

## Seminars in Immunopathology

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Review Article

Special Issue on the Lymphoid Liver - by Dr. Podda, Dr. Selmi, and Dr. Gershwin

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Editors

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Cliona O'Farrelly cliona.ofarrelly@ucd.ie

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Hiromi Ishibashi hiishibashi-gi@umin.ac.jp

4. The immunobiology of bile duct cells

Ruth Lan

5. Mononuclear cells in liver injury and fibrosis

Fabio Marra f.marra@dmi.unifi.it

6. Hematopoietic stem cells and the liver

Jan Nolte jan.nolte@ucdmc.ucdavis.edu

7. Biliary atresia

Giorgina Mieli-Vergani giorgina.vergani@kcl.ac.uk

8. Autoimmune hepatitis

Diego Vergani diego.vergani@kcl.ac.uk

9. Primary biliary cirrhosis

Ulrich Beuers U.H.Beuers@amc.uva.nl

10. Primary sclerosing cholangitis

Christopher Bowlus clbowlus@ucdavis.edu

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Silvia Fargion silvia.fargion@unimi.it

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### Authors

Hiromi Ishibashi      Department of Hepatology Nagasaki University Graduate School of Biomedical Sciences, and Clinical Research Center, National Hospital Organization(NHO) Nagasaki Medical Center, Omura, Nagasaki, Japan

Atsumasa Komori      Department of Hepatology Nagasaki University Graduate School of Biomedical Sciences, and Clinical Research Center, National Hospital Organization(NHO) Nagasaki Medical Center, Omura, Nagasaki, Japan

Kiyoshi Migita      Department of Hepatology Nagasaki University Graduate School of Biomedical Sciences, and Clinical Research Center, National Hospital Organization(NHO) Nagasaki Medical Center, Omura, Nagasaki, Japan

Minoru Nakamura      Department of Hepatology Nagasaki University Graduate School of Biomedical Sciences, and Clinical Research Center, National Hospital Organization(NHO) Nagasaki Medical Center, Omura, Nagasaki, Japan

Shinji Shimoda      Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Communicating author: Hiromi Ishibashi, M.D.

Professor, Department of Hepatology Nagasaki University Graduate School of Biomedical Sciences, Director General, Clinical Research Center, National Hospital Organization(NHO) Nagasaki Medical Center and

Kubara 2-1001-1, Omura, Nagasaki, 856-8562, Japan,

TEL: +81-957-52-3121, FAX:+81-957-53-6675, e-mail: hiishibashi-gi@umin.ac.jp

**Abstract** (100 words)

The liver is an organ consisting of the largest reticulo-endothelial cell network in the body and playing an important role in host defense against invading microorganisms. The organ is comprised of parenchymal cells and many different types of non-parenchymal cells, all of which play significant role. Even biliary epithelial cells are not only the target in autoimmune liver diseases but also have central role in orchestrating several immune cells involved in both innate and acquired immunity. Tissue damage caused by various agents results in fibrosis, inflammation, necrosis, fibrosis and eventually distortion of normal hepatic architecture, cirrhosis, and functional deterioration.

**Key Words:** 5

innate immunity, Kupffer cells, cytokine, chemokine, Toll-like receptor

## Introduction

The liver has a particularly intriguing immunological milieu consisting of a largest reticulo-endothelial cell network in the body and being a major source of many components of the innate immune response including acute phase and complement proteins as well as inflammatory cytokines and chemokines. The organ is also a significant site of immune-mediated damage initiated by infectious, autoimmune and malignant stimuli. Recent studies have demonstrated that the liver is also an important site of innate immune system. The innate or natural immune system is the first rapid line-defense against environmental threats such as microbial infection and physical or chemical injury. Sequential activation of innate and adaptive immune response is crucial for elimination of microorganisms and for immune response orchestrated by dendritic cells linking innate and adaptive arms of immune system. Unique repertoires of dendritic and lymphoid cells including NKT cells and regulatory T lymphocytes modify the immune response in the liver. Non-immune cells of the liver including endothelial cells, hepatocytes and biliary epithelial cells also contribute to local immunological potential. All of these elements play roles, together and independently, determining the outcome of immunological stimulation within the liver. In addition, immune response upon exposure to exogenous or autogenic agents varies depending on the host genetic backgrounds. The genetic basis of immune response will offer new approaches to understanding the pathophysiology, diagnosis and management of patients with liver diseases.

## Liver architecture

The liver is the largest organ comprising about 1/50 of the adult body weight. Structurally and histologically, the liver can be divided into 5 tissue systems: 1) vascular system, 2) hepatocytes and hepatic lobule, 3) hepatic sinusoidal cells, 4) biliary system, and 5) stroma. The organ is composed of many different cell types which are divided into parenchymal cells (hepatocytes) and nonparenchymal cells (Table 1). It has been estimated that the hepatocyte population accounts for approximately 78% of the liver tissue volume, while non-parenchymal cells constitute about 6.3% in which about 2.8% are endothelial cells, 2.1% Kupffer cells, and 1.4% hepatic stellate cells. The extracellular space represents approximately 16% of the liver tissue volume [10].

