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Immune interactions with the liver

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To my mother and late father,

To Zafir, Karrar and Fatima
&
To the rest of my family

ABSTRACT

The liver has an important role in the induction of immune tolerance and can be a target for immune mediated damage. The following studies are performed to address the role of liver sinusoidal endothelial cells (LSEC) in tolerance induction and to understand some of the mechanisms involved in the pathogenesis of primary sclerosing cholangitis (PSC).

We performed study I and II to investigate the role of LSEC in liver immunity. We hypothesized that LSEC would be the ideal cells responsible for tolerance induction and may also play a role in liver allograft rejections. We were interested in studying if antibodies to LSEC (LSEC Ab) may alter LSEC function, leading to liver allograft rejection.

Study III and IV address the role of biliary epithelial cell antibodies (BEC Ab) in the immunopathogenesis of PSC. BEC are the targeted cells that are damaged in PSC. Earlier we reported that PSC patients have high frequency of BEC Ab. We hypothesized that BEC Ab may play a role in the inflammatory process during PSC. We therefore investigated the correlation between the chronic inflammation and the high frequency of BEC Ab. We studied the functional role of BEC Ab and innate immunity, Toll like receptors (TLR). The male predominance in PSC and the high levels of BEC Ab prompted us to investigate the functional relationship between the sex hormones (estradiol) and BEC Ab.

In study I, we provide evidence that LSEC is one of the major cell types in the liver that contribute to peripheral tolerance through the induction of apoptosis of activated T cells. The apoptosis is mediated via the production of the immunosuppressive cytokine transforming growth factor beta (TGF-β) and needed caspase 3 and 8 activity.

In study II, we showed that the presence and binding of antibodies to LSEC are associated with acute liver allograft rejections. Antibodies to LSEC may facilitate acute liver allograft rejections by inducing CD86 expression and downregulating the production of TGF-β and therefore upregulating alloreactive T cell proliferation.

In study III, we found that PSC BEC Ab upregulate TLR-4 and -9 on BEC which in turn induced the production of interleukin-1β (IL-1β), interleukin-8 (IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF). TLR upregulation specifically phosporylated ERK1/2 and the transcription factors ELK-1 and NF-κB. TLR-4 and -9 ligation induced the production of TNF-α and IFN-γ. The adaptive immune response (BEC Ab) may facilitate the innate immune responses by induction of TLR on the bile duct epithelium in PSC patients.

In study IV, we demonstrate that PSC patients have significantly high levels of estradiol which correlates with presence of BEC Ab. Our *in vitro* results show that estradiol binds to estrogen receptor beta ERβ (upregulated by BEC Ab), which then decreases BEC proliferation and induces BEC apoptosis. Immunohistochemical staining of diminishing ducts in livers from PSC patients expresses ERβ. This is one mechanism by which increased levels of estradiol and BEC Ab play a role in the pathogenesis of PSC

In summary, our findings indicate that LSEC play a role in peripheral tolerance by inducing apoptosis of activated T cells. Antibodies to LSEC may contribute to liver allograft rejections by modulating cellular immune responses. Antibodies to BEC and the high estradiol levels play a major role in PSC pathogenesis.

LIST OF PUBLICATIONS

I. **Karrar A**, Broomé U, Uzunel M, Qureshi AR, Sumitran-Holgersson S. Human liver sinusoidal endothelial cells induce apoptosis in activated T cells: a role in tolerance induction. Gut. 2007 Feb;56(2):243-52. Epub 2006 Jul 13

II. Sumitran-Holgersson S, Ge X, **Karrar A**, Xu B, Nava S, Broomé U, Nowak G, Ericzon BG. A novel mechanism of liver allograft rejection facilitated by antibodies to liver sinusoidal endothelial cells. Hepatology. 2004 Nov;40(5):1211-21.

III. **Karrar A**, Broomé U, Södergren T, Jaksch M, Bergquist A, Björnstedt M, Sumitran-Holgersson S. Biliary epithelial cell antibodies link adaptive and innate immune responses in primary sclerosing cholangitis. Gastroenterology. 2007 Apr;132(4):1504-14. Epub 2007 Jan 25.

IV. **Azza Karrar**, Annika Bergquist, Meghnad Joshi, Hanns Ulrich Marschall, Ulrika Broomé, Suchitra Sumitran-Holgersson. Abnormally increased levels of bioavailable sex hormones estrogen and testosterone in patients with primary sclerosing cholangitis. Submitted

CONTENTS

LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomysin D
Ab	Antibody
Ac-LDL	Acetylated low density lipoprotein
AIF	Apoptosis inducing factor
AIH	Autoimmune hepatitis
AMA	Anti-mitochondrial antibodies
ANA	Anti-nuclear antibodies
ANCA	Atypical anti-neutrophil cytoplasmic antibodies
APAF-1	Apoptosis activating factor-1
APC	Antigen presenting cell
Bcl-2	B cell lymphoma 2
BDL	Bile duct ligation rat model
BEC	Biliary epithelial cells
CD31	Platelet /endothelial cell adhesion molecule (PECAM)
CD62E	E-selectin
CD105	Endoglin
CD144	Vascular endothelial (VE) cadherin
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non integrin
DNA	Deoxyribonucleic acid
E2	17-beta estradiol
EC	Endothelial cell
EGF	Epidermal growth factor
ELISA	Enzyme-linked immosorbent assay
ERK/1 2	Extracellular regulated kinases 1/2
ERα/β	Estrogen receptor alpha / beta
FADD	Fas- associated death domain
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAEC	Human aortic endothelial cells
HBV /HCV	Hepatitis B virus /C virus
HDL	High density lipproteins
HLA	Human leuckocyte antigen
IBD	Inflammatory bowel disease
ICAM-1/3	Intercellular adhesion molecule-1/3
IFN-α//β/γ	Interferon α//β/γ
Ig	Immunoglobulin
IGF1	Insulin -like growth factor1
IL-	Interlukein
IRAK	IL-1 receptor-assciated kinase
IRF-3	IFN regulatory factor 3
KC	Kupffer cells
LDL	Low density lipoproteins
LEC	Lung epithelial cells

LFA-2 /3	Leukocyte factor antigen-2 /3
LPS	Lipopolyscarride
LSEC	Liver sinusoidal endothelial cells
L-SIGN	Liver/lymphnode-specific ICAM-3 grabbing non integrin
MCC	Mixed cell culture
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatability complex
MICA/B	MHC class I-related antigen A/B
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NK cells	Natural killer cells
NOS2	Nitric oxide
PAMP	Pathogen associated molecular patterns
pANNA	Peripheral anti-neutrophil nuclear antibody
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cells
PDL-1/ 2	Programm death ligand 1, 2
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PI	Propidium iodide
PI3-Kinase	PI3- kinase/ (phosphatidylinositol-3' kinase
pIgR	Polymeric immunoglobulin receptor
PRP	Pattern recognition receptor
PS	Phospholipid phosphatidylserine
PSC	Primary sclerosing cholangitis
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPTEC	Renal proximal tubular epithelial cells
SCGE	Single cell gel electrophoresis
SHBG	Sex hormone binding protein
SLE	Systemic lupus erythematosus
SMA	Anti-mooth muscle antibodies
SR-AI/II	Scavenger receptor class A
SR-B1	Scavenger receptor B1
TGF- β	Transforming growth factor beta
TH1/ 2	T helper 1 /2
TLR	Toll like receptors
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF-a	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TReg	T regulatory cells
UC	Ulcerative colitis
VAP-1	Vascular adhesion protein 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

1 BACKGROUND

1.1 THE IMMUNE SYSTEM

The immune system is divided into a primitive innate immune system and acquired or adaptive immune system. The innate immune response is non specific, provides first line defense against infection and does not confer long lasting or protective immunity to the host. The main functions of the innate immune response is the recruitment of immune cells eliminating pathogens, the production of cytokines, activation of the complement system, and activation of the adaptive immune response. Components of the innate immune system include epithelial barriers, phagocytes: neutrophils, monocytes/ macrophages and natural killer (NK) cells. Recognition of microbes by the innate immune system occurs through pattern recognition receptors (PRR). Toll like receptors, TLR are an evolutionarily conserved family of cell surface molecules, which are key to innate immunity. TLR are a type PRR and recognize microbial components, known as pathogen associated molecular patterns (PAMP) [1-3].

The adaptive immune system is further divided into humoral and cellular components. Humoral immunity refers to antibody production by B cells. The effector functions of antibodies include pathogen and toxin neutralization, complement activation, opsonin promotion of phagocytosis and pathogen elimination [1]. Cell-mediated immunity is most effective in removing virus-infected cells, and defends against fungi, cancerous cells and intracellular bacteria. Effector functions of cellular immunity include activating cytotoxic T-lymphocytes, activating macrophages and NK cells enabling them to destroy intracellular pathogens; and stimulating immune cells to secrete a variety of cytokines. Cell-mediated immunity also plays a major role in transplant rejection.

The immune system has to be tightly regulated, turned on in response to a threat (foreign antigen) and turned off again when the threat has been removed. In addition, the immune system needs to be regulated so that it does not respond against self (immunological tolerance). Self tolerance can be divided into central tolerance and peripheral tolerance. Central tolerance to immature lymphocytes is initiated in primary lymphoid organs (the bone marrow for B cells and the thymus for T cells). Peripheral tolerance occurs when mature self reactive lymphocytes encounter self antigens in the peripheral tissues they are shut off or killed. Normal healthy individuals possess mature circulating self reactive lymphocytes. Since the presence of these self-reactive lymphocytes does not result in autoimmune reactions their activity is regulated by mechanisms of peripheral tolerance namely anergy (functional unresponsiveness), suppression by regulatory T cells and deletion (apoptotic cell death). The liver is considered as one of the organs responsible for induction of peripheral tolerance. Breakdown of this peripheral tolerance can lead to activation of self reactive clones of B-and T-cells, generating responses against self antigens. These reactions can cause serious damage to cells and organs and the diseases that results from such an aberrant immune response is termed autoimmune disease. Autoimmune diseases are therefore a result of loss of tolerance. The mechanisms of induction of autoimmunity are not fully

understood and it is unlikely that autoimmunity develops from one single event but rather from a number of different events.

1.2 LIVER IMMUNITY

1.2.1 Liver structure

The liver appears to be an immune-privileged organ which favors the induction of peripheral tolerance rather than induction of immunity. The liver's unique location and anatomy are relevant for its role in the immune system. A main anatomical characteristic is its dual blood supply. Oxygen-rich blood is supplied via the hepatic arteries and protein and antigen rich blood is supplied via the portal vein. More than 1.5 L of blood passes through the liver within a minute and about 10^8 peripheral blood lymphocytes pass through the liver within 24 hours. The liver has a dual role in induction of tolerance of T cell response and also in supporting intra-hepatic priming of T cell responses [4, 5]. Lymphocytes can transmigrate through a monolayer of liver sinusoidal endothelial cells (LSEC) to enter the space of Disse and the hepatocytes [6]. The liver must balance activation of the immune system by antigens together with prevention of damage to hepatocytes and peripheral tissues from the resulting immune response. The hepatic microanatomy allows such a balance.

The liver consists of functional units called hepatic lobules. Hepatocytes are arranged in thin layers that radiate from the central vein to the periphery of the lobule. At the corners of each lobule is the portal area, composed of branches of the hepatic portal vein, hepatic artery and a bile duct. Bile drains from the hepatocytes by the many small bile ducts that unite to form the main bile duct of the liver, the hepatic duct. The blood vessel in the middle of each lobule receives blood from the hepatic portal vein and hepatic artery via the sinusoids and drains the blood into the hepatic vein (Figure 1). The liver consists of various non-parenchymal cells such as Kupffer cells (KC), SEC, stellate cells, dendritic cells (DC), biliary epithelial cells (BEC) and liver-associated lymphocytes which play a role in immunologic surveillance within the hepatic sinusoids. The focus of this thesis will be immune interactions with SEC and BEC.

Liver allografts are accepted spontaneously and in some species immunosuppression is not required. This tolerance is so powerful that it can protect other subsequent donor organs like hearts or skin grafts from acute and chronic rejections [7, 8]. LSEC trap and induce apoptosis and can be antigen presenting cells resulting in deletion of antigen specific T cells. The liver also participates in the induction of oral tolerance. LSEC are active in uptake and cross-presentation of oral antigens from portal venous blood and are engaged in the induction of tolerance of T cells towards these antigens [4, 9].

Autoimmune liver diseases include autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [10]. This thesis focuses on PSC and includes studies addressing some of the immune mechanisms involved in immunopathogenesis of PSC.

Figure 1: The liver lobule

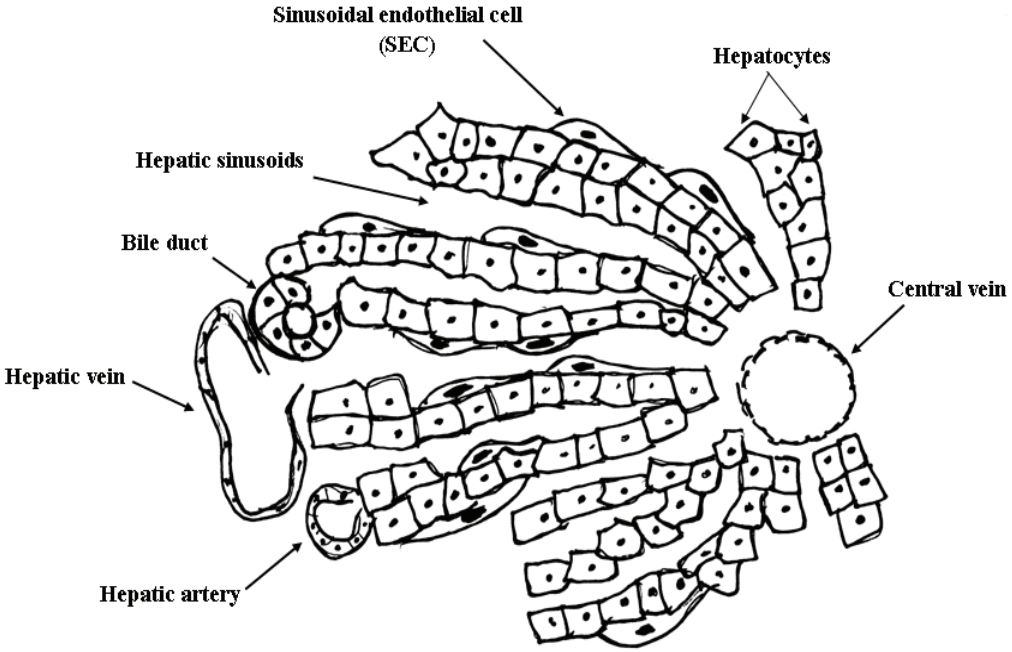


Figure 1. Schematic illustration of the liver lobule. A lobule is made of hepatocytes, between them are the hepatic sinusoids that act like capillary spaces allowing the blood to flow around the liver cells. The portal area is composed of branches of a hepatic vein, a hepatic artery and a bile duct.

