fields, will be of importance.

PSC patients have an increased risk of developing CCA. The intratumoral composition of immune cells has been shown to be important for the ability of cancer cells to establish and progress (85). Therefore it is of interest to characterize and quantify cells of immune and non-immune origin in CCA patient livers. High numbers of Tregs and myeloid-derived suppressor cells are associated with a poor prognosis for patients with different cancers (85), whereas infiltration of cytotoxic T cells into the tumor area is considered to be more advantageous (102).

In paper III we investigated the intrahepatic cellular composition of patients with CCA. We studied samples from both within the tumor as well as outside the tumor using immunohistochemistry and quantitative image analysis. Histopathological parameters, such as tumor location, degree of tumor differentiation or a coexisting PSC disease were examined for an eventual impact on the expression of immune cell markers.

The mixture of immune cells within tumors in our study was characterized by a selective loss of Kupffer cells and MAIT cells, high numbers of Tregs and a retained cytolytic capability of

the CTLs. Moreover, the expression of IL-33, a cytokine associated with a Th2 cellular response, was significantly lower within tumors. This distinct intratumoral phenotype was unaffected by tumor location, degree of differentiation, or an underlying PSC.

The liver is an organ that harbors many immune cells, whose activity is controlled by the action of immunosuppressive cytokines, such as TGF-β and IL-10 (6). We know little about the ability of these immune cells to recognize and combat tumor cells. Little is also known about Tregs and their involvement in CCA. The immunosuppressive functions of Tregs has been reported in several forms of cancer, and the outcome of their activities depends a lot on which cells they are targeted against. In some instances Tregs may suppress the tumor cells, which can be beneficial for the host (73). We observed that the Treg specific marker Foxp3 was significantly increased within tumor areas of CCA patients, which is in line with previous reports (74). Moreover, the levels of Tregs were strongly associated with the expression of Caldesmon, the phenotypic marker for smooth muscle cells which expression is lost in some PSC livers, on which we reported in paper II (103). Caldesmon is involved in many cellular processes (104) and at the moment there is no explanation for its association with infiltration of Tregs. TGF-beta, which is a cytokine produced by Tregs have previously been shown to downregulate the expression of Caldesmon (105). This is contrary to our results, which instead showed an increased expression of Caldesmon with increased levels of Tregs.

We observed significantly fewer MAIT cells in the tumor areas than in the surrounding, non-tumorous tissue. As already mentioned in the discussion of paper II, MAIT cells are T cells with an innate phenotype, whose frequencies are reported to be high at mucosal sites in the body, including the liver. They have been shown to respond to IL-7, released by hepatocytes during inflammation (14), but to date there is no knowledge about an eventual involvement of

MAIT cells in cancer. One can speculate on the reason for the observed reduction in tumor areas in CCA. One possibility may be that the MR1 molecule, that is required for presentation the antigen to MAIT cells, is lost on the tumor cells. Another possibility is that MAIT cells are kept outside the tumor area because of the action of immunosuppressive cytokines. Since MAIT cells have the ability to immediately exert effector functions against bacteria, and given that the liver is constantly exposed to gut-derived microbial compounds, it is plausible that MAIT cells may have a role in the pathogenesis of CCA. Further studies will be of importance to investigate this.

In addition to the scarce knowledge about MAIT cells in CCA, there are conflicting results on the role of cytotoxic T cells in CCA. Earlier studies have reported them to be unchanged or reduced in numbers in CCA tumors (74). In the present study, we could not detect any significant difference between the total number of $CD8^+$ CTLs outside and inside the tumor area. Additionally, their cytolytic functions appeared to be preserved, since the expression of perforin was at the same level inside as outside the tumor area.

IL-33 is a cytokine that, in experimental models, has been reported to promote fibrosis (33) as well as to drive the malignant transformation of BECs (106). Further support of this is the report of increased IL-33 levels in sera and tumors of HCC patients (107). Our results are in contradiction to this, as we observed significantly less expression of IL-33 within the CCA tumors than in the surrounding, non-tumorous tissue. The majority of the included samples in our study were assessed as having mild fibrosis, and it is reasonable to believe that levels of IL-33 may increase by severity of fibrosis.

From our earlier report in paper II on an increased number of T cells localizing to fibrotic areas in PSC patients (103), we would have expected to see higher numbers of infiltrating

immune cells in CCA patients with a coexisting PSC than in CCA patients without PSC. However, only small differences were detected. A possible explanation to this is that the included PSC patients in paper III were all histologically assessed as having a mild disease, except for their cancer, whereas the patients described in paper II mainly consisted of individuals with end-stage liver disease. We therefore speculate that the intrahepatic immune composition would have looked different if the PSC patients would have had a more severe disease.