### *Vascular system*

The liver receives portal blood enriched with nutrients absorbed by intestine from splanchnic circulation via portal vein. The portal blood also contains substances secreted by pancreas, intestine and spleen. Hepatocytes take up, metabolize, biotransform and store a great variety of incoming substances. They also de novo synthesize and secrete substances to other organs in the body. The role of the liver is to provide appropriate amounts of solutes needed for adequate functioning of distant organs such as brain, heart, and kidneys. The interaction between blood and liver cells occurs at the level of the liver cell plate. In addition to the blood supply by portal vein, the liver is also perfused by hepatic artery which carries blood with a high oxygen content. This completes a perfusion circuit encompassing the splanchnic-sinusoidal-systemic circulation. There is another circuit to which the liver actively contributes, the entero-hepatic circulation.

### ***Hepatocytes and hepatic lobule***

The hepatic lobule is the structural and functional unit of the liver (Fig. 1) [53]. It consists of a roughly hexagonal arrangement of plates of hepatocytes which extend forming liver cell plates of 1-cell-thick by 15-25 hepatocytes in length. Between the two cell-plates, blood flows from the portal tract to the terminal hepatic venule, forming so called "sinusoid". All the hepatocytes, seems to be apparently homogeneous by light microscopy. Though, there are some functional differences between periportal hepatocytes which locate closer to portal venule and centrilobular ones located closer to central hepatic venule [9]. The portal tract contains a portal venule, a hepatic arteriole, and bile ducts. Blood flows from the portal vein into hepatic sinusoids, perfuses the liver cell plate and flows out into the hepatic venule in the central acinus reaching systemic circulation.

Two structural characteristics are critical for liver functions to be accomplished: (a) hepatocytes located in different positions between the portal tract and the hepatic venule express different genes and attain distinct functional capabilities, and (b) given this functional compartmentation, the sequential perfusion of hepatocytes in the liver cell plate, from portal to hepatic venule, allows progressive qualitative modification of the sinusoidal blood composition as it traverses the liver.

### ***Hepatic sinusoidal cells***

Nonparenchymal cells encompass endothelial cells, Kupffer cells, hepatic stellate cells (or Ito or fat-storing cells), and Pit cells, all of which are located in sinusoids and called as "hepatic sinusoidal cells" [69]. Endothelial cells form the

walls of the hepatic sinusoids (Fig. 2). The extended processes of the endothelial cells have pores or fenestrations through which solutes can apparently move freely into the perisinusoidal space of Disse. Alcoholics or cirrhotics who have developed liver fibrosis show disturbances in solutes exchange between blood and hepatocytes due to loss of the endothelial cell fenestrations concomitantly with the appearance of endothelial cell basal membranes. Kupffer cells are intravascular tissue macrophages which remove relatively large particles from the circulation, while endothelial cells take up rather small particles. Hepatic stellate cells (or Ito or fat-storing cells) are responsible for the storage of vitamin A and play a major role in the development of hepatic fibrosis in response to injury [17]. Pit cells which account for a small proportion of the non-hepatocyte liver cells are natural killer cells located beneath endothelial cells and fibroblasts.

### ***Biliary system and biliary epithelial cells***

Hepatocytes secrete bile into the bile canaliculi. Their flow is parallel to the sinusoids, but is in opposite direction to the blood flows (Fig. 3). Via biliary secretion, the liver excretes substances in feces and participates in intestinal functions such as intestinal absorption of fats by supplying bile acids. At the ends of the bile canaliculi, bile flows into bile ducts, which are true ducts lined with epithelial cells. Biliary cells form conduits (biliary system) carrying bile into the gall bladder and small intestine with bile flowing from hepatocytes near the hepatic venule to portal tract bile ducts. Bile duct cells also contribute to bile formation (ductular component of bile formation). Biliary epithelial cells represent about 3.5% of the liver nuclear population.

## **Distortion of normal hepatic architecture: Cirrhosis**

Cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by dense fibrous scar tissue as well as regenerative nodules formation which result in widespread distortion of normal hepatic architecture. It is most commonly caused by hepatitis B and C, alcohol-induced liver injury, autoimmune liver diseases and fatty liver disease but may have many other possible causes and be cryptogenic in some cases.

Loss of liver tissue due to injury results in fibrosis, regeneration and hyperplasia of liver cells and arterial growth (angiogenesis) induced by growth regulators which include cytokines and hepatic growth factors; e.g., hepatocyte growth factor, epithelial growth factor, transforming growth factor- $\alpha$ , tumor necrosis factor. Hormones, including insulin, glucagon, and change of intrahepatic

blood flow patterns determine localization and peculiarities of nodules formation.