1.2.2 Liver sinusoidal endothelial cells (LSEC)

The liver's first contact with pathogens and/or signals is at the level of the sinusoids. SEC are found in the spleen and bone marrow, as well as in the liver, and in all these sites they have a minimal basement membrane and lack classical tight junctions. Hepatic sinusoidal endothelium differs from sinusoidal endothelium in the other beds by its discontinuous nature, being interspersed with KC and by the presence of open fenestrations arranged in sieve plates [11]. LSEC function as a protective barrier despite being fenestrated. These fenestrations are approximately 100 to 120 nm in diameter and the total area of the fenestrae accounts for approximately 10% of the entire surface of the LSEC [6]. They allow the hepatocytes, to interact with circulating blood and its

leukocyte population (Figure 2). The fenestrations act as a ‘dynamic filter’ allowing macromolecules in blood controlled access to hepatocytes. The number of fenestrations per endothelial cell decreases in disease following viral infection [12] or cirrhosis [13].

The fenestrated sinusoid is highly selective about what passes into contact with the liver. Substances do not freely pass through into the hepatic microcirculation. Passage is receptor mediated and allows the liver control over systemic exposure to toxic substances [14, 15]. In general, LSEC can be regarded as a "selective sieve" for substances passing from the blood to hepatocytes and act as a "scavenger system" which clears the blood from many different macromolecular waste products.

To study human LSEC, cells must be isolated, cultured and passaged. LSEC are characterized using antibodies to endothelial, immune recognition and several other specific sinusoidal endothelial markers. LSEC share many cell surface receptors with leukocytes, including PECAM (CD31), CD4, CD11b, and CD11c, which may contaminate endothelial cell preparations in culture [11]. During cirrhosis and chronic hepatitis, LSEC develop a more vascular morphology and produce a basement membrane in a process known as ‘capillarisation’ [16]. This is associated with increased expression of CD31 and loss of fenestrations. Lalor PF et al. [17], showed that CD31 is expressed by both normal and diseased LSEC. The same author has reported expression of functional E-Selectin (CD62E) on cultured cytokine stimulated human LSEC [17].
Other markers reported to be used for characterization of LSEC include von Willbrand factor (vWF) expression which has been reported in passaged, cultured LSEC as well as normal and diseased samples. Also most studies suggest that LSEC in normal liver lacks VE-cadherin (CD144) or expresses it at low level. It seems likely that the relative lack of VE-cadherin on LSEC is a consequence of the absence of classical adherens junctions between LSEC [17]. CD105 (endoglin) is a receptor for transforming growth factor beta (TGF-β) and modulates TGF-β signalling by interacting with TGF-β receptor I and /or II. CD105 is expressed on LSEC but again is not a tissue or cell specific maker [17].

1.2.2.1 Scavenger receptors

LSEC constitute a powerful scavenger system in the body and they express five endocytic receptors: scavenger, mannose, hyaluronan, collagen and IgG FC receptors [15, 18, 19]. Many of the scavenger receptor proteins can be used to determine the phenotype of LSEC. The ability to bind Ulex lectin and take up Ac-LDL is often used to characterize LSEC despite its lack of specificity [17].

Two members of the link family Stabilin-1 and -2 are constitutively expressed by LSEC [20]. Most of the scavenger functions assigned to this molecule relate to endocytosis of hyaluronic acid and Ac-LDL. LSEC also express LYVE-1 (hyaluronan receptor) constitutively [21].

Another HDL/LDL receptor, CD36, is strongly expressed on LSEC where it fulfils multiple functions including acting as a scavenger receptor for lipid [22] and as an adhesion receptor for red blood cells infected with malaria parasite [23].

DC-SIGN* (CD209) is expressed on DC and L-SIGN† is constitutively expressed on LSEC, but not on dendritic cells or on endothelium in other tissues. L-SIGN is thus an excellent marker of liver endothelium. L-SIGN on endothelial cells acts as an attachment factor for HCV but does not mediate HCV entry directly but rather enhances infection of hepatocytes in proximity [24].

1.2.2.2 Antigen presenting receptors

LSEC have the ability to phagocytose particles and to present antigen to lymphocytes [15, 25, 26]. They do not need to undergo maturation for acquisition of the antigen-presenting function but can simultaneously and efficiently endocytose, process and present antigen to T cells. The LSEC in mice expresses all molecules necessary for antigen presentation. LSEC constitutively express MHC class I and II [19, 27] together with CD80, CD86 and CD40 and CD54 and can function as antigen presenting cell (APC) for CD4+ and CD8+ T cells [14]. In humans, LSEC express CD58, which is the ligand for CD2, the molecule that triggers the alternative pathway of T-cell activation [28]. Human LSEC also express vascular adhesion protein 1 (VAP-1) and leukocyte rolling depends upon the SEC expression of VAP-1. Interaction occurs between the receptor ligand pairs CD54–CD11a and CD106–CD49d. Because both CD54 and CD106 are constitutively expressed on LSEC, adhesion of leukocytes to LSEC can occur in the liver under physiological situations [17].

LSEC has been described as a new type of antigen presenting cells that induces immune tolerance in naive T cells [29, 30]. In mice LSEC are capable of stimulating naive CD4+ T cells. However, following priming by antigen-presenting LSEC, CD4+ T cells fail to subsequently differentiate towards a T helper1 (TH1) phenotype but, instead, become T regulatory cells (TReg), expressing interleukin (IL-) IL-4 and IL-10 upon restimulation [19, 31].

There is evidence that antigen presentation is important for generating immunological tolerance to gut-derived antigens. Local antigen presentation by LSEC provides a priming site for naive CD8+ T cells [32]. LSEC have the capacity to present exogenous antigen on Major histocompatibility complex (MHC) class I molecules to CD8+ T cells, a process termed cross-presentation. Cross-presentation in LSEC occurs with extraordinary efficiency, requiring only minute amounts of antigen and occurring within 60–120 min after exposure to an antigen. Stimulation of naive CD8+ T cells by LSEC results first in the proliferation of T cells and the release of cytokines and finally leads to antigen-specific tolerance, as demonstrated by a loss of cytokine expression at the same time as the failure of CD8+ T cells to develop into cytotoxic effector T cells [33].

* DC-SIGN: Dendritic Cell-specific ICAM-3 grabbing nonintegrin

† L-SIGN: The related Liver/lymph node specific ICAM-3 grabbing nonintegrin)
(ICAM-3 : intercellular adhesion molecule-3)

Figure 2. The liver sinusoid

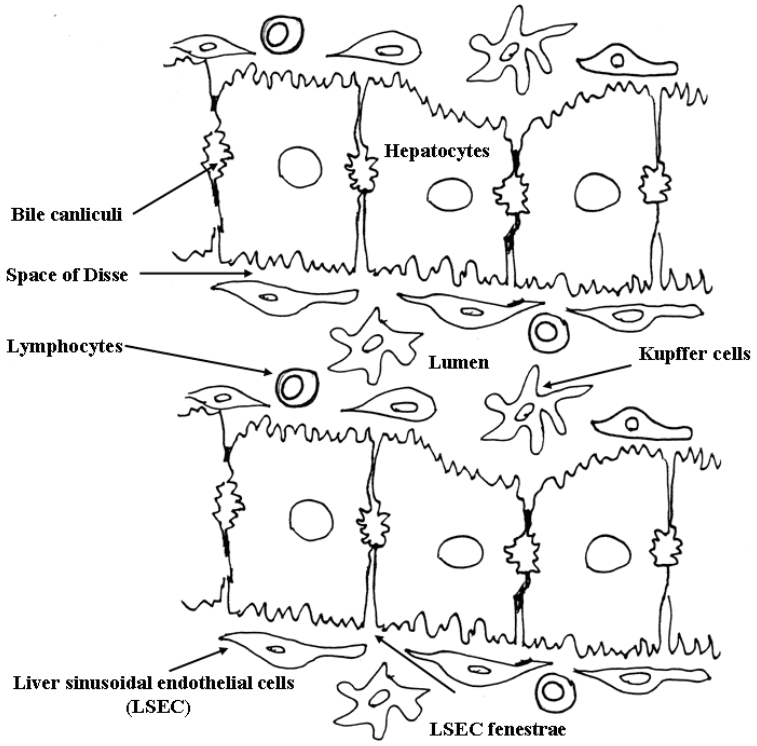


Figure 2: The liver sinusoid. The sinusoid is lined by liver sinusoidal endothelial cells (LSEC). LSEC are fenestrated and lack a basement membrane. Kupffer cells and lymphocytes exist mainly in the sinusoidal lumen. Kupffer cells can make direct contact with hepatocytes. The subendothelial space is known as the space of Disse.

1.2.3 Biliary epithelial cells (BEC), cholangiocytes

1.2.3.1 Cholangiocytes and immune system regulation

Cholangiocytes or BEC are the epithelial cells lining the biliary tree. The intrahepatic biliary tree is a network of interconnecting ducts of increasing diameter from the duct of Hering to the extra hepatic bile ducts. Cholangiocytes represent 3 to 5% of the total population of liver cells [34, 35]. Cholangiocytes appear to have an active role in both innate and adaptive immune responses.

Cholangiocytes produce inflammatory mediators following interactions with PAMP by TLR expressed on their cell surface. Immortalized mouse cholangiocytes from small intrahepatic ducts express CD14 and TLR-4 and secrete IL-12 and TNF-α after binding to lipopolysaccharide (LPS) [36]. The expression of cytokines, chemokines, and

chemokine receptors by cholangiocytes could result in the recruitment and activation of T cells, macrophages, and NK cells to protect against bacterial antigens [37]. Cholangiocytes regulate the biliary immune defense and induce protective responses against pathogens in bile [38].

The biliary epithelium is the target of inflammatory mediators during infections, autoimmune liver diseases, and neoplastic development [39]. Human cholangiocytes constitutively express and secrete IL-8 and monocyte chemotactic protein-1 (MCP-1). These factors are chemotactic agents for neutrophils, monocytes, and T cells, and promote their infiltration into portal tracts [40]. Adhesion molecules expressed on the cholangiocyte surface permit the adhesion and recognition of lymphocytes and subsequently, the activation of cytotoxic effector lymphocytes. Thus, such interactions modulate the intensity and localization of the inflammatory reactions in the biliary system. The presence of leukocyte factor antigen-3 (LFA-3) on the cell surface of cholangiocytes allows the interaction with CD2 molecules expressed on cytotoxic T lymphocytes and natural killer cells, leading to cell cytotoxicity [41].

Cholangiocytes and the immune system are interconnected and represent two systems that work together to eliminate pathogens. Cholangiocytes express TNF-α and IL-6 receptors and cultured cholangiocytes secrete IL-6 which could be involved in injury to BEC. Studies have shown that human cholangiocytes respond to LPS stimulation with secretion of IL-6, a factor that triggers cholangiocyte proliferation by an autocrine mechanism [42, 43]. IL-6 can stimulate terminal differentiation of B cells and immunoglobulin (Ig) secretion, and TNF-α increases cytotoxic activities of T cells and induces the expression of adhesion molecules and human leucocyte antigen (HLA) on cholangiocytes [44].

T cells can be activated by a mechanism that involves CD40 expressed on cholangiocytes. The CD40/CD40L and the LFA-2(CD2)/LFA-3 (CD58) system trigger the production of IL-12, which plays an important role in the cytotoxic response of cholangiocytes. CD40 and CD40L expression in cholangiocytes are increased, respectively, by stimulation with IFN-γ and activation of LFA-2 on T cells [45]. Furthermore, a significant increase of ICAM-I, HLA-1, and HLA-II expression in cholangiocytes from normal and PBC liver was evident after stimulation with proinflammatory cytokines, such as TNF-α and IFN-γ [44].

1.2.3.2 Cholangiocytes as possible antigen presenting cells (APC)

The other immune feature attributed to cholangiocytes is their potential ability to act as APC. In support of this concept, studies demonstrate that cholangiocytes express MHC II, and such expression is increased in injured cholangiocytes and after stimulation with IFN-γ and IL-1 [44]. Increased damage of the bile duct epithelium is closely associated with an increased number of MHC II-specific lymphocytes. During liver rejection, MHC II specific lymphocytes infiltrate the allograft and play a role in the destruction of the biliary epithelium [46]. These findings suggest that cholangiocytes may act as APC by activating lymphocytes to respond to specific portal antigens.

In contrast, other studies do not support the role of cholangiocytes as APC. The functions of APC allow activation and proliferation of T cells when recognizing the antigens. This requires MHC class II molecules in the presence of specific adhesion molecules expressed on the cell surface, such as B7-1 (CD80) and B7-2 (CD86). CD80 and CD86 are not expressed by human resting cholangiocytes and their expression is not induced by the proinflammatory cytokines, IFN-γ and TNF-α, either alone or in combination. Accordingly it has been suggested that cholangiocytes cannot act as APC because they are missing the costimulatory molecules [41]. It is possible that antigens are presented by cholangiocytes in an inefficient manner, which may result in specific T-cell anergy or deletion. The anergic T cells may function as suppressor cells, thus inhibiting subsequent T cell activation [47]. Nevertheless, cholangiocytes normally express low levels of B7 family members programmed death ligand 1 (PD-L1) (B7-H1) and PD-L2 (B7-DC) that interact with PD-1, a receptor expressed on activated T cells [48].