Furthermore, it was not possible to relate our findings with clinical outcome in paper III, since the groups of CCA patients, both with and without PSC were relatively small. Future studies should aim to include more samples and use additional techniques, such as flow cytometry, for a more detailed phenotypic characterization of the intratumoral compartment of CCA. Our data in this study is mainly descriptive, and forthcoming studies should focus in revealing mechanisms behind the reduced levels of MAIT cells in CCA tumors, as well as the decreased expression of IL-33. This, and more detailed characterization studies, might contribute to a better understanding of the pathogenesis of CCA, as well as leading to the finding of CCA-specific biomarkers.

6 GENERAL CONCLUSIONS

Patients with primary sclerosing cholangitis have circulating IgA and IgG antibodies reactive against biliary epithelial cells (paper I).

Autoreactive IgA antibodies in patients with primary sclerosing cholangitis are associated with reduced survival (paper I).

The intrahepatic cell composition in patients with primary sclerosing cholangitis is heterogeneous but generally characterized by a numerous infiltration of T cells, mainly concentrated to the fibrotic areas. Loss of intrahepatic smooth muscle cell markers is associated with increased numbers of T cells and a more extensive bile duct proliferation (paper II).

Primary sclerosing cholangitis livers have lower expression of MHC class I than controls, whereas the levels of $NKp46^+$ NK cells are similar. Instead, NK cells are fewer in numbers in severely fibrotic livers as compared to livers with mild fibrosis (paper II).

The tumor microenvironment in livers with cholangiocarcinoma is characterized by high numbers of $Foxp3^+$ regulatory T cells and a conserved presence of cytotoxic lymphocytes (paper III).

Innate immune cells, such as mucosal-associated invariant T cells and Kupffer cells are selectively reduced within cholangiocarcinoma tumors as compared to surrounding tissue (paper III).

The tumor microenvironment has low expression of the Th2-associated cytokine IL-33. These phenotypic characteristics of cholangiocarcinoma tumors are not influenced by tumor location, degree of differentiation, or a coexisting primary sclerosing cholangitis (paper III).

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Primär skleroserande kolangit, PSC, är en ovanlig kronisk leversjukdom som karakteriseras av inflammation och ärrbildning (fibros) i gallgångarna. En hög andel av patienterna utvecklar med tiden skrumplever. Det finns ett starkt samband mellan PSC och inflammatorisk tarmsjukdom, IBD, och i Sverige har drygt 80 procent av alla patienter med PSC en samtidig IBD. I Skandinavien förekommer PSC hos drygt 8 av 100 000 individer och två tredjedelar av dem som drabbas är män. Det är vanligast att sjukdomen debuterar i yngre medelåldern.

Patienter med PSC har ofta diffusa symptom till en början, som trötthet, klåda, feber och högersidig buksmärta. Allt eftersom inflammationen och ärrbildningen fortskrider, förvärras de kliniska symptomen till följd av kolestas, som hindrar gallflödet från levern ut till tarmen. Den enda botande behandling som finns för patienter med PSC är levertransplantation. Tiden från sjukdomsdiagnos fram till att behov för levertransplantation uppstår varierar mellan 12 och 18 år.

Förutom risken att utveckla skrumplever och leversvikt, löper patienter med PSC en hög risk att utveckla cancer i levern. Särskilt kolangiokarcinom (gallgångscancer) är vanligt förekommande och drabbar 10-15 procent av patienterna. En tredjedel av patienterna får sin cancerdiagnos inom ett år från det att PSC sjukdomen blivit fastställd. Överlevnaden efter diagnos av gallgångscancer är kort, i medeltal mindre än 6 månader. Varför just patienter med PSC har så hög risk att utveckla kolangiokarcinom är inte känt, men inflammationen i levern är troligtvis en starkt bidragande orsak.

Etiologin bakom PSC är okänd och det behövs bättre kunskap om de immunologiska mekanismer som leder till att sjukdomen utvecklas. PSC uppvisar vissa klassiska egenskaper som förknippas med autoimmunitet, så som genetiska variationer inom MHC, eller HLA, som det också ofta benämns. MHC regionen är ur immunologiskt perspektiv ett viktigt

område i vårt DNA, och innehåller gener som styr vårt immunförsvar. Andra autoimmuna egenskaper i PSC är att patienterna ofta har cirkulerande antikroppar i sitt serum, vilka reagerar mot kroppsegna proteiner som om de vore främmande ämnen.

PSC har dock inte alla de egenskaper som räknas som klassiska för en autoimun sjukdom. Bland annat så drabbas fler män än kvinnor av PSC, och immunsuppresiv behandling har ingen effekt.

Den kunskap som finns om genetisk känslighet för PSC och som är kopplad både till områden inom och utanför HLA regionen, samt observationer om hög invandring av T lymfocyter i levern hos PSC patienter, gör att man oftast talar om PSC som en immunmedierad sjukdom.