Portal hypertension is the most common complication of cirrhosis. Angiogenesis produces new vessels within the fibrous sheath that surrounds nodules. These new vessels connect hepatic artery and portal vein to hepatic venules, thereby restoring intrahepatic circulatory pathways. Such interconnecting vessels provide relatively low-volume, high-pressure venous drainage and as a result portal vein pressure increase. Such distortions in blood flow contribute to portal hypertension.

Progressive loss of hepatic architecture impairs hepatic function, leading to hepatic insufficiency which manifest as coagulopathy, renal failure, and hepatic encephalopathy. Hepatocellular carcinoma frequently complicates cirrhosis, particularly cirrhosis resulting from chronic hepatitis B and C.

## **Liver cells in innate immune response**

The liver has a number of important functions in systemic and local host defense including both innate and adaptive immunity, and inflammatory reaction. The organ is perfused with antigen-rich blood from the gastrointestinal tract, cytokine-rich blood from the spleen and oxygen and metabolite-rich blood from the systemic artery through a network of sinusoids. The parenchymal cells (hepatocytes) secrete acute phase proteins such as C-reactive protein, anti- $\alpha$ 1-antitrypsin, ceruloplasmin or haptoglobin in response to IL-6 secreted from Kupffer cells, thus controlling systemic and local inflammatory reactions. Each of non-parenchymal cells plays important role in normal physiology and homeostasis, and also participates in systemic as well as in local inflammation and immune response [39].

Innate immunity can detect infection through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) that recognizing specific structures, called pathogen-associated molecular patterns (PAMPs) that are expressed by invading pathogens [21]. There are many different cell types in the liver which express a variety of TLRs: parenchymal cells and non-parenchymal cells which include biliary epithelial cells, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, hepatic dendritic cells, NK cells, and NKT cells [56,57]. TLRs are the key components of the innate immune system, which activate multiple inflammatory pathways and coordinate systemic defense against pathogens. In addition to TLRs, cytoplasmic pattern recognition receptors, such as nucleotide-binding oligomerization domain (NOD)-like receptors and the RNA

helicase family can detect microbial components that enter the cell's cytoplasm and induce innate immunity [34]. The best defined PAMPs include LPS found on gram-negative bacteria and peptidoglycan found on gram-positive bacteria.

### ***Kupffer cells***

Kupffer cells reside within the lumen of the liver sinusoids, therefore, they are the first cells to be exposed to materials absorbed from the gastrointestinal tract [46]. These cells are resident macrophages of the liver and constitute 80~90% of the tissue macrophages presenting in the body. Kupffer cells are the principal liver cells for phagocytosis, antigen presentation and production of proinflammatory cytokines. Activation of Kupffer cells by pathogenic agents results in release of inflammatory mediators, growth factors, and reactive oxygen species (ROS) [8]. This activation appears to be required for the normal physiological functioning of the liver, such as removal of or tolerance to pathogens, as well as occurs in acute hepatic injury [66]. Understanding the role of Kupffer cells in these diverse responses is a key to understanding mechanisms of liver physiology and pathology.

Kupffer cells express a variety TLRs, which participate in liver injury. The TLR4 protein has been detected on Kupffer cells and is likely involved in uptake and clearance of endotoxins, production of cytokines and ROS. Expression of functional TLR2 has also been reported in Kupffer cells and activation of TLR2 leads to production of proinflammatory cytokines [42]. Kupffer cells-derived cytokines play a key role in modulation of other cells. In response to LPS, Kupffer cells produce TNF- $\alpha$  and IL-10, which downregulate receptor-mediated antigen uptake and MHC class II-expression on LSEC and DCs and decrease T cell activation [55]. Kupffer cells are involved in the pathogenesis of liver injury through the release of biologically active substances. Activated Kupffer cells are the major source of inflammatory mediators including cytokines, superoxide, nitric oxide, eicosanoids, and chemokines [52], while in the non-inflamed liver, Kupffer cells secrete anti-inflammatory mediators, such as IL-10, endogenous prostanoids and TGF- $\beta$ [26]. Activated Kupffer cells exposed to pro-inflammatory mediators such as LPS or bacterial products, secrete pro-inflammatory cytokines (TNF- $\alpha$ , IFN- $\alpha$ ), chemokines (MCP-I, IL-8) and reactive oxygen/nitrogen species which contribute to liver injury [61]. Kupffer cells also stimulate profibrogenic response by production of TGF- $\beta$ 1, matrix metalloproteinases, platelet-derived growth factor, and ROS. Since Kupffer cells are the first cell to encounter gut-derived toxins including LPS, they are adapted to less respond to LPS, which is called "LPS tolerance" under the physiological environment.