1.2.3.3 IgA production by cholangiocytes

Cholangiocytes secrete and transport Igs [49], which protect against pathogens derived from the systemic circulation or the portal blood. IgA in bile plays a major role in the biliary mucosal immune defense. In humans, IgA is synthesized by plasma cells in proximity to bile ducts, and secreted into bile after binding to polymeric Ig receptor (pIgR) located on the basolateral membranes of cholangiocytes [50]. IgA may protect biliary ducts by preventing the attachment of pathogens or their toxins to the cholangiocyte surface [39].

Another function of IgA is their capacity to form immune complexes with circulating antigens. This reduces the possibility of a systemic response caused by pathogens present in systemic circulation, and prevent chronic inflammation derived from antigens continuously present in the gastrointestinal mucosa [51]. Finally, IgA bind intracellular pathogens during the transcytosis process into cholangiocytes [39].

1.3 ROLE OF THE LIVER IN PERIPHERAL TOLERANCE

The liver is constantly bathed in antigens, either from the gastrointestinal system via the portal circulation or systemically from other areas in the body. As a result, the liver has developed a mechanism that allows it to balance “unnecessary” immune activation with “appropriate” immune reaction. This mechanism is known as tolerance [4, 26].

In the liver, clearance of antigen from the blood occurs mainly by SEC through an efficient receptor-mediated endocytosis. Other hepatic cells, such as DC, KC, and perhaps hepatocytes, may contribute to tolerance induction by deletion of T cells through the induction of apoptosis [14]. Apoptosis of activated T cells is prevalent in the liver. T cells undergoing apoptosis may have been activated within the liver itself by APC or may be T cells activated in the periphery that migrate in a nonspecific fashion to the liver. In the liver, they die because they are already programmed to do so, or because they are trapped by a non antigen specific mechanism and receive an apoptotic signal from liver cells [52].

It was shown by Crispe et al [53] that interaction of naive CD4+ T cells with LSEC results in differentiation of the T cells to a TReg phenotype. This is probably because LSEC normally produce IL-10, which favors the regulatory pathway of CD4+ T-cell differentiation. Furthermore, interaction of naive CD8+ T cells with SEC results in partial activation of the T cells, followed by passive cell death. The reason for this is unknown, but it is proposed that continuous exposure to trace amounts of intestinal LPS results in the differentiation of both LSEC and liver DC to a state that promotes T cell tolerance in CD4+ and CD8+ T cells. The apoptotic CD8+ T cells are then endocytosed by KC.

Several investigations have shown that passage of an antigen through the liver via the portal circulation is a necessary precondition for the induction of tolerance. Portosystemic shunting results in a loss of this tolerance induction [14]. This phenomenon of tolerance has been the subject of transplantation research for the last years in the hope of being able to induce tolerance of newly transplanted grafts in the recipient.

1.3.1 Liver transplantation tolerance

The field of transplantation immunology has taken interest in the tolerance-inducing capacity of the liver, because donor-specific tolerance is the main goal of transplantation immunology. There are several observations that support the concept of peripheral tolerance induction in liver transplantation. Allogeneic liver grafts have been shown to directly mediate alloantigen specific tolerance, thereby allowing the transplantation of other organs from the same donor [7, 54, 55]. Prolongation of allograft survival as a consequence of venous drainage of the allograft into the portal vein and induction of tolerance following portal-venous injection of donor lymphocytes [56-58].

Early studies showed that pigs survived indefinitely after receiving orthotopic liver allografts from an unrelated pig without the need for immunosuppression [55]. This unaided (without immunosuppression) tolerance in the porcine liver grafts, unfortunately, does not occur in all species. In humans and dog models, immunosuppression is needed to control rejection of liver allografts and to improve graft survival.

Interestingly, animal experiments have shown that with or without immunosuppression, the livers undergo a rejection both histologically and biochemically, but instead of progressing to destruction, the organs recovered spontaneously [8]. This suggests that too much immunosuppression may prevent this tolerance-like effect from occurring. The liver antigen specific tolerance effect is believed to be mediated via liver-resident APC (KC, LSEC and DC). In-vivo impairment of KC abrogates specific tolerance [59]. LSEC play a role in tolerance induction by inducing the differentiation of naive T cells into IL-4 and IL- 10 producing cells with regulatory function [31]. Thus, intrahepatic APC modulate the effector functions of lymphocytes that respond to the antigens. In addition, in the liver transplant situation, donor lymphocytes appear to play a major role for graft acceptance and tolerance [60, 61].

1.3.1.1 Liver transplantation rejections

Histopathological characteristics of acute rejections are lymphocyte infiltration of portal tract associated with the bile duct damage and inflammation of portal and hepatic venular endothelium. Cellular immunity especially CD4+ and CD8+ T cell mediated immune responses are believed to be the major mechanisms underlying acute liver allograft rejections [62].
In host secondary lymphoid organs, host T cells could be activated by donor and recipient APC through two distinct pathways of allorecognition. In the direct pathway, recipient T cells recognize intact donor MHC molecules with peptide on the surface of migrating donor APC. While, in the indirect pathway, recipient T cells recognize donor derived allopeptides processed and presented in the context of recipient MHC on host APC [62]. The direct pathway has been considered to play a primary role in initiating alloimmune responses during the early post transplantation period and lead to acute rejection [63]. In contrast, the indirect pathway has been shown to play roles in initiating CD4+T cell responses and mediating chronic rejection during a later phase of transplantation. However, the importance of the direct and indirect pathways during the acute phase of organ transplantation in initiating graft rejection or tolerance is not fully clear. The survival of the transplanted organ is influenced by T cell recognition pathways, the induced effector function and the alloreactive T cell repertoire [62].

The effector mechanisms involved in antibody mediated rejections include complement activation, endothelial cell (EC) activation, activation of NK cells and cytotoxic T cells. EC can be a target of humoral immunity following transplantation. This has been studied in other organ transplantation [64-66]. The relevant antigens could be allogenic MHC molecules. Antibody binding to endothelium will result in apoptosis /proliferation of EC, proinflammatory cytokines activation. Very little is known about the role of antibodies in liver allograft rejections

1.3.1.2 Induction of oral tolerance by the liver

The involvement of the gastrointestinal immune system in the induction of tolerance towards dietary antigens has been documented and strategies to induce tolerance via oral application of antigens are already employed to treat autoimmune disease [67].

The liver receives blood containing dietary antigens from the gastrointestinal tract via the portal vein and it may function as a second security line to ensure tolerance towards dietary antigens. This view is supported by experimental evidence. It has been observed that portosystemic shunting, i.e. deviation of the portal-venous blood from the liver directly into the systemic circulation, results in loss of tolerance towards dietary antigens [68]. Spillover of dietary antigen into the systemic circulation appears to be a physiologic event, as spleen cells removed 6 h after feeding can present dietary antigen to T cells [9].

Antigens derived from the gastrointestinal tract are usually accompanied by LPS. Antigens present in low concentrations may not be presented and thus may be ignored by the immune system; only those dietary antigens present in higher concentrations would be presented by LSEC and lead to generation of TReg. In addition, LPS appears to be an important factor of the local tolerogenic microenvironment in the gut and the

liver. This becomes obvious in the observation that mice which are unresponsive to LPS lack tolerance to dietary antigens [69].
Li et al [70] have demonstrated that the liver is able to induce oral tolerance when fed high dose of ovalbumin and that this tolerance probably involved LSEC and DC. Importantly, cross-presentation of oral antigens by LSEC leads to induction of CD8+ T cell tolerance, indicating a role for the liver in oral CD8+ T cell tolerance [71].

1.3.2 Balance between induction of immunity or tolerance

Peripheral tolerance induced in the liver may limit immunity at extra hepatic sites. It is proposed that LSEC are involved in limiting or terminating the immune responses by the liver [14]. Generally, to keep immune responses local it is necessary to clear antigen from the systemic circulation followed by elimination of circulating effector T cells reactive with this antigen. Clonal deletion by apoptosis may be used by the liver to eliminate effector T cells from the systemic circulation. One example is a study showing a significant number of T cells found in the liver three days after influenza-induced pneumonia and viremia. The T cells in the liver were specific for influenza antigens but are no longer functionally active in terms of IFN-γ expression and showed signs of apoptosis [72].

The liver is a site of infection by pathogens that infect hepatocytes; such as HBV, HCV and sporozoites of *Plasmodium malariae.* Certain common features of infection by these pathogens suggest that both the physiologic function and the unique immunologic environment of the liver are abused by these pathogens for successful chronic infection [14].

In a study by Breiner and Knolle using animal model of infection with HBV it was found that LSEC, rather than hepatocytes themselves, play the key role in the initial uptake of viral pathogens into the liver. Viruses initially scavenged by LSEC are released to infect adjacent hepatocytes. The uptake into LSEC may protect the virus from the hostile environment in the liver sinusoid; KC, passenger leukocytes, NK cells and complement molecules. Those viral particles may become processed and presented to T cells and may lead to tolerance induction [73]. Hepatocytes release pathogen-related antigens; which will first be presented by LSEC to cells of the immune system. It is proposed that tolerance ensues from this encounter. Then, the pathogen will gain further time to replicate before antigen is taken up by conventional APC and effector T cells will be generated in lymphatic tissue. This is exemplified by the pre-erythrocytic phase of malaria [74]. In summary, hiding in hepatocytes behind the LSEC barrier or generating tolerant T cells by first antigen encounter on LSEC may provide a pathogen with time to replicate.

A break in tolerance mechanism is proposed by Crispe et al [53]. In an infected liver, a virus-infected hepatocyte secretes type 1 IFNs (IFN-α/β), which act on LSEC causing them to secrete IL-12 and promote the differentiation of CD4+ T cells to inflammatory TH1 cells. In parallel, type 1 IFNs act on other parenchymal cells, causing them to secrete IL-15, a survival factor for CD8+ T cells. The type 1 IFN initiate a cytokine/chemokine cascade that promotes T-cell recruitment.

1.4 APOPTOSIS

Apoptosis, the process of programmed cell death, plays a crucial role in the immune system in both maintenance of self tolerance and homeostasis. Failure to undergo apoptosis may lead to autoimmunity. TGF-β is a multifunctional cytokine with many biological activities. The numerous cell and tissue roles of TGF-β include cell-cycle control, the regulation of early development, differentiation, extracellular matrix formation, hematopoesis, angiogenesis, chemotaxis, and immune functions [75]. TGF-β represses growth of many epithelial cell types, whereas the growth of mesenchymal cells is stimulated. TGF-β -mediated growth inhibition and apoptosis can be correlated with its function as a tumor suppressor [75, 76]. The apoptosis- inducing capacity of TGF-β has been investigated in many cell types as will be discussed

In general there are two overlapping signaling pathways leading to apoptosis termed the intrinsic and extrinsic pathways.The intrinsic pathway is mediated by mitochondrial dysfunction and by endoplasmic reticulum stress. Intracellular stress can lead to damage of the mitochondrial inner membrane, resulting in the production of proapoptotic factors cytochrome *c* and apoptosis inducing factor (AIF) from the mitochondrial. In the cytosol, cytochrome *c* forms a complex with apoptosis activating factor-1 (Apaf-1) to activate procaspase 9, which in turn activates downstream effector caspases, such as caspase 3, 6 and 7. The process is regulated by the relative proportions of anti-apoptotic protein expression (Bcl-2, Bcl-xl) and pro-apoptotic (Bax, Bid, Bak or Bad) of the Bcl-2 (B-cell lymphoma-2) within a cell.
The extrinsic pathway is mediated either by cell surface receptors, including Fas and TNF-α receptor- 1 (TNF-R1), or by perforin and granzyme B released from activated, cytotoxic lymphocytes. Upon activation by their respective ligands (FasL and TNF-α); both Fas and TNF-R1 form a complex with adapter proteins and forms Fas- associated death domain (FADD). Aggregation of this complex initiates cleavage of procaspase 8 into its active form, which subsequently activates downstream effector caspases such as caspase 3. Activation of procaspase 8 can also lead indirectly to cytochrome *c* release from the mitochondria, thereby linking the extrinsic and intrinsic pathways. Cytotoxic lymphocytes express FasL and release granules containing granzyme B and perforin, which allows granzyme B to enter target cells. Granzyme B then directly cleaves critical cellular proteins and activates procaspases [77]. Apoptosis is tightly controlled and regulated via several mechanisms including the effects of cytokines such as TNF-α and TGF-β, and the influence of pro- and anti-apoptotic mitochondria-associated proteins of the Bcl- 2 family.

1.4.1 TGF-β signaling pathways

The TGF-β signal transduction pathway is best reviewed by Massague et al [78]. TGF-β binds to its membrane-bound receptors, TGF-β receptors 1 and 2 (TGF-β RI, TGF-β RII), which form a complex. TGF-β RI is phosphorylated by TGF-β RII. Upon this activating phosphorylation, TGF-β RI phosphorylates the receptor-activated SMAD proteins (SMAD2 and SMAD3), which form a complex with the co-SMAD, SMAD4 and enter the nucleus for transcriptional regulation thereby involving other components of the transcriptional machinery. An autoregulatory feedback loop is

established by the induction of inhibitory SMAD proteins (SMAD6 or SMAD7) that prohibit the activation of receptor-activated SMAD.

TGF-β1-induces hepatocyte apoptosis and SMAD molecules are involved in the transduction of the TGF-β1 signal [78]. TGF-β1 may inhibit the (nuclear factor-kB) NFκB factors, known to promote cell survival. TGF-β1 may also decrease Bcl-xL, an antiapoptotic member of the Bcl-2 gene family, without changing the expression of proapoptotic Bax or Bad [79]. TGF-β1-induced apoptosis is associated with the activation of several caspases, including caspase 1, caspase 2, and caspase 3. In additionTGF-β induces cell death in a hepatoma cell line and this is accompanied by the activation of caspase-family. The broad-spectrum caspase inhibitor ZVAD-FMK blocks TGF-β induced cell death in a concentration- dependent manner. Application of the more specific caspase inhibitor DEVD-FMK has revealed that the activation of caspase 3 is necessary for TGF-β induced cell death [80].