De leverceller som angrips och förstörs vid den immunmedierade attacken i PSC är gallgångscellerna. Biliära epitelceller eller kolangiocyter är andra benämningar för dessa celler. Det finns två huvudsakliga hypoteser om varför immunförsvaret aktiveras vid PSC. Den ena är att immuncellerna går till attack mot ett antigen av ännu okänt ursprung (t ex härstammande från virus, bakterie eller parasit), eller ett autoantigen (kroppseget ämne som immunförsvaret uppfattar som främmande). Den andra teorin är att inflammationen uppkommer till följd av en känslighet hos de biliära epitelcellerna för gallans toxiska sammansättning.

En av anledningarna till att det är svårt att studera PSC är att patienterna vanligen får sina första symptom när sjukdomen pågått under en längre tid. Det är då omöjligt att utröna om de immunologiska förändringar som syns i levern vid denna tidpunkt kan sammankopplas med den ursprungliga skadan, eller om de är orsakade av sekundära effekter, till följd av den kroniska inflammationen. Att för forskningsändamål få tillgång till levervävnad från patienter med tidig sjukdom är svårt, och begränsas ytterligare av att PSC är en ovanlig sjukdom. Det finns risker med att ta en leverbiopsi och därför görs detta bara i undantagsfall, till exempel

för att bättre kunna prognostisera patienten, eller vid misstanke om annan, samtidig leversjukdom som går att behandla.

Att upptäcka tidiga förändringar hos patienter med PSC skulle kunna bidra till identifiering av sjukdomsspecifika proteiner, vilket vore värdefullt både ur diagnostiskt och prognostiskt hänseende. All forskning som bidrar till att öka förståelsen för de inflammatoriska processerna i PSC och varför tumöruppkomst ofta sker, är dock av största vikt för att kunna utveckla behandlingsmetoder.

Det övergripande målet med denna avhandling var att förbättra kunskapen om sjukdomsframkallande mekanismer i PSC och den PSC-relaterade cancerformen kolangiokarcinom. I de tre studierna har levervävnad från friska kontroller använts för att kunna jämföra med prover från PSC patienter. Detta kliniska jämförelsematerial har erhållits från patienter som opererat bort en bit av levern på grund av cancer, och där vi då kunnat få en bit av den friska vävnaden. I vissa fall har även frisk vävnad erhållits från donerade levrar, som på grund av tekniska orsaker inte kunnat användas för transplantation.

I den första studien har vi undersökt förekomsten av antikroppar i serum från PSC patienter, och antikropparnas reaktivitet mot gallgångsceller som vi isolerat från friska, humana levrar. Serum som samlats från totalt 81 PSC patienter undersöktes och patienterna kunde följas under en sammanlagd tidsperiod av 16 år, från provtagning och fram till att datum för dödsfall eller transplantation inföll. Vi fann att en majoritet av patienterna hade antikroppar av både IgA och IgG klass, med kapacitet att binda till de isolerade och odlade cellerna. Vidare såg vi att patienter med autoreaktiva IgA antikroppar generellt hade högre serumnivåer av IgA. Förekomsten av autoreaktiva IgA antikroppar visade sig vara associerat med en sämre överlevnad för patienterna.

I studie II har vi med immunhistokemi och kvantitativ bildanalys karakteriserat levervävnad från 17 PSC patienter och 17 kontroller. Vi har jämfört levervävnaden med avseende på

förekomst och frekvens av olika cellpopulationer, av immunologiskt och icke-immunologiskt ursprung. Levervävnaden från PSC patienter erhölls i samband med att patienterna genomgick levertransplantation. Vi fann att det fanns stora olikheter inom PSC patientgruppen och att vissa fenotypiska mönster var relaterat till svårighetsgrad av sjukdom. Sammantaget kännetecknades levrarna från PSC patienter av hög infiltration av T lymfocyter, företrädesvis lokaliserad till områden med fibros. Därutöver observerades att levrar med svår grad av fibros hade färre NK celler än levrar med mild fibros. Andra fenotypiska skillnader som observerades, och som kan tänkas ha betydelse för immunsvaret, var att PSC patienter hade lägre uttryckta proteinnivåer av MHC klass I på cellytan.

I studie III har vi använt samma experimentella och analysbaserade metoder som i studie II, men undersökt sammansättningen av immunceller i levervävnad från patienter med kolangiokarcinom. Vi har jämfört patienter med kolangiokarcinom, med och utan PSC, men vi har även jämfört sammansättningen av celler i tumöromården med hur det såg ut i den friska, kringliggande levervävnaden.

Sammanfattningsvis har studierna visat att autoreaktiva antikroppar finns vid PSC med förmåga att binda till biliära epitelceller. Leverinflammationen vid PSC varierar mellan individer och kännetecknas framför allt av en massiv T cellsinfiltration, koncentrerad till fibros. Tumörområdet i kolangiokarcinom kännetecknas av infiltration av immunsuppressiva T celler och ett minskat antal Kupffer och MAIT celler. Dessa fynd utgör en viktig kunskapsbas för fortsatta studier av mekanismer som driver den inflammatoriska processen.

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