### ***Hepatic stellate cells (HSCs) and other liver sinusoidal cells***

HSCs are located in the space of Disse and are the principal cellular sources for the production of extracellular matrix proteins, such as collagen type I, III, and IV in the liver. Upon TLR4 ligation, TLR4 signaling induces upregulation of proinflammatory molecules including chemokines (CCL2, CCL3, and CCL4) and adhesion molecules (VCAM-1, ICAM-1, and E-selection). TLR4 signaling also enhances profibrogenic signaling such as TGF- $\beta$  signaling [58].

LSECs express TLR4 and TLR4 signaling induces production of TNF- $\alpha$  and ROS. Innate immune response in LSEC is also modulated by “LPS tolerance”. Other cell types involved in innate immunity in the liver are hepatic dendritic cells (DC), plasmacytoid DC (pDC) and conventional DC (cDC), liver NK cells, and NKT cells. Hepatic DC are professional antigen-presenting cells (APC) in the liver. pDCs are also the principal cells producing IFN- $\alpha$  in response to the ligands for TLR7 and TLR9, while cDC produces TNF- $\alpha$  and IL-6 in response to TLR4, TLR7, and TLR9 [56,57].

Hepatocytes and biliary epithelial cells (BEC) express almost all TLRs at mRNA and protein levels. The ligation of TLR4 and 2 on both hepatocytes and BEC by LPS and lipopeptides, respectively, induces TLR signaling through NF $\kappa$ B and p38/c-jun N-terminal kinase (JNK) resulting in proinflammatory cytokine production such as TNF- $\alpha$ , IL-6, IL-12 [14,56,57,72].

## **TLRs and liver diseases**

The interplay between TLRs and their exogenous and/or endogenous TLR ligands is involved in pathogenesis of various liver diseases [56,57]. Since the liver is constantly exposed to microbial products from the enteric microflora that are carried through the portal circulation, innate immune response to TLR ligands is normally regulated partly through the modulation of TLR signals, namely “liver tolerance” [49,56]. Therefore, a breakdown of this “liver tolerance” and/or excessive activation of TLR signaling may possibly be involved in the pathogenesis of various chronic inflammatory liver diseases such as alcohol-induced liver diseases, nonalcoholic steatohepatitis (NASH), hepatic fibrosis, ischemia/reperfusion liver injury, hepatocellular carcinoma (HCC) and hepatic autoimmune disorders including autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC).

### ***Alcohol-induced liver injury***

Excessive alcohol intake injures intestinal epithelial barrier causing increased intestinal permeability followed by elevated LPS levels in the portal circulation [51]. The LPS then activates TLR4 on Kupffer cells to produce proinflammatory cytokines, such as TNF- $\alpha$ , leading to hepatocyte damage. Chronic alcohol consumption upregulates hepatic TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, and CD14 mRNA expression and sensitizes to the corresponding TLR ligands to enhance TNF- $\alpha$  production [12].

### ***Non-alcoholic steatohepatitis (NASH)***

NASH is characterized by lipid accumulation in hepatocytes and inflammatory cell infiltration, which leads to hepatic fibrosis. In methionine/choline-deficient (MCD) diet-induced animal model of NASH, TLR4-signaling and Kupffer cells play pivotal roles in the pathogenesis of NASH [54]. The loss of TLR4 attenuates hepatic lipid accumulation and hepatic fibrogenic markers, such as collagen  $\alpha$ 1 and TGF- $\beta$ 1 in MCD diet-induced steatohepatitis, indicating the importance of TLR4 in NASH [57].

### ***Hepatic fibrosis***

Hepatic fibrosis results from chronic liver injury, which is caused by a variety of liver diseases including viral hepatitis, autoimmune hepatitis, cholestasis (PBC, PSC), alcohol-induced liver injury and NASH. In these diseases, TLR4 signaling is considered to initiate fibrogenesis by inducing proinflammatory and profibrogenic cytokines of Kupffer cells, which then activate HSCs. Endogenous CpG-DNA from damaged hepatocytes activates HSCs to produce collagen via TLR9, while endogenous DNA also provide a stop signal for migrating activated HSCs as soon as they sense apoptotic DNA [65]. CD14, LBP, TLR4, and Myd88 are critical for hepatic fibrogenesis induced by bile duct ligation and CCl<sub>4</sub> in mice [58]. The injection of TLR3 ligand poly-I:C inhibits HSCs activation mediated by IFN- $\gamma$  from NK cells, which attenuating hepatic fibrosis. Chronic ethanol consumption abolishes this anti-fibrotic effect of TLR3, implying the mechanism by which alcohol induces liver fibrosis [57]. The genetic determinant for liver fibrosis is recently identified on TLR4 SNP [10].