Shima and co-workers [81] have detected the activation of caspase 8, 9, and 3 in response to TGF-β administration to a hepatoma cell line. The apoptotic process is accompanied by a decreased level of Bcl-xL protein. Interestingly, the potent growth factor, epidermal growth factor (EGF), completely abolishes the TGF-β induced apoptosis of this cell line by an unknown mechanism, as Bcl-xL levels remain unaffected [81].

The study by Roberts et al [82] provides strong evidence that TGF-β signaling interacts with other signaling pathways to regulate cellular survival. In primary hepatocytes, TNF-α and EGF antagonizes the pro-apoptotic effect of TGF-β [82]. Pre-treatment of hepatocytes with EGF or TNF-α suppresses TGF-β induced cell death. Various survival signals are involved in this process. The suppression of TGF-β induced apoptosis by TNF-α is mediated by ERK1/2 (extracellular regulated kinases 1/2) and p38 MAP kinase.

Data from cell-culture experiments and in vivo studies show of TGF-β mediated apoptosis is important in the maintenance of B- and T-cell homeostasis. TGF-β is shown to induce growth arrest and apoptosis in lymphocytes of human. Inman and coworkers [83] found that TGF-β induces cell death of the Burkitts lymphoma cell line and it involves the activation of caspases.

Figure 3. TGF-β and apoptosis

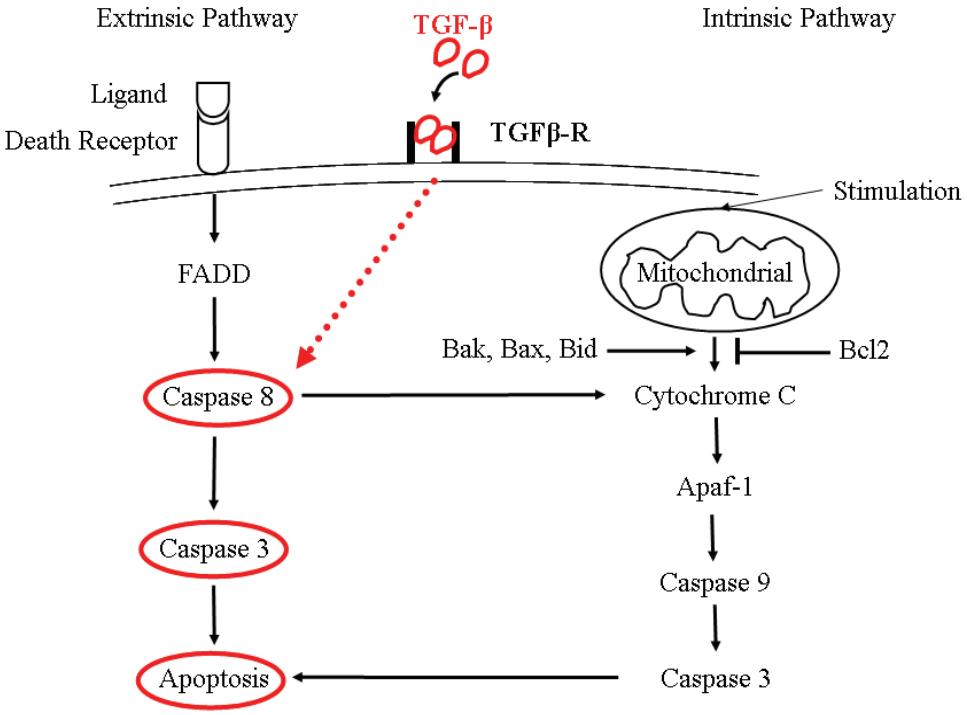


Figure 3: Intrinsic and extrinsic pathways of apoptosis. The extrinsic pathway is triggered by death receptor engagement, leading to the formation of FADD which initiates a signaling cascade mediated by caspase-8 activation. Caspase-8 initiates caspase-3 activation and also stimulates the release of cytochrome c by the mitochondria. Caspase-3 activation leads to the degradation of cellular proteins necessary to maintain cell survival and integrity. The intrinsic pathway occurs when various apoptotic stimuli trigger the release of cytochrome c from the mitochondria (independently of caspase-8 activation). Cytochrome c interacts with Apaf-1 and caspase-9 to promote the activation of caspase-3. The apoptotic process is controlled by the gene expression of Bak, Bax, Bid, BcL-2, and Bcl-xL. We showed that TGF-β induced caspase 8 and caspase 3 activities which induced T cell apoptosis. (Apaf-1: apoptosis-activating factor 1; FADD, Fas-associated death domain protein)

1.5 TOLL-LIKE RECEPTORS (TLR) AND THE LIVER

To date, 12 members of the TLR family have been identified in mammals. TLR can be further divided into several subfamilies, each of which recognizes related PAMP. The subfamily of TLR-1, -2, and -6 recognizes lipids, whereas TLR-7, -8, and -9 recognize nucleic acids. TLR-4 recognizes a very divergent collection of ligands such as LPS, fibronectin, and heat-shock proteins [3, 84].

TLR are expressed on various immune cells, including macrophages, DC, B cells, specific types of T cells, and even on non-immune cells such as fibroblasts and epithelial cells. Expression of TLR is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stress. Furthermore, TLR may be expressed extra- or intracellularly. While TLR-1, -2, -4, -5, and -6 are expressed on the cell surface, others TLR -3, -7, -8, and -9 are found almost exclusively in intracellular compartments such as endosomes and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible. TLR activate the same signaling molecules that are used for IL-1 receptor signaling. Stimulation of cells with a TLR ligand recruits adaptor proteins such as myeloid differentiation factor 88 (MyD88), to the cytoplasmic portion of the TLR. This results in the triggering of downstream signaling cascades and production of proinflammatory cytokines and chemokines [2, 3, 85].

1.5.1 TLR signaling pathways

The engagement of TLR by microbial components triggers the activation of signaling cascades, leading to the induction of genes involved in antimicrobial host defense. Upon ligation and recognition of PAMP, TLR interact with MyD88 which then recruits protein kinases including IL-1 receptor-associated kinase (IRAK). IRAK, in turn, activates TNF receptor associated factor (TRAF) and downstream effectors including p-38, ERK1/2, JNK[3]. Activation of the kinases lead to the nuclear translocation of nuclear transcription factors such as NF-κB to the nucleus which regulates host-cell responses to pathogens such as subsequent transcriptional activation of genes encoding pro- and anti-inflammatory cytokines and chemokines. MyD88 is critical to the production of inflammatory cytokines induced by the TLR family. The MyD88-dependent pathway signals via MyD88, IRAK, and TRAF6 and leads to NFκB activation [85]. The MyD88-independent pathway may be initiated as a result of the LPS-TLR-4 signaling pathway. Stimulation with TLR-3, -4, -7, and -9 ligands, induces type I IFN production in addition to proinflammatory signals [3].

TLR-4 recognition of LPS requires a co-receptor known as MD-2. LPS binds to CD14, expressed on the cell surface of cells and then LPS is then transferred to MD-2, which associates with the extracellular portion of TLR-4 [86]. Bacterial genomic deoxyribonucleic acid (DNA) is recognized by TLR-9 [87, 88]. Since TLR-9 resides in the endosome, bacterial DNA must be delivered to this intracellular compartment, where the acidic and reducing conditions lead to the degradation of double-stranded DNA into multiple single-stranded CpG-motif-containing regions that subsequently interact directly with TLR-9. DNA pathways downstream from TLR-9 are completely dependent on the adapter protein MyD88.

Figure 4. TLR-4 and -9 signaling

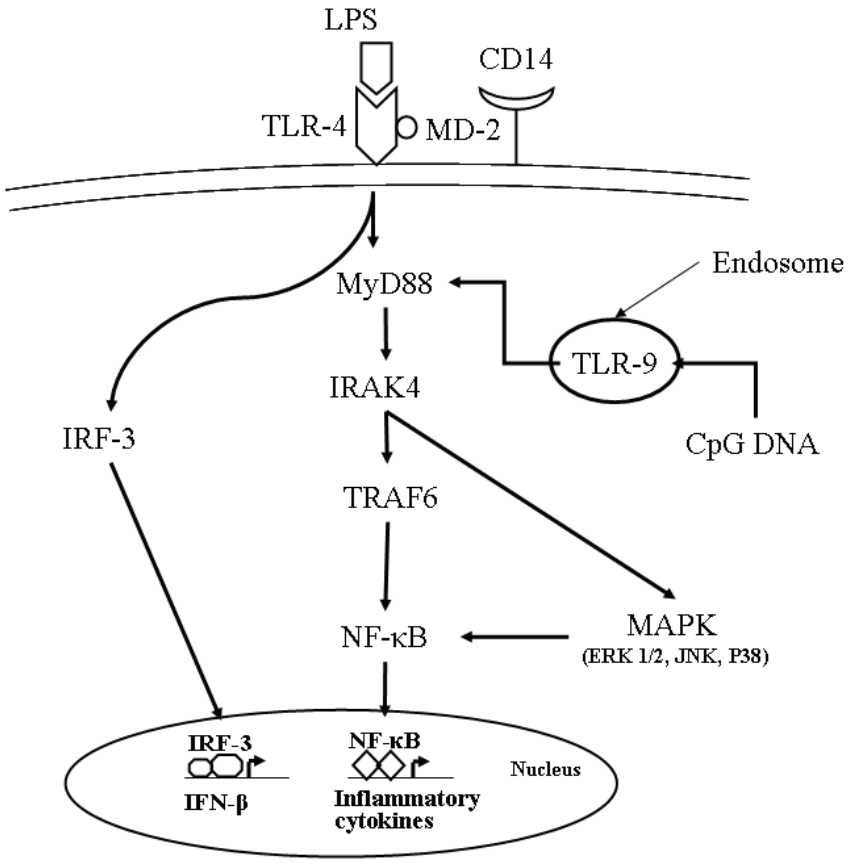


Figure 3: Signaling pathways triggered by TLR. TLR-4 ligands such as LPS induces the production of inflammatory cytokines as well as IFN-β. The induction of inflammatory cytokines is dependent on the adaptor molecules MyD88 which will recruit IRAK. This in turn may lead to the activation of the MAPK signaling pathways or activation of TRAF leading to NFκB translocation. This will result in production of inflammatory cytokines. The induction of IFN-β is independent of these molecules and may be regulated through the nuclear translocation of IRF-3. Ligation of CPG-DNA with TLR-9 occurs inside the endosome. Cytokine induction through TLR-9 depends on MyD88.
(IRAK: IL-1 receptor associated kinase, TRAF: TNF receptor associated factor, IRF-3: IFN regulatory factor 3).

1.5.2 TLR and cholangiocytes

To guard against the infection of pathogens, the biliary tract is equipped with several defense mechanisms. Cholangiocytes express PRR, among them, TLR [89-91]. Activation of TLR induces intracellular signaling cascades which result in the expression of several adhesion molecules, inflammatory mediators, cytokines /chemokines, and antimicrobial peptides initiating epithelial immune responses [39]. Human bile contains PAMP, which might be secreted from hepatocytes that take them up from portal blood inflowing in the gastrointestinal tract and infrequently from the duodenum through the extrahepatic bile duct. Cholangiocytes play a key role in the defense response in the biliary tree [89-91].

Studies by Harada et al have shown that BEC express TLR, which recognize PAMP in normal or diseased livers. Physiologically, however, PAMP do not elicit an inflammatory response in biliary lining epithelial cells of intrahepatic bile ducts. This failure to respond to PAMP, especially LPS, could be due to 'endotoxin tolerance.' This tolerance can be considered a protective mechanism that prevents damage to the cells by avoiding excessive inflammation. It also suppresses the immune response of intestinal epithelium against the resident intestinal bacterial flora under physiologic conditions [92].

Chen et al explored the role of TLR signaling in cholangiocyte responses to parasitic infection. They found that normal human cholangiocytes express all known TLR and parasitic infection of cultured human cholangiocytes recruits both TLR-2 and TLR-4 to initiate epithelial host responses. TLR-2 and TLR-4 are signaling via the activation of IRAK and phosphorylation of p-38 and NF-κB. This implicates an important role of TLR in cholangiocyte defense responses to infection in the biliary tree [90, 93]. Furthermore activation of TLR has been demonstrated in cholangiocytes during bacterial and viral infection [92-94].

Yokomuro S et al [36] investigated the innate immunity of the intrahepatic biliary tree, by examining expression of TLR and intracellular signaling in BEC in response to bacterial components by using cultured murine biliary cells. They showed that cultured BEC expressed TLR and related molecules. LPS binds directly to the surface of these cells and forms the receptor complex of CD14, TLR-4, and MD-2 in an association with MyD88 in BEC, mediating the activation of NF-κB and synthesis of the proinflammatory cytokine, TNF-α. Furthermore our group [95] has shown that antibodies to BEC are able to upregulate TLR-2 and TLR-3 which may have a role in post liver transplant cholangitis.

1.5.3 TLR and autoimmunity

T and B cells specific for self-antigens can be found in every healthy person but are not sufficient to result in the development of autoimmune diseases. When systemic autoimmune diseases develop, the targets of the autoimmune response are not random but are restricted to a small subset of antigens.
Autoimmunity and TLR correlation is best established for the TLR that detect nucleic acids, including TLR-3, -7 and -8 activated by ribonucleic acid (RNA), and TLR-9

activated by unmethylated CpG DNA. One specificity of these TLR is the detection of the pathogen in endosomal compartment; from which self nucleic acids are normally excluded. In addition, the methylation and suppression of CpG DNA in vertebrate DNA prevents it from having the same immune stimulatory activity as bacterial DNA. Nevertheless, self RNA and DNA retain some ability to autoactivate TLR-driven immune responses. Recent studies reveal that inappropriate activation of the TLR-7, -8, and/or -9 pathways can lead to autoantibody production [96].