### ***Autoimmune hepatitis (AIH)***

In mouse model of AIH induced by lymphocytic choriomeningitis virus infection, TLR3 but not TLR9 signaling plays a critical role in development of hepatocyte damage and inflammation via IFN- $\alpha/\beta$ , TNF- $\alpha$  and CXCL9 induction [33]. However, there has been no evidence for involvement of TLR signals in the

development of human AIH. Multiple conditions can cause sensitization to endotoxin-induced liver injury including drugs, toxins, metabolic factors, and pathogens. This sensitization via upregulation of TLRs is mediated by bone-marrow derived immune cells but not by liver parenchymal cells [19].

### ***Primary biliary cirrhosis (PBC)***

Monocytes from PBC patients appear to be more sensitive to the ligands for TLR2, TLR3, TLR4, TLR5, and TLR9, producing higher levels of proinflammatory cytokines, particularly IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  [40]. In PBC patients B cells are characterized by high expression of TLR9, namely CpG, stimulating B cells to significant production of immunoglobulin M and anti-mitochondrial antibodies, indicating that occurring hyper responsiveness of B cells via TLR9 accelerate B-cell mediated autoimmunity in PBC [25,44]. The increased expression of TLR3 and type 1 IFN mRNA is found in both the portal tract and parenchyma of PBC-diseased livers derived from early stage PBC patients, indicating the involvement of TLR3-type 1 IFN signaling pathway in the pathogenesis of PBC [62]. The marked increase of TLR3 proteins in small bile ducts of PBC-diseased liver indicate the involvement of TLR3 in pathogenesis of the bile duct damage in PBC, although the real endogenous or exogenous ligand for TLR3 is still unknown in PBC (Fig. 4) [47]. The expression of TLR4 is also increased in PBC-diseased livers [64]. These observations strongly indicate the involvement of TLR-signals in pathogenesis of PBC.

### ***Primary sclerosing cholangitis (PSC)***

Primary sclerosing cholangitis (PSC) is characterized by progressive inflammation and fibrosis of the medium to large sized hepatic bile ducts. High frequency of anti-BEC antibodies presence is found and the binding of anti-BEC antibodies to BECs induce production of proinflammatory cytokines and upregulation of TLRs. BECs expressing higher levels of TLR4 and TLR9 respond to their ligands interaction by production of higher levels of inflammatory cytokines, thus leading to destruction of BECs in PSC [7,24].

In addition to the liver diseases mentioned above, TLR signaling is also considered to be involved in the pathogenesis of ischemia/reperfusion liver injury, liver regeneration and development of HCC.

In conclusion, adequate strength of TLR signaling induces “beneficial” responses, such as microorganism clearance, regeneration, protection from cell death, and adjuvants for vaccination, whereas excessive TLR signaling triggers

“harmful” responses, such as suppression of regenerative responses, chronic inflammation, necrosis, fibrosis, and induction of autoimmune liver diseases [50]. In order to identify the molecular target for the treatment of liver diseases, further studies are needed to clarify the role of innate immunity in the pathogenesis of these conditions.

## Liver cells in hepatic inflammation

In the course of hepatic inflammation, where hepatocytes are the main target of immune-mediated destruction, non-parenchymal liver cells contribute to pro-inflammatory and/or immuno-modulatory functions. With distinct mode of actions, i.e. secretion of lymphocyte chemotactic factors, ability to support adhesion and to promote onward migration, antigen presentation, and T cell instruction, these cells exert substantial influence on inflammatory settings, as well as on basal normal state, where continual immuno-surveillance is in operation by professional immune cells.

### *Hepatocytes*

As hepatocytes are indeed the major cell type in the liver, they might represent primary modulators of hepatic immunity, especially in the setting of chronic liver injury, where non-injured hepatocytes in close proximity are predisposed to inflammatory mediators as bystanders. A line of evidence may support this idea. 67. Wiegand et al [67] recently demonstrated that MHC-II expressing hepatocytes induced Th2-biased differentiation of uncommitted CD4+ T cells, and that suppressed the ability of previously differentiated Th1 to secrete IFN- $\gamma$  in vitro. Accordingly in vivo, they found that MHC-II expression by hepatocytes was associated with impaired IFN- $\gamma$  response and impaired lymphocytic choriomeningitis virus clearance [67]. MHC-II expressing hepatocytes in inflamed milieu may have strong influence on the chronicity of hepatitis, by instructing infiltrating CD4+T cells to differentiate into a less inflammatory phenotype [67]. Application of immuno-modulatory properties of hepatocytes is still challenging. Recent report clearly described that ectopic expression of neural autoantigen myelin basic protein (MBP) in the liver, either in liver-specific MBP transgenic mice or in transient gene transfer in vivo, induced protection from autoimmune necro-inflammation in a mouse model of multiple sclerosis, via generation of MBP-specific CD4+ CD25+ Foxp3+ Tregs [38].

### ***Sinusoidal endothelial cells***

Lymphocyte recruitment to the liver, especially within the hepatic sinusoids, is characterized by special features: in addition to the classical endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), other adhesion receptors appear to play more specific roles for lymphocyte recruitments to hepatic sinusoids [59]. These non-classical adhesion molecules in the LSEC include certain scavenging receptors, such as mannose receptor and common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1), VAP-1, a 170kDa homodimeric glycoprotein that has monoamine oxidase activity, and CD44. CXCL9-11 and CXCL16, chemokines, secreted not only by LSEC but also by inflamed cholangiocytes and hepatocytes, and subsequently presented on sinusoidal endothelia, are also required for recruitment/adhesion of lymphocytes and transmigration across LSEC [59].

LSEC are specialized organ-resident APC, contributing to peripheral immune tolerance. With scavenger activity, they have been reported to have capacity to present exogenous antigens on both MHC-II and MHC-I molecules to CD4+ or CD8+T cells, respectively. Diehl et al [6] recently demonstrated that cognate interaction with naïve CD8+T cells induced tolerogenic maturation of LSEC, characterized by the increased expression of co-inhibitory B7-H1: in contrast to dendritic cells (DC), tolerogenic maturation of LSEC was cell-autonomous, not controlled by exogenous mediators (such as transforming growth factor  $\beta$ , IL-10). Tolerization of CD8+T cells by matured LSEC is a unique, non-deletional process, dependent on B7-H1/programmed death 1 (PD-1) interaction.

### ***Hepatic stellate cells (HSC) and activated liver myofibroblasts (aLMF)***

HSC perform potent APC function for stimulation of CD4+/ CD8+T cells as well as NKT cells. Accordingly, mode of antigen presentation of HSC was demonstrated to be through either MHC-II/MHC-I, or CD1d, the latter of which presents lipid antigens. Additional work in mice clearly confirmed that antigen presentation by HSC promoted protection against *Listeria monocytogenes* infection in the liver. IFN- $\gamma$  induced amplification of APC proteins, along with B7-H1 production, in turn adds immunomodulatory functions to HSC, giving rise to B7-H1 dependent T cell apoptosis in mice. HSC transdifferentiate into aLMF through the interaction with inflammatory cells, resulting in transformation into prominent fibrogenic cells in the liver [68]. Holt et al [18] recently observed that aLMF played a direct role in regulating the infiltration and positioning of

lymphocytes through G-protein coupled receptor-dependent and -independent fashion *in vitro*, apparently relevant in chronic liver disease. In murine models of liver fibrosis, apoptosis of aLMF by macrophages is followed by spontaneous resolution of inflammation. Very recently, senescent aLMF in murine liver were demonstrated to exhibit gene expression profile consistent with reduced secretion of extracellular matrix components, enhanced secretion of extracellular matrix-degrading enzymes, and enhanced immune surveillance [29]. Consequently, senescent aLMF were poised for selective target of natural killer cells, resulting in fibrosis reversion with aLMF clearance. Finally, stellate cell-mediated T cell instruction was proposed by Winau et al [68]. HSC plays a pivotal role in vitamin A homeostasis, storing vitamin A and converting retinol into retinoic acid. Generation of induced regulatory T cells from naïve CD4+T cells in the periphery is dependent on retinoic acid as well as on TGF- $\beta$ . Contrarily, retinoic acid inhibits the TGF- $\beta$ /IL-6-inducing differentiation of inflammatory TH-17 cells [68]. Taken into account that HSC are capable of producing retinoic acid, TGF- $\beta$ , and IL-6, it is plausible to have a scenario that HSC play a vital role in the instruction of regulatory T lymphocytes in the liver.

### ***Biliary epithelial cells***

Biliary epithelial cells that line the intrahepatic biliary tract are the primary site of innate immunity against microbials in bile. We reported that unstimulated conditioned medium of human cholangiocytes *in vitro* were already rich in multiple humoral factors, including ELR+CXC chemokines, such as IL-8/CXCL8, growth-related oncoprotein (GRO), epithelial neutrophil chemoattractant (ENA-78), known chemoattractants with wide range of non-leukocytic activities [27]. Moreover, human cholangiocytes were found to be permissive in TLR2, 4 and 3 dependent pathways *in vitro*, the former of which caused increase in the secretion of IL-6, monocyte chemoattractant protein-1 (MCP-1), and IL-8, upon activation with LPS or LTA, respectively [72].

## **Biliary epithelial cells in immunological inflammation**

Several hepatobiliary diseases, especially PBC and PSC, appear to be mediated by a breakdown of self-tolerance, in which the immune reaction occurs against autoantigens expressed on biliary epithelial cells. PBC is one of organ-specific autoimmune diseases characterized by appearance of autoantibodies specific for epitopes of 2-oxo-acid dehydrogenase multi-enzyme complexes of mitochondria and histologically chronic non-suppurative destructive

cholangitis (CNSDC). Liver-infiltrated mononuclear cells (LMNC) around small bile ducts are believed to destroy BECs. On the other hand, PSC may be mediated by an immune response against endothelial cells of the peribiliary capillary plexus, with secondary reactions to BEC antigens.