The immune system in systemic lupus erythematosus (SLE) is specifically directed against RNA- and DNA-associated antigens. Immune complexes containing self-RNA and/ or -DNA are endogenous triggers for TLR-7 and/or TLR-9, respectively and may initiate autoreactivity in SLE or rheumatoid arthritis (RA) [96-98]. In addition to stimulating TLR-9, the DNA in lupus sera forms immune complexes with anti-chromatin or anti-DNA antibodies, and these complexes stimulate B cells via TLR-9 and the B-cell receptor, inducing and spreading autoimmunity.

TLR-9 activation on the surface of intestinal epithelial cells was reported to have a role in suppressing gut inflammation and maintaining homeostasis [99]. One group of investigators reported that in a mice model for inflammatory bowel disease that is genetically deficient in TLR-9 are unusually susceptible to acute colitis. Endogenous or exogenous TLR-9 activation promotes the development of TReg cells that protect against gut inflammation. These studies suggest that in this anatomic region, TLR-9 activation has a constitutive role in preventing inflammation [99, 100].

1.6 AUTOIMMUNE LIVER DISEASES WITH SPECIAL FOCUS ON PRIMARY SCLEROSING CHOLANGITIS (PSC)

Three chronic liver disorders are usually grouped under the heading of autoimmune liver diseases: AIH, PBC and PSC. All three are associated with hypergammaglobulinaemia and circulating autoantibodies against a wide range of tissue antigens. AIH fulfils most of the criteria for an organ-specific autoimmune condition such as female predominance, HLA association, response to immunosuppressive treatment but, whether PBC and PSC are autoimmune diseases are still under debate [101]. During the past years, our group has tried to explore the immune mechanisms involved in the pathogenesis of PSC.

1.6.1 Autoimmune hepatitis (AIH)

AIH is a disease affecting the hepatic parenchyma. The characteristic morphological changes are interface hepatitis, with a dense inflammatory infiltrate composed mainly of CD4+ T cells and plasma cells spilling out of the portal tracts. Approximately 80 % of patients with AIH present with significant titers of antinuclear (ANA) and/or anti-smooth muscle (SMA) auto antibodies [101]. AIH shows a striking response to immunosuppressive therapy with corticosteroids and azathioprine. Liver transplantation is seldom required.

1.6.2 Primary biliary cirrhosis (PBC)

PBC is predominantly affecting the small interlobular bile ducts. 95% of PBC patients have anti-mitochondrial antibodies (AMA) which are important diagnostic markers [102]. These specific AMA are directed against pyruvate dehydrogenase. There is no beneficial effect of immunosuppressive treatment with steroids. Treatment with ursodeoxycholic acid leads to biochemical and possibly histological benefits in PBC. PBC is a well established indication for liver transplantation with good results when liver failure occurs.

1.6.3 Primary sclerosing cholangitis (PSC)

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the intra- and/or extrahepatic bile ducts. It is characterized by a concentric obliterative fibrosis that leads to bile duct strictures. The disease often progresses to biliary cirrhosis and hepatic failure. Approximately 13% of patients develop cholangiocarcinoma [103]. PSC is closely associated with inflammatory bowel disease (IBD); especially ulcerative colitis (UC). Approximately 70-80% of PSC patients have an associated IBD. There is at present no effective medical treatment preventing development of cirrhosis and liver failure. PSC has become a major indication for liver transplantation with good results. There is no single sensitive and specific diagnostic marker for PSC. Diagnosis is made from a combination of clinical, biochemical, radiological and histological features. The diagnosis relies on cholangiography.

The aetiology and pathogenesis of PSC is not fully understood and growing evidence supports that immune mechanisms are involved in the pathogenesis. Circumstantial evidence that PSC may be immune-mediated is supported by several findings. PSC have an independent association with many other autoimmune diseases. Saarinen et al., [104] studied 119 patients with PSC, 24% of the patients with PSC had one or more autoimmune disorders outside the liver and colon. PSC is also associated with autoantibodies and HLA haplotypes that are closely related to IBD. Lymphocytic infiltration into areas of portal damage is also suggestive of an autoimmune mediated destructive process.

However, PSC is not a classical autoimmune disease; it occurs with a 2:1 male predominance, compared with the female predominance found in other classical auto-immune diseases. Moreover, PSC does not have the classical response to immunosuppressive treatment. Therapeutic interventions in PSC include mechanical endoscopic manipulation of the bile ducts, treatment of cholangitis and treatment with ursodeoxycholic acid [105-107].

1.6.3.1 Cellular and humoral immunity in PSC

Periportal mononuclear cell infiltration with at least 80% T lymphocytes is found in most liver biopsy specimens of PSC patients. Some of these T cells show evidence of activation, as indicated by expression of HLA-DR [108]. Berg and colleagues have classified PSC as TH1 mediated disease as the TH2/TH1 ratio was found to be 1:4.

TH1 cells produce proinflammatory cytokines, including IFN- γ, IL-2, and potentially TNF-α [108].

Patients with PSC have a predominantly TH1 response [109] with high levels of TNF-α occurring in all stages of disease [110]. TNF-α is a potent stimulator of the immune system and may contribute to destruction of hepatocytes and BEC. Liver-derived T cells from PSC patients produce higher levels of TNF-α as compared to those derived from patients with PBC or AIH. Spengler and colleagues [109] showed the presence of relatively high levels of TNF-α in culture supernatants of Phytohaemagglutinin (PHA) stimulated T cell lines from liver biopsies in patients with different stages of PSC. In addition, Bo X et al [110] found significantly higher levels of TNF-α and IL-1β but significantly lower levels of IL-2, IL-10, and IFN-γ in the supernatants of mitogen stimulated liver derived lymphocytes obtained from PSC livers as compared with controls.

Significantly more patients with PSC have autoantibodies to surface antigens expressed on BEC than patients with PBC, AIH, or normal controls. These induce increased expression of CD44 on the BEC and increased production of IL-6 by BEC [111]. BEC Ab may be both IgM and IgG. IL-6 induces BEC proliferation in vitro and suppresses BEC apoptosis.

Atypical antineutrophil cytoplasmic antibodies (ANCAs) are present in the serum of 33- 88% of patients with PSC. However, they are not specific for PSC and are found in UC (60–87%), and AIH (50–96%). The target antigen in PSC for these atypical ANCAs is probably a neutrophil nuclear envelope protein. Recently, Terjung [112] suggested the term peripheral antineutrophil nuclear antibody (pANNA). These antibodies in PSC are not directed against a single antigen. The importance of auto-antibodies in the development of PSC is also not known. The majority of studies show no association between titers of antibodies and clinical parameters in PSC. Titers of ANCAs remain unchanged after a transplant in PSC and after a colectomy in UC, suggesting they are not an epiphenomenon resulting from chronic inflammation. Presence of a monoclonal antibody to a colonic epithelial protein in UC cross-reacts with epithelial cells lining the extrahepatic ducts of patients suffering from PSC with UC, suggesting that there may be a common antigen involved in the pathogenesis of these two conditions [105, 113, 114].

1.6.3.2 Immunogenetics of PSC

PSC is not attributable to one gene locus and is probably acquired through inheriting a combination of genetic polymorphisms that act together to cause susceptibility to disease. It was reported that first-degree relatives of patients with PSC run an increased risk of PSC, indicating the importance of genetic factors in the etiology of PSC [115]. The study by Bergquist et al., revealed more than a 10-fold increased risk of PSC among first-degree relatives of patients with PSC. First-degree relatives of PSC patients without IBD are also at an increased risk of UC, which might indicate shared genetic susceptibility factors for PSC and UC [115]. A study published by Karlsen et al., showed that none of the IBD candidate genes investigated was linked with PSC susceptibility [116].

Several associations have been made with HLA haplotypes, as well as other non MHC genes with PSC development. There is an increased frequency of the haplotype HLA-B8-DR3 in patients with PSC compared with healthy controls [105]. A study by Donaldson [117] showed a secondary association with DR2 in DR3-negative patients. An increase in HLA-DR6 has also been observed in patients with PSC [118].
The MIC genes (MHC class I chain-related antigen A and B) are expressed in non diseased liver and thymic and gastrointestinal epithelia [119]. MICA has been identified as a ligand for γδT-cells and NK (CD56+) cells. Increased numbers of both γδT cells and NK cells have been documented in PSC livers [120]. Norris demonstrated an association between the MICA*008 allele and PSC. MICA*002 has a strong negative association with disease and is the functional opposite of MICA*008 [121]. The genetic variants of the NKG2D receptor are associated with increased risk of cholangiocarcinoma as found by Melum et al. Patients who were homozygous for the non risk alleles are unlikely to develop cholangiocarcinoma [122]. Genetic susceptibility to PSC might be correlated to polymorphism within the TNF genes particularly within the TNF-2 allele. Individuals with haplotype TNF-2 may produce high levels of TNF-α [123].

1.6.3.3 Animal models of PSC

Animal model of sclerosing cholangitis have been reported and are best reviewed by Vierling et al. [124]. No animal model for PSC fulfils all of the attributes of an ideal model. Future studies in animal models, however, should enhance our understanding of the pathogenesis of PSC and provide new insights for the development of novel therapies. *Although the work of this thesis does not involve animal work, it is interesting to discuss some of the important animal models and compare their relevance to PSC in humans.*

Rat model of small bowel bacterial overgrowth
Models involving reactions to enteric bacterial cell-wall components or colitis appear to be the most promising for studies of human PSC with its strong association with IBD and the production of pANCA. The sequence of histopathological events in these models indicates an initiation phase involving secretion of proinflammatory cytokines, especially TNF-α, by activated macrophages. Subsequent peribiliary infiltration with CD4+ and CD8+ T cells indicates a role for the adaptive immune response, although the antigen specificity of the T cells remains unknown [125, 126]. This rat model of small bowel bacterial overgrowth in susceptible rat strains explains the role of the innate immune response in the pathogenesis of sclerosing cholangitis. It provides clear proof that bacteria that are normally present in the colon may provide a stimulus in susceptible hosts when the integrity of the colonic epithelium is compromised, as occurs in UC and Crohn's disease.

Models of toxic, infectious or intra-luminal biliary injury
Retrograde instillation of TNBS (2,4,6-trinitrobenzene sulfonic acid) into the distal biliary tract induces cholangitis and stimulates a similar dynamic inflammatory and immunological response, including production of ANCA autoantibodies [127]. The inflammatory and immunological events in portal tract include inflammation, cytokine

production, diffuse focal stricturing of the intra- and extra-hepatic bile ducts, portal fibrosis; and development of ANCA and SMA autoantibodies. The TNBS model provides proof that biliary injury and inflammation can induce ANCA production. In mouse models, there is successful elimination of disease with the use of anti-IFN-γ, anti-TNF-α or anti-IL-12 [128]. The predominant role of TNF-α in the pathogenesis of mucosal inflammation was shown in TNF-α transgenic mice where TNBS treatment caused severe, lethal colitis [129]. In addition patients with sever UC are successfully treated with anti-TNF-α.

Experimental biliary obstruction

PSC of the small intra-hepatic ducts results in ductopenia, and involvement of larger intra- or extra-hepatic ducts contributes to biliary obstruction. Bile duct obstruction in rats may results in a sequence of events; increase in LPS levels and hence activation of KC and macrophages. Secretion of chemokine and cytokine by BEC will lead to recruitment and activation of neutrophils, monocytes and T cells. TNF-α will induce loosening of BEC tight junctions and regurgitation of bile. Bile ducts ligation will result in ductular (cholangiocytes) proliferation [130]. The may result in biliary fibrosis and cirrhosis unless obstruction is relieved. Models of biliary obstruction provide important insights to help discriminate between inflammatory and immunological mechanisms leading to sclerosing cholangitis and those that are the secondary consequences of duct obstruction and cholestasis. Bile duct ligation rat model is used to study estrogen and its effects on cholangiocytes.

1.7 ESTROGEN

1.7.1 Synthesis, production and metabolism

Estrogens are steroid hormones that are synthesized from cholesterol and function as the primary female sex hormone. The three major naturally occurring estrogens are estradiol, estriol and estrone. The most potent and dominant estrogen in humans is 17-beta estradiol (E2), but lower levels of the estrone and estriol are also present. In premenoposal women, most of the estrogens are produced in the ovary while, in men and postmenoposal women is produced by aromatization of androgens in peripheral tissue including muscle, fat, liver and brain. Estradiol is produced by the Sertoli cells of the testis and there is evidence that it prevents apoptosis of male germ cells. When estrogens are released in the circulation most of it is bound to plasma proteins and is then transported to the target tissues. In plasma estradiol is largely bound to sex hormone binding globulin (SHBG), and to a lesser extend to albumin. Only a small fraction is free and biologically active. Estradiol is conjugated in the liver by sulfate and glucuronide formation and as such excreted via the kidneys. Some of the water-soluble conjugates are excreted via the bile duct, and partly reabsorbed after hydrolysis from the intestinal tract. This enterohepatic circulation contributes to maintaining estradiol levels.

1.7.2 Functions and mechanism of action

Estrogens act on a wide range of target tissues in the human body. Estrogens are required for female sexual maturation and affects growth, differentiation and function

of the female reproductive system. In addition, in both men and women they have a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous systems [131]. In addition estrogens play biological activities in several organs, for example in liver regeneration [132]. In the skeleton, estrogens prevent bone resorption and estrogen replacement therapy is known to reduce osteoporosis in post menopausal women [133].
In target tissues, estrogens may exert opposite actions and heterogeneous effects, promoting the resistance to apoptotic damage, modulating reparative processes and controlling inflammation. Recent findings suggest a protective effect against uncontrolled or neoplastic cell proliferation. By acting on both estrogen receptors-alpha (ERα) and beta (ERβ) subtypes, estrogens affect their targeted cells [134-136].