### ***Cell populations within and around BECs in PBC***

Cytokines produced by lymphocytes infiltrating around CNSDC are closely associated with the progression of bile duct injury in PBC because BECs bear several cytokine receptors against interleukin (IL)-4, IL-6, interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$  [13]. In addition, BECs themselves also produce TNF- $\alpha$  and IL-6. It has been demonstrated that T cells are predominant cell type of the inflammatory cells within the portal tracts in PBC [28,70]. Moreover, in the development of cholangiopathy, the infiltration of immune cells within the biliary epithelial layer and the direct adhesion between BEC and immune cells are key events leading to cell-mediated cytotoxicity and apoptosis of BECs [15,71]. A number of proinflammatory cytokines are known to be elevated in the local portal tract microenvironment in PBC, contributing to development of chronic inflammatory reaction around the bile ducts, and BECs as well as immune cells actively participate in this inflammatory process. Immunoreactivity and autoimmunity are regulated at least by three different types of CD4+helper T cells; Th1, Th2 and Th17 subsets, principally subdivided by distinctive cytokine production and effector functions. Th1 cells which secrete IL-2, IFN- $\gamma$ , involved in the cell-mediated response provide help to cytotoxic CD8+T lymphocytes, activate natural killer cells, and produce delayed hypersensitivity reactions. In contrast, Th2 clones secrete IL-4 and IL-10, while Th17 cells which produce IL-17 are now considered as commanders for autoimmunity [3]. Presence of predominant Th1 cytokine profile is demonstrated in PBC [2]. Cytokine profiles determined primarily from stimulated peripheral blood and liver-derived T lymphocytes may be misleading for defining a Th1/Th2 cytokine profile in PBC [37,41]. *In situ* hybridization study reveals that IFN- $\gamma$  mRNA-expressing mononuclear cells are more commonly detected primarily around damaged bile ducts in PBC livers than IL-4 mRNA-expressing cells and that the level of IFN- $\gamma$  mRNA expression is highly correlated with the degree of portal inflammatory activity [16]. A recent study has reported that CD8+ and CD4+ (in particular CD4+ CD28-) T cells are markedly increased as intraepithelial lymphocytes within damaged bile ducts in PBC [20]. Since these unique CD4+CD28- T cells proliferate in target tissues of autoimmune diseases and are associated with Th1/Th2 balance in the regulation of spontaneous autoimmune diseases by possessing high expression of IFN- $\gamma$  and

auto-reactive and cytolytic function, CD4+CD28-T cells may be involved in the pathogenesis of auto-immune-mediated bile duct damage of PBC [22].

Additionally with these three CD4+T cells (Th1, Th2 and Th17), regulatory T cells must be mentioned. Regulatory T cells have two types; natural occurring CD4+CD25+Foxp3+T cells and acquired IL-10-producing Th3 cells. Autoimmunity will occur when regulatory T cells decrease functionally or numerically. Recently it is reported that natural occurring regulatory T cells are decreased around CNSDC in PBC [32].

### ***Chemokine and bile ducts***

Leukocyte migration depends on existence of a chemoattractant gradient created by a large family of molecules known as chemokines. Because of their role in inflammation, chemokines and their receptors are known to play a crucial part in directing the movement of mononuclear cells throughout the body, engendering the adaptive immune response and contributing to the pathogenesis of a variety of diseases [4]. The migration and accumulation of leukocytes in the target organs are a critical step in the pathogenesis of autoimmune diseases [43,45].

Chemokines provide a sustained inflammatory bridge between innate and acquired immunity [31]. BECs are one of the sources of chemokines, and BECs spontaneously produce GRO- $\alpha$ /CXCL1, ENA-78/CXCL5, GCP-2/CXCL6, IL-8/CXCL8 (Figure 5) [27,60]. Fractalkine (CX3CL1), consisting of a membrane-bound form and a soluble chemotactic form, is produced by several epithelial cells and is associated with cell adhesion and the chemoattractant for its receptor (CX3CR1)-expressing cells such as CD8+ and CD4+T cells. In PBC, the expression of CX3CL1 is upregulated in injured bile ducts of PBC, and the CD4+ and CD8+ lymphocytes expressing CX3CR1 are found in portal tracts and within the biliary epithelial layer of injured bile ducts.

Defense against invading pathogens by cells of the innate immune system involves the rapid recognition of conserved PAMPs through members of TLR protein family [30,35]. BECs locate in the pathway from the gut to the liver and constitutively express transcripts encoding several TLRs [5,72]. Moreover, the expression levels of TLR-3 and -4 are high in the portal tract in PBC [62,64] and stimulation with TLR3, BECs induce MIP-1 $\alpha$ /CCL3, MIP-1 $\alpha$ /CCL4, RANTES/CCL5, and IP-10/CXCL10.