1.7.3 Estrogen, estrogen receptors and the biliary tree

In cholestatic liver diseases, the course of these diseases is characterized by a balance between damage (loss) of bile ducts and compensatory proliferation of the residual ducts. The terminal decompensated stages are characterized by the inefficiency of proliferation to balance for the loss of intrahepatic bile ducts [35, 38, 137-141]. Recent studies indicate that estrogens play a role in the pathophysiology of PBC since both experimental and clinical studies indicate that they modulate cholangiocyte survival and death. PBC specifically affects females with a clinical presentation typically occurring during the peri- and post-menopausal period; involving endocrine dysfunctions [135, 136, 142].

During cholestasis, in bile duct ligation rat model (BDL), findings indicate that estrogen serum levels are increased and this could play a role in sustaining cholangiocyte proliferation. Rat cholangiocytes express both ERα and β subtypes. Cholangiocyte proliferation after BDL is associated with a marked increase in the expression of ERβ. Furthermore the high estradiol serum levels may potentiate the effect of growth factors driving cholangiocyte proliferation in the BDL rat model.
In addition, ovariectomy prevents cholangiocyte proliferation in BDL rats and causes a decrease of bile duct mass associated with lower expression of ERβ. When E2 was administered in BDL and ovariectomy rats, bile duct proliferation was restored and ER expression in cholangiocytes returned similar to controls [138, 140].

It was found that chronic liver allograft rejection leading to ductopenia is more frequently from male donor into female recipient. In Turner syndrome, in which liver morphology resembles that of a newborn liver, there is bile duct pathology which is improved by estrogen replacement treatment. The progression of polycystic liver disease is significantly influenced by female sex hormones, pregnancies and estrogen replacement treatment [143-145]. Marked alterations of estrogen hepatic metabolism occur in cholestasis leading to enhanced estradiol serum levels which could influence disease progression [138, 145].

Cholangiocytes lining interlobular bile ducts of PBC patients, express both ERα and ERβ subtypes. Interestingly, in stages I-III of PBC, ERα expression increases and correlates with proliferating bile ducts indicating that the expression of this receptor

subtype is a typical feature of proliferating cholangiocytes. Furthermore, in stage IV of PBC, when the maximal degree of ductopenia is reached, cholangiocytes are negative for ERα [141]. In PBC, pathogenesis could be related to a defect in proliferative response of cholangiocytes to estrogens. These findings could have important therapeutic implication, since the modulation of ERs could delay the progression of PBC toward ductopenia [141]. To our knowledge the role of estrogen in the pathogenesis of PSC has not been studied.

1.7.4 Estrogen receptors signaling

Ligand-dependent estrogen signaling begins with the binding of estrogen to ER. Cellular signaling of estrogens is mediated through ERα and ERβ both belonging to the nuclear receptor (NR) family of transcription factors. ERα and ERβ have overlapping yet unique roles in estrogen signaling. ERα and β are found intracellularly, and not on the cell membrane [146].

Evidence suggests that there are several distinct pathways by which estrogens, via ERs, can regulate biological processes. In the classical model of ER action, ligand-activated ERs bind specifically to DNA at estrogen-responsive elements (EREs) through their DNA binding domains and this induces transcription at other sites. Estrogen also modulates gene expression by a second mechanism in which ERs interact with other transcription factors, through a process referred to as transcription factor cross talk [147].
In addition, estrogen may elicit effects through non-genomic mechanisms where estrogen has been claimed to bind to ERs localized on the plasma membrane of target cells. It has been suggested that these effects may be the result of estrogen activation of MAPK and ERK signaling [148, 149]. However, such membrane-associated ER proteins have so far not been conclusively identified and the mechanistic details of activation through these non-genomic pathways remain to be characterized. Recently, Galluzzo et al., showed that ERβ is necessary for the p38-dependent activation of the downstream pro-apoptotic cascade [150].

The effects of steroid hormones upon cytokine production are suggested to be mediated by the NF-kB which in turn regulates the expression pro-inflammatory genes [151]. It has been shown that the steroid/receptor complex can physically interact with NF-kB and inhibits its activity [151]. Via this mechanism estrogens, can inhibit proinflammatory cytokine expression in immune cells expressing the respective receptor.

Evidence supports that estrogen has non genomic effects because of the rapid effects of estrogen which have been observed. These effects occur within seconds to minutes after estrogen treatment. Studies have suggested that these effects may be the result of estrogen activation of MAPK and ERK signaling [148]. Recently it has been shown in cholangiocytes that estrogens activate intracellular signaling cascades ERK1/2, PI3-kinase/AKT (phosphatidylinositol-3' kinase/AKT)] typical of growth factors such as insulin like growth factor (IGF1), nerve growth factor and vascular endothelial growth

factor (VEGF), thus potentiating their action. In addition, estrogens stimulate the secretion of different growth factors in proliferating cholangiocytes [152].

1.7.5 Estrogen and the immune system

The thought that estrogens can influence the clinical course of PBC, as for most autoimmune disorders, came from several studies showing that estrogens are able to modulate the humoral and cellular immune response [153] through the inhibition of TH1 proinflammatory cytokines (IL-12, TNF-α and IFN-γ). By switching the immunological response toward the TH2 profile, estrogens stimulate the production of TH2 anti-inflammatory cytokines (such as IL-10, IL-4 and TGF-β) thus potentiating the anti-inflammatory response [154]. In addition, estrogens can prevent oxidative stress in hepatocytes which are injured by cholestasis [137].
Through the ERα, estrogens can positively modulate the growth factors. This further supports a possible therapeutic role of ERα positive modulators in cholestatic liver diseases since Alvaro et al., demonstrated that IGF- 1 and estrogens play additive effect on cholangiocyte proliferation [155] .

E2 seems to exert effects on cell growth and apoptosis [156]. E2 is thought to play a dual pro- and anti-inflammatory role in chronic inflammatory diseases related to low and high concentrations of estrogens. [157]. Men with RA had aberrations in all sex hormones analysed, although only estradiol consistently correlated with inflammation [158, 159]. In another study, E2 was found to increase IgG and IgM production by peripheral blood mononuclear cells (PBMC) from SLE patients, which leads to elevated levels of polyclonal IgG. In vitro it has been shown that estrogen increases IgG and IgM production by PBMC. Testosterone inhibited IgG and IgM [160, 161]. In line with these results, it has also been shown that estrogen increased and testosterone decreased autoantibody production of PBMC in patients with SLE [162, 163].

Also studies in animals have shown an effect of estrogens on B cell development: estrogens increase bone marrow progenitor B cells in mice by protecting the progenitor cells from apoptosis and increase survival in splenic B cells. These estrogen effects on B cell development may decrease negative selection in naive immature B cells and enhance the survival of autoreactive B cells and may, therefore, be involved in the higher incidence of autoimmune diseases in women [164].
We need a better understanding of the gender and sex hormone influences on B cell functions and counts in humans, in order to produce, novel therapeutic approaches for humoral autoimmune diseases.

1.8 SUMMARY

The role of the liver in tolerance induction has always been in focus. It was not known exactly which cell types were involved in tolerance effect by the liver. LSEC have been suggested to play an active role in tolerance induction and immunological response within the liver. Therefore it was important to study if LSEC have the capacity to interact with T cell and antibodies and what is their role in tolerance induction and liver allograft rejections.

BEC are the cells that are targeted during PSC. We were interested in studying the mechanisms involved in PSC pathogenesis and to study the role played by human bBEC Ab in the immune mediated disease – PSC.
We were therefore interested in elucidating some of the mechanisms underlying liver tolerance –liver allograft rejections and immunopathogenesis of PSC.

2 AIMS

The overall aim of the thesis is to study the role of LSEC and BEC and antibodies to LSEC and BEC in immune response by the liver.

The specific aims were:

1. To investigate the role of LSEC in peripheral tolerance induction. Specifically we were interested in studying the ability of LSEC to regulate apoptosis of activated T cells.

2. To detect the presence of LSEC Ab in liver transplant recipients and investigate their clinical correlations and functional capacity in allograft liver rejections.

3. To investigate the functional role of BEC Ab as a possible major link between adaptive and innate immune response (TLR molecules) in PSC patients.

4. To investigated the functional relationship between presence of BEC Ab and sex hormones in PSC patients.

3 METHODOLOGICAL CONSIDERATIONS

The methods used have been described in details in paper I-IV. Some of the methods are discussed below with some general considerations.

Patients and samples

Informed consent was obtained from all patients prior to blood or tissue sampling. All studies were approved from the ethics committee at Karolinska University Hospital.

In paper I, CD3+ cells were isolated from normal healthy controls who are known not to have any disease. In paper II, the patients included were 95 consecutive liver transplant patients performed at our centre during 1999-2000. At our centre (Karolinska-Huddinge University Hospital), standard indications for liver transplantation, surgical techniques and immunosuppression protocols were followed. In paper III and IV, the diagnosis of AIH, PBC, UC and PSC were based on accepted histological, clinical and cholangiographic criteria [165].
After blood sampling, the patients' sera were separated and stored at -70˚C until further use.
In paper II sera were tested for the presence of LSEC antibodies. Sera from 5 patients with rejections and who had antibodies to LSEC were pooled together for IgG isolation. IgG fractions were also purified from 5 patients without rejections and no LSEC Ab.
In paper III and IV sera from PSC patients were tested for the presence of BEC antibodies. All the sera that had antibodies to BEC were pooled together for IgG isolation.

Isolation of LSEC

In paper I and II we used LSEC isolated from 3 whole livers that were not used for transplantation for one reason or another. We followed standard protocol with modifications aiming at a better pure, specific and homogenous population of LSEC. For paper II we used LSEC isolated from one of those livers. To reduce the potential of contaminating with vascular endothelium, excision of visible large vascular structures from the liver tissue prior to enzymatic digestion is done.We followed the basic protocols, chopping the liver to small pieces, followed by enzymatic digestion and mechanical disruption of the pieces for detachment of LSEC, followed by separation on percoll gradient. In our experience use of a mild enzyme such as dispase and incubation at 4˚C overnight gave a good yield of cells.
LSEC can be passeaged to 5-7 times with stable phenotype and morphology as determined by flow cytometry and electron microscopy. To obtain a more specific population of LSEC we now routinely include a step of immunomagnetic depletion to remove common contaminating cell populations, such as BEC, followed by positive selection of endothelial cells using antibody against DC-SIGN (L-SIGN). To date there is no known single molecule that is only expressed on hepatic sinusoidal and no other type of endothelia. Since many classical endothelial markers are widely expressed and shared by other EC. However, characterization is done by using a better defined set of phenotypic makers (endothelial markers, and immune recognition markers). We also use negative control markers that are not expressed by LSEC, such as smooth muscle marker and a fibroblast marker. Furthermore, the use of electron microscopy for

phenotyping for the presence of fenestrae and lack of basement membrane is also needed.
Isolated cultures of hepatic SEC present a valuable tool for the study of liver physiology and pathophysiology.

Isolation of BEC
Isolation of BEC from livers was performed after a simple digestion using a stronger digesting enzyme, collagenase. We found that the type of collagenase used and length of time for digestion was important in obtaining a good yield of these cells. In our experience the use of collagenase IV and incubation time of 45 minutes in incubator at 37˚C were good conditions for a good yield of BEC. We also routinely included a step of magnetic beads separation in order to obtain as much as possible a pure population of BEC. The cells were isolated using magnetic beads coated with HEA-125, a pan epithelial cell marker expressed on BEC but not on hepatocytes. To differentiate between isolated BEC and contaminating vascular epithelial cells further characterization with cytokeratins is done. BEC can be passeaged up to 7-8 times with stable phenotype and morphology as determined by flow cytometry.

Detection of IgG against LSEC and BEC using flow cytometry
The detection of IgG in patients' sera against LSEC or BEC can be performed using different methods. The most common are flow cytometry assay and enzyme linked immunosorbent assay (ELISA). However, in our group we routinely use the flow cytometry and we developed and optimized our technique for detection of IgG in sera against endothelial and epithelial cells. The target cell and antibody complexes can be detected by flow cytometry. The advantage with flow cytometry is it that it is rapid and also quantitative. It is consistent between the experiments and the same setting can be used for all experiments so that all results are comparable after analysis. For the flow cytometric assay, trypsinized and single cell suspended endothelial/ epithelial cells (target cells) were incubated with patient's serum followed by a secondary fluorescent-labeled $F(ab')_2$ goat anti-human IgG antibody against Fc part and followed by detection by flow cytometry. To test whether the antibodies are tissue specific for the targeted cells, we tested the sera to human lung epithelial cells (LEC), renal proximal tubular epithelial cells (RPTEC) and even lymphocytes from PSC patients. The use of these control cells helped us in determining the specificity of antibodies used in these studies. LEC were commercially purchased while the (RPTEC) was isolated at our lab, both cell lines were cultivated in their respectively recommended media.

Isolation of PSC IgG
Sera from patients who are known to have antibodies to LSEC or BEC were pooled and total IgG fractions were isolated using goat anti-human IgG (Fc-chain specific) agarose beads according to the established procedure. In our experience, filtering the patients' sera before adding to the column will minimize the clogging of the column. Bound IgG was eluted and neutralized and protein concentrations were roughly estimated using BCA protein assay kit (Nordic Biolabs AB, Stockholm, Sweden). Control IgG fragments was isolated in the same way from pooled sera of normal controls. IgG fractions were then dialysed, lyophilized and resuspended in distilled water and concentration was determined by standard Mancini method.

Apoptosis detection

For detection of apoptosis we used a combination of techniques. Annexin V staining for the analysis of plasma membrane integrity was used. Comet assay for analysis of DNA fragmentation, detection of protease activity (caspase 3 and 8 activity) and western blots for the detection of protein expression of genes controlling apoptosis (Bcl-2 family, Bcl-2, Bcl-xl, Bax, Bid, Bak and Bad)

AnnexinV/PI:

Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Annexin V is an early marker of apoptosis. One of the earliest indications of apoptosis is the translocation of the membrane phospholipids phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed, binding sites on PS become available for Annexin V. Annexin V can be conjugated to a fluorochrome such as Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE), and used for flow cytometric analysis of cells undergoing apoptosis.

Because PS translocation also occurs during necrosis, Annexin V is not an absolute marker of apoptosis. Therefore, it is often used in conjunction with vital dyes such as 7-amino-actinomysin D (7-AAD) or propidium iodide (PI), which binds to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is lost, as occurs in the later stages of apoptosis or in necrosis. Cells that are negative for both Annexin V and the vital dye are not apoptotic. Cells that are Annexin V positive and PI negative are in early apoptosis. In our studies we included the cells that were Annexin V positive only. Cells that are positive for both Annexin V and the PI are either in the late stages of apoptosis or are already dead. Annexin V and a vital dye can be used to monitor the progression of apoptosis: from cell viability, to early-stage apoptosis, and finally to late-stage apoptosis and cell death.

***Comet assay*:**

The Comet Assay or single cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells [166]. It is a biochemical technique for detecting DNA single strand breaks, alkali-labile sites. The image obtained looked like a "comet" with a distinct head, comprising of intact DNA and a tail, consisting of damaged or broken pieces of DNA hence the name "Comet" assay.

The concept underlying the SCGE assay is that undamaged DNA has an organized association with matrix proteins in the nucleus. When DNA is damaged, this organization is disrupted, and the strands of DNA lose their compact structure, relax, and expand out of the cavity into the agarose. When the electric field is applied the DNA, which has an overall negative charge is drawn towards the anode. Undamaged DNA strands are too large and do not leave the cavity, whereas the smaller the fragments, the farther they are free to move. The amount of DNA that leaves the cavity is a measure of the amount of DNA damage in the cell. In our experience it is important to be careful with the voltage and the time (1 V/cm for 12-20 minutes) was applied. However in our studies we were interested in documenting number of the cells that were undergoing apoptosis. The slides were stained with SYBR green and examined using fluorescence microscopy and apoptotic cells which formed a comet were counted.

Calculations of bioavailable estradiol and testosterone:

In paper IV we measured sex hormones and SHBG levels in sera of PSC patients and control groups, using ELISA. Estrogen and testosterone circulate in the plasma nonspecifically bound to albumin and specifically bound to SHBG and in small percentage as free or unbound. The free and the nonspecifically bound fraction are commonly referred to as the bioavailable fraction. The bioavailable fraction reflects the hormone available at the cellular level since the hormones are biologically readily available and dissociate from the albumin and SHBG to enter the cell and exert their action. This bioavailable fraction reflects more accurately the clinical situation than the total hormone levels. Several methods have been used to measure free or bioavailable estrogen and testosterone levels in plasma, but they are rather tedious and time consuming.

A reliable value of free or bioavailable sex hormones can be calculated from values of total sex hormone, SHBG and albumin values using second degree equations. In our experience we found no difference in the analysis (*P* values and significance level) when calculating free or bioavailable sex hormone levels). However, for convenience we present the data of bioavailable levels of hormones.

4 RESULTS AND DISCUSSION

The results from the four papers are briefly presented and discussed.

4.1 PAPER I

Human liver sinusoidal endothelial cells induce peripheral tolerance by induction of apoptosis of activated T cells

The liver is considered a site for trapping, apoptosis and phagocytosis of activated T cells. LSEC may be one of the major cell types in the liver that actively participates in peripheral T cell deletion and in the homoeostasis of the immune system.
Therefore in paper I, we investigated the role of LSEC in induction of apoptosis of activated T cells.

We found that activated T cells proliferated the least when cocultured with LSEC in comparison to when cocultured with the control cells; human aortic endothelial cells (HAEC) and PBMC. This intrigued us to assess for apoptosis.

Our results showed that 30% of activated peripheral T cells underwent apoptosis when cultured with stimulated LSEC. No significant apoptosis was observed when non-activated or activated T cells were cocultured with HAEC.

Other studies have shown that LSEC are emerging as a cell type that has a central role in several biological processes. This is reflected by their strategic location, innate scavenger capacity, specialized morphology and phenotype that enables trapping, apoptosis and antigen presentation to activated and naive T cells [31]. Furthermore, interaction of naive $CD8^+$ T cells with sinusoidal endothelium results in partial activation of the T cells, followed by passive cell death [53].

Our results from time kinetics studies assessing activated T cells after coculture with LSEC showed that apoptosis started early in coculture and after 4 hours 15% of T cells were already apoptotic. Using transwell assays, we showed that activated T cells required contact with LSEC in order to undergo apoptosis. The reason for this is not clear. Since only a fraction (30%) of the activated T cells and not all activated T cells undergo apoptosis, it is possible that contact with LSEC may induce a phenotypic change in activated T cells that are already programmed for cell death.

We next studied the mechanism of induction of apoptosis by LSEC. We tested for the presence of cytokines in the mixed cell culture (MCC) to identify a possible soluble factor responsible for induction of apoptosis. We tested for some inflammatory cytokines as well as cytokines that are known to have immunomodulatory effects such as IL-2, IL-4, IL-10, TNF-α and TGF-β.

Human liver sinusoidal endothelial cells induce apoptosis of activated T cells via the cytokine TGF-β

Our results show that TGF-β is constitutively produced by LSEC and the levels of TGF-β were further significantly increased in the MCC supernatants of activated T cells with LSEC. This could be due to the apoptotic T cells producing TGF-β. This

finding is similar to other studies where they showed that apoptotic T cells themselves can secrete TGF-β and thereby contribute to the immunosuppressive milieu of the liver [167].
Our results documented that LSEC induced apoptosis of T cells via the cytokine TGF-β. It is known that TGF-β is a multifunctional growth factor involved in the regulation of proliferation, extracellular matrix production and degradation, cell differentiation and apoptosis induction [168]. The role of LSEC in tolerance induction has been documented by several studies. It was shown by Crispe et al., that LSEC produce IL-10 and that upon interaction of LSEC with CD4+ T cells results in their differentiation into a TReg phenotype [53]. LSEC play a role in tolerance induction by inducing the differentiation of naive T cells into IL-4 and IL- 10 producing cells with regulatory function [31].

In addition, we found caspase activity in the activated T cells after coculture with LSEC. Our results show that LSEC induced caspase 3 and 8 activity in the apoptotic T cells. Similar findings were shown by other studies; It was found that caspase activity participated in TGF-β induced apoptosis in human hepatoma cell lines [80]. TGF-β-induced apoptosis can occur via the release of cytochrome c and the subsequent oligomerisation of Apaf-1, which initiates the caspase cascade [169].

Neutralizing TGF-β antibodies partially reduced apoptosis of T cells
We found that when blocking apoptosis with anti-TGF-β alone or with general pan caspase inhibitor reduced the apoptosis by approximately 50% and strongly downregulated the proapoptotic molecule Bak and upregulated the antiapoptotic molecule Bcl-2. Furthermore we showed that there was upregulation of Bcl-2 and downregulation of Bak in T cells blocked with anti-TGF-β antibodies, indicating that TGF-β induces apoptosis by downregulating Bcl-2 and upregulating Bak. The fact that only approximately 50% of the activated T cells are rescued from apoptosis by anti-TGF-β antibodies indicates that LSEC may induce cell death by additional pathways which may be TGF-β and caspase-independent. We have observed strong cell surface expression of the molecules PDL-1 and -2 and galectin-1 on LSEC. These are important molecules which may play a role in other apoptotic mechanisms induced by LSEC.
Therefore our results show that LSEC induces peripheral tolerance by inducing apoptosis of activated T cells (Figure 3a).

4.2 PAPER II:

Acute liver rejections may be mediated by humoral immune response. Specifically LSEC antibodies may have immune modulatory functions on LSEC. In paper II we investigated the role of LSEC Ab in liver allograft rejections.

LSEC Ab induces the expression of CD86 and downregulates TGF-β production by LSEC

In this study, we showed that LSEC Ab facilitate the antigen presenting capacity of LSEC by induction of costimulatory molecules. Our in vitro experiments showed that LSEC Ab from patients with liver transplant rejections play a role in acute rejection

process indirectly by regulating the function of effector T cells. LSEC Ab induced the expression of the costimulatory molecule CD86 on LSEC. It was previously shown that human LSEC express only MHC class I and II and CD40 but do not express the costimulatory molecules CD80 and CD86 [29] in contrast to the rat LSEC that constitutively express these costimulatory molecules [33]. Therefore, it was proposed that LSEC may function as APC that induces T cell tolerance. To our knowledge this is the first study that demonstrates that antibodies to LSEC may induce the expression of the costimulatory molecule CD86 on LSEC (Figure 3b). Cellular immunity especially CD4+ and CD8+ T cell mediated immune responses are believed to be the major mechanisms underlying acute liver allograft rejections [62]. It is possible that in transplant patients, LSEC present the donor antigens to the recipient lymphocytes thus cause tissue damage and liver graft rejections.

We showed in paper I that TGF-β is constitutively produced by LSEC. It was previously shown that TGF-β has many functions among them suppressing T cell proliferation [170]. Interestingly, our results in the present study show that LSEC Ab downregulated TGF-β levels, thereby enhancing the cellular immune responses in addition to the upregulation of CD86. Therefore increased T cell proliferation during liver allograft rejections could be a result of combined antigen presentation by LSEC and decreased TGF-β levels (Figure 3b).

Our group has previously shown that antibodies binding to LSEC can change the morphology and phenotype of LSEC to a vascular one [16]. LSEC antibodies are able to capillarise LSEC by inducing the production of basement membrane, loss of fenestrae and formation of tight junctions. LSEC Ab further induced the expression of markers such as FactorVIII RAg and CD31 that normally LSEC do not express. Capillarization of LSEC in liver allograft has been reported during rejections and graft failure [171]. Other consequence of the morphological changes of LSEC is the development of cirrhosis caused by ischemic atrophy of hepatocytes. Furthermore, the formation of the basement membrane will interfere with the bidirectional exchange of molecules. This will affect the liver physiology, such as decreased compliance with increased resistance to blood flow and this may in turn contribute to development of portal hypertension [172]. We have also shown that LSEC Ab may facilitate the antigen presenting capacity of LSEC and hence mediate liver allograft rejections.

4.3 PAPER III

Isolated IgG from PSC patients bind BEC

In paper III, we investigated the role of PSC IgG isolated from PSC patients who were shown to have IgG that binds BEC. We investigated the role of BEC Ab in the immunopathogenesis of PSC.

In this study we confirmed previous results from our group that PSC IgG isolated from PSC patients bind BEC. In addition, in this study we documented that the $F(ab')_2$ part of the isolated PSC IgG binds specifically to BEC. In a previous study our group has shown that BEC Ab induced BEC to produce increased levels of the inflammatory cytokine IL-6 and the adhesion molecule CD44 which is involved in homing of

leukocytes [111]. In this study we describe the inflammatory role played by BEC Ab in the pathogenesis of PSC.

Isolated IgG from PSC patients upregulates TLR-4, and -9 and MyD88 on BEC
We show that BEC did not demonstrate protein expression for the TLR tested (TLR-1, -2,-3,-4,-9). Our group has previously demonstrated that normal human BEC express mRNA for TLR-1, -2, -3, -4, and -9 and this finding was confirmed in the present study. BEC Ab induces expression of TLR-2 and TLR-3 on cultured BEC and these antibodies correlate with post–liver transplantation cholangitis [95].
Here, we showed that PSC IgG stimulates the expression of TLR-4 and -9 on BEC, reflecting the varied and unique specificities of the BEC Ab in different inflammatory responses.

Cytokine and chemokine levels in supernatants of PSC IgG–treated BEC stimulated with LPS or CpG DNA
We found that granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, and IL-8 were significantly increased in the supernatants of BEC when stimulated with PSC IgG (BEC Ab). Furthermore PSC IgG treated-BEC stimulated with LPS or CpG DNA showed further increased levels of both TNF-α and IFN-γ. The production of inflammatory cytokines TNF-α and IFN-γ by TLR expressing BEC has important implications as documented by these studies. Spirli et al showed that TNF-α- and IFN-γ synergistically could stimulate BEC to generate nitric oxide via NOS2 induction [173]. In liver tissue from patients with PSC, bile ducts strongly express NOS2 which can cause ductular cholestasis and also inhibit cholangiocellular bile formation [174].

Furthermore, our data show that BEC express functional TLR-4 and TLR-9. This tallies with results from studies by Harada K et al., [36] who examined the expression of TLR on BEC and their signaling pathways. They showed that LPS formed a complex with TLR-4 with the associated molecules CD14, MD-2 and MyD88 on cultured BEC which was associated with NFκ- B activation and synthesis of TNF-α.

PSC IgG (BEC Ab) selectively phosphorylated ERK-1/2 in BEC and the transcription factor ELK-1 and NF-κB.
The major function of MAPK signaling pathways is to regulate gene expression in response to extracellular stimuli. ERK1/2 kinases function in a protein kinase cascade that plays a critical role in the regulation of cell growth, differentiation, and development [175, 176]. Consistently with this, we investigated whether PSC IgG could cause activation of various MAPK on human BEC. We found that ERK1/2 MAPK were phosphorylated after stimulation with PSC IgG. In addition, phosphorylation of the transcription factor ELK-1 and NFκB was also observed.

Expression of TLR-4 and -9, on bile ducts of PSC liver biopsy sections
In vivo, we showed that 58% of liver biopsies from PSC patients stained positive for TLR-4 and -9. Expression of TLR-4 and -9 on bile ducts of patients with PSC indicate that these receptors may recognize circulating LPS and viral DNA, causing local inflammation, which may correspond to liver disease flares in these patients who experience recurrent bacterial and viral infections. These findings provide a critical role for BEC-Ab in epithelial cell response to microbial pathogens and activation of

inflammatory responses. In a proposed animal model for PSC, abnormal accumulation of the bacterial product LPS in biliary epithelia has been shown [177]. Interestingly, expression of both TLR-4 and TLR-9 has been reported in intestinal epithelium of patients with UC [178]. Because PSC is closely associated with ulcerative colitis, this observation implicates an important role for microbes/TLR in the pathogenesis of PSC.

The expressed TLR- 4 and -9 on the bile ducts of PSC patients may recognize circulating LPS and viral DNA causing more local inflammation. This may lead to direct biliary epithelial cell injury or altered biliary function and sclerosing cholangitis. Thus, PSC BEC-Ab may play a potentially important role in the pathogenesis of PSC by linking innate and adaptive immune response (Figure 3c).

4.4 PAPER IV

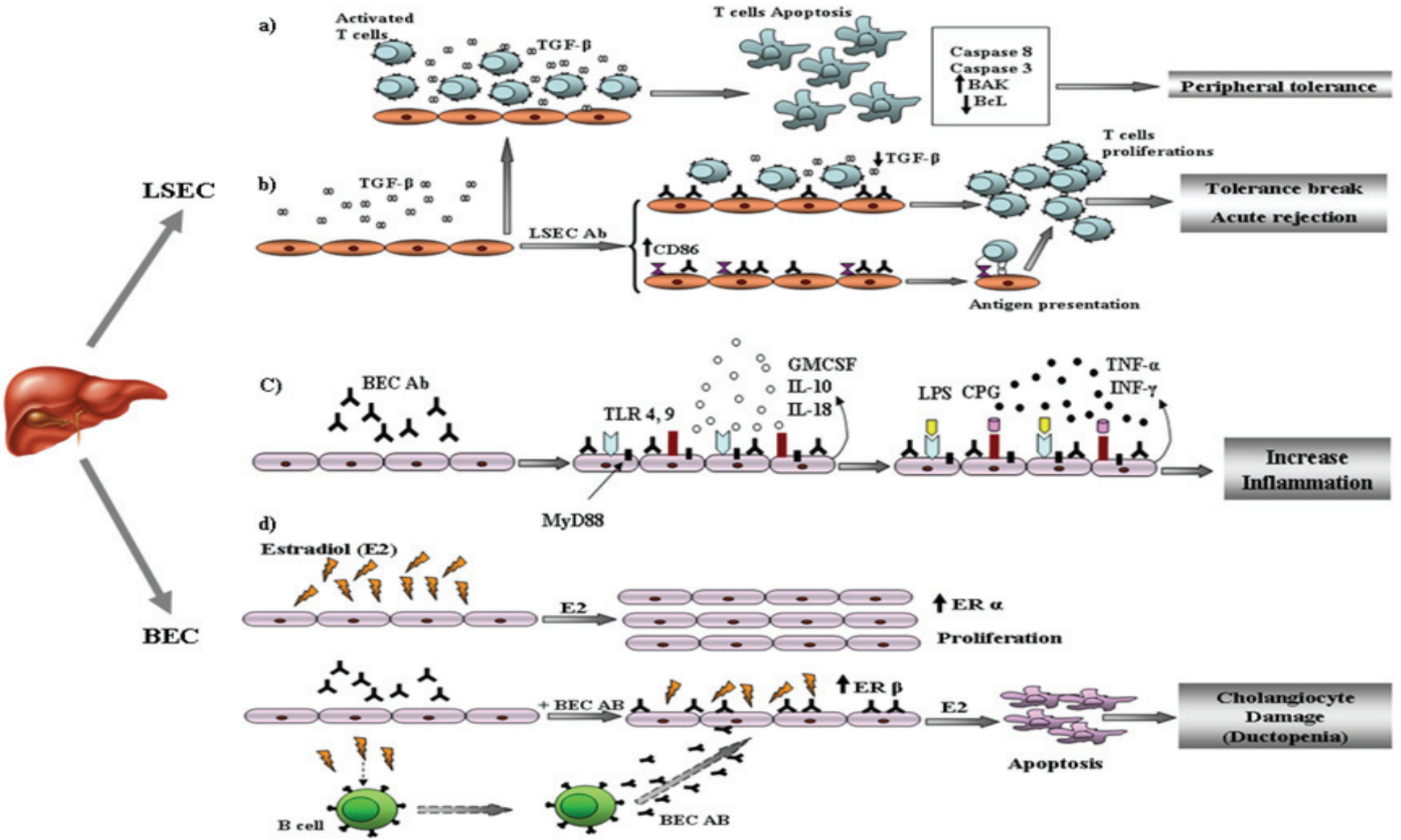
PSC patients have significantly higher levels of bioavailable estradiol
In this study, we report for the first time that PSC patients have significantly high levels of bioavailable estradiol and testosterone compared to other liver disease patients (AIH and PBC) and healthy controls. It is known that PSC is a male dominated disease that occurs with a ratio of male to female 2:1. We found that PSC patients with UC and those without did not differ significantly in their estradiol and testosterone levels. In addition, there was no correlation between bioavailable estradiol, testosterone and cholestasis. This supports the theory that the high bioavailable estradiol levels in PSC do not represent a secondary effect of cholestasis/cirrhosis but may be an important player in the pathogenesis of the disease leading to increased cholestasis over time. Hence we wanted to investigate the possible role of estradiol on BEC.

PSC patients with BEC Ab have significantly high levels of bioavailable estradiol
One striking finding in this study is that PSC patients with BEC Ab have significantly higher levels of bioavailable estradiol compared to patients without BEC Ab. This intrigued us further to investigate the causal relationship between presence of BEC Ab and sex hormones. Mizoguchi Y et al., [179] documented that estrogen stimulates antibody production by B cells and enhances their resistance to apoptosis. Thus, in PSC patients, the high levels of estradiol may directly induce BEC Ab production by B cells. We hypothesize that the high levels of estradiol prolong the survival of already existing B cells resulting in the production of autoantibodies with various specificities, including autoantibodies against BEC (Figure 3d).

Estradiol binds ERβ upregulated by BEC Ab and increases apoptosis of BEC
Our *in vitro* results documented that upon addition of exogenous estradiol, an increase in the proliferative capacity of BEC was induced. However, treatment of BEC with PSC IgG fractions followed by addition of estrogen induced cell death of BEC. Staining of freshly isolated and cultured BEC demonstrated the expression of both the estrogen receptors ERα and ERβ on the BEC. However, treatment of BEC with purified PSC IgG increased the expression of ERβ but not ERα. We further demonstrate that when estrogen binds to this receptor, it decreases proliferation and instead increases apoptosis of BEC- a finding consistent with the immunohistochemical

staining of diminishing ducts in livers from PSC patients which expressed ERβ but not ERα.
Thus, we demonstrate one mechanism by which estrogen and the immune system interplay in the complex loop of disease processes leading to cholangiocyte damage in PSC (Figure 3d).
Estrogens have been shown to target the biliary tree, where they modulate the proliferative and secretory activities of cholangiocytes. By acting on both ERα and ERβ subtypes, and by activating either genomic or non-genomic pathways, estrogens play a key role on growth factors and cytokines, which modulates the proliferative response of cholangiocytes to damage [152]. It has been shown in cell lines [180-182] that estrogen in the presence of ERα elicits proliferation, but in the presence of ERβ, inhibits proliferation, demonstrating dual but opposite effects of the same hormone.
However it is not known which has occurred first in PSC patients, is it the auto antibodies or is it the high estradiol levels. Furthermore what is the gut-liver connection?
We need a better understanding of the gender and sex hormone influences on B cell function in humans, in order to produce, novel therapeutic approaches for humoral autoimmune diseases.

Figure 5. Immune interactions with the liver



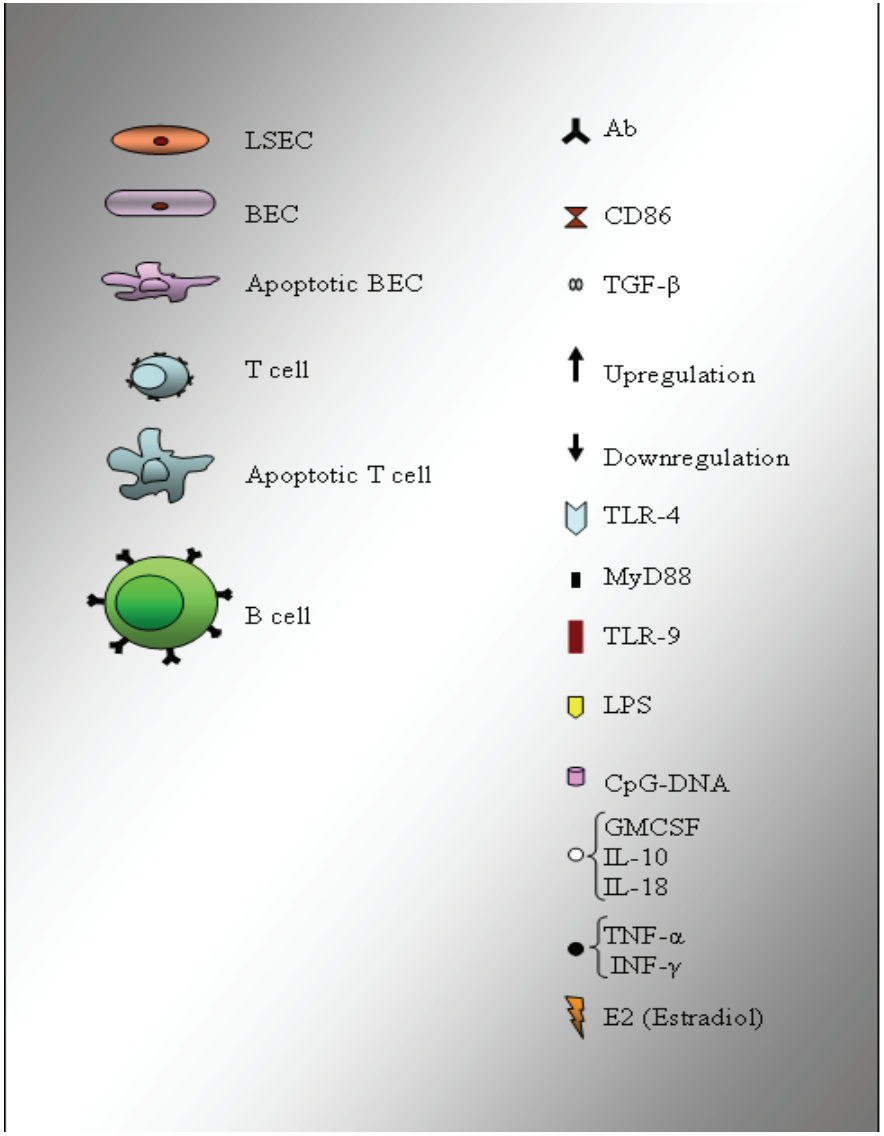


Figure 3 a) Tolerance effect by liver sinusoidal endothelial cells. TGFß is constitutively produced by LSEC. LSEC induced apoptosis of activated T cells via the cytokine TGF-β. The apoptosis was associated with caspase 3 and 8 activity and upregulatation of Bak and downregulation of Bcl-2.

b) Tolerance break by LSEC antibodies leading to liver allograft rejection
LSEC Ab downregulated TGF-β levels production by LSEC, thereby increasing T cell proliferation. LSEC Ab were able to induce the expression of CD86 on LSEC. Increased T cell proliferation during liver allograft rejections could be a result of combined antigen presentation by LSEC and decreased TGFβ levels.

c) BEC antibodies link innate and adaptive immune response in PSC
BEC Ab upregulates TLR-4, -9 and MyD88 on BEC. GMCSF, IL-1β, and IL-8 were increased in the supernatants of BEC when stimulated with BEC Ab. Furthermore, BEC Ab treated-BEC when stimulated with LPS or CpG DNA showed increased levels of TNF-α and IFN-γ. The upregulated TLR and production of cytokines may lead to increased inflammatory response in PSC patients.

d) Interplay between BEC antibodies and estradiol in the pathogenesis of PSC
Addition of exogenous estradiol to cultured BEC, increased their proliferation and ERα. Treatment of BEC first with BEC Ab followed by addition of estradiol decreases the proliferation and induced apoptosis of BEC. Estradiol binds ERβ and induces apoptosis. In PSC, we hypothesize that the high levels of estradiol prolong the survival of already existing B cells resulting in the production of autoantibodies (BEC AB)

5 CONCLUSIONS

- LSEC are one of the major cell types in the liver that contribute to peripheral tolerance through the induction of apoptosis of activated T cells. The apoptosis is mediated via the production of the immunosuppressive cytokine TGF-β and signals via caspase 3 and 8. We showed that LSEC participate in peripheral deletion of activated T cell and thus contribute to the homeostasis of the immune system.

- LSEC Ab are significantly associated with acute rejections in liver transplantation. LSEC induced CD86 expression on LSEC and downregulated TGF-β levels which in turn increased T cell proliferation. Our results show a novel mechanism of liver allograft rejection facilitated by LSEC Ab.

- PSC IgG stimulated the expression of TLR-4 and -9 on BEC which upon ligation induced the BEC to produce several inflammatory cytokines/ chemokines. Thus the adaptive immune response BEC Ab may facilitate the innate immune responses by induction of TLR on BEC in PSC.

- PSC patients, have significantly increased levels of estradiol. Estradiol binds ERβ upregulated by BEC Ab which in turn leads to decreased proliferation and apoptosis of BEC. Therefore increased levels of estradiol and BEC Ab may be a driving force in the pathogenesis of PSC leading to ductopenia/cholestasis.

6 FUTURE PRESPECTIVE

- **Capillarized LSEC do not induce apoptosis of activated T cells: A break in tolerance induction in autoimmune liver diseases**. We will investigate the functional role of LSEC-Ab in AIH and PBC patients. We have shown in our results that patients who have LSEC Ab have decreased levels of TGF-β and increased levels of T cell proliferation. Our group has also shown capillarization of LSEC is induced by LSEC Ab. Therefore we hypothesized that T cells from autoimmune LD will proliferate with capillarized LSEC.

- **Circulating inflammatory endothelial and epithelial cells as a disease activity marker in autoimmune liver diseases.** In autoimmune liver diseases, endothelial /epithelial cells may detach from the site of inflammation (liver) and be released in the peripheral blood. We will investigate whether circulating inflammatory endothelial /epithelial cells may be used as a disease activity marker in autoimmune liver diseases (AIH, PBC and PSC).

- **Estradiol signaling:** We intend to study the signaling pathways used by estradiol in BEC. Also investigating the aromatase activity in PSC patients and investigate whether levels of this enzyme correlate with the high levels of estradiol in PSC patients.

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