It is reported that damaged BECs in PBC and, to a lesser degree and frequency, in other hepatobiliary diseases, expressed HLA-DR antigens [48], and that the bile ducts in PBC liver tissues frequently expressed increased levels of CD40 associated with apoptotic BECs [1]. There were also some studies dealing



with the differences of surface markers of BEC from PBC patients by immunohistochemical studies [63]. It was previously found that IFN- $\gamma$  stimulates BECs to express HLA DR [23], and it is now shown that TLR3 ligands stimulate BECs to express HLA DR and CD40, indicating that the cultured circumstance of special condition makes BECs to change to the PBC phenotype. It is now suggested that PBC does not occur as a result by changed BECs, but BECs would change as a result of the developing PBC [60].

(4860 words)

## Abbreviations

AIH : autoimmune hepatitis  
aLMF : activated liver myofibroblasts  
APC : antigen-presenting cell  
BEC : biliary epithelial cells  
cDC : conventional DC  
CLEVER-1: common lymphatic endothelial and vascular endothelial receptor-1  
CNSDC : chronic non-suppurative destructive cholangitis  
DAMPs : damage associated molecular pattern  
DC : dendritic cells  
ENA-78: epithelial neutrophil chemoattractant  
GRO : growth-related oncoprotein  
HSCs : Hepatic stellate cells  
ICAM-1: intercellular adhesion molecule-1  
IFN : interferon  
IL : interleukin  
HCC : hepatocellular carcinoma  
JNK : jun N-terminal kinase  
LPS : lipopolysaccharide  
LMNC : liver infiltrated mononuclear cells  
LSEC : liver sinusoidal endothelial cells  
MBP : myelin basic protein  
MCD : methionine/choline-deficient  
MCP-1: monocyte chemotactic protein-1  
NASH : non-alcoholic steatohepatitis  
NOD : nucleotide-binding oligomerization domain  
PAMPs : pathogen-associated molecular patterns  
PBC : primary biliary cirrhosis  
pDC : plasmacytoid DC  
PRRs : pattern-recognition receptors  
PSC : primary sclerosing cholangitis  
ROS : reactive oxygen species  
TLR : toll-like receptor  
TNF : tumor necrosis factor  
VCAM-1: vascular cell adhesion molecule-1

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## Tables

Table 1. Cells comprising liver

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Parenchymal cells

hepatocyte

Non-parenchymal cells

sinusoidal endothelial cells

Kupffer cells

hepatic stellate cells (Ito or fat-storing cells)

Pit cells (NK cells)

hepatic dendritic cells

NKT cells

biliary epithelial cells

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## Figure Legends

Fig. 1 Blood flows through the sinusoids and empties into the central vein of each lobule.

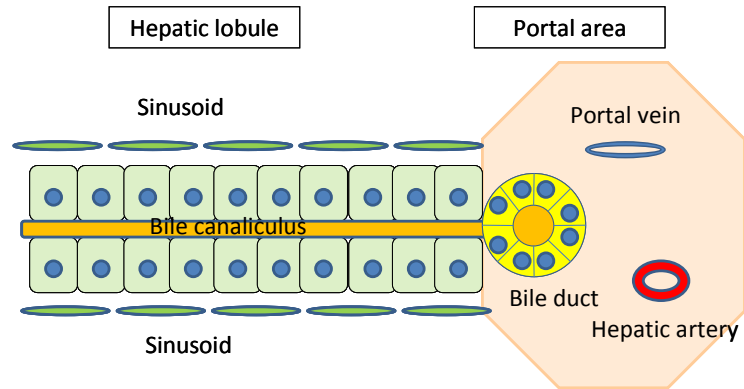


Fig. 2 Hepatocytes secrete bile into the canaliculi

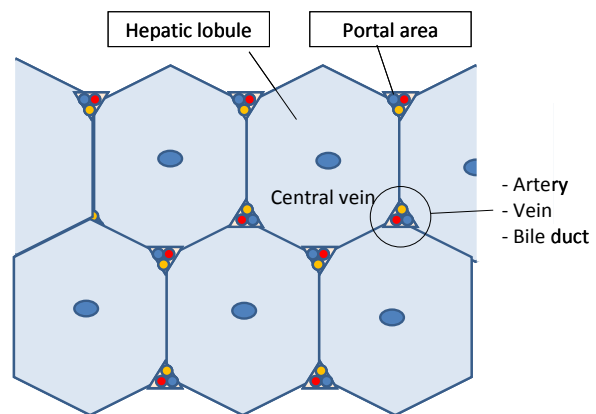


Fig. 3 The hepatic lobule is the structural unit of the liver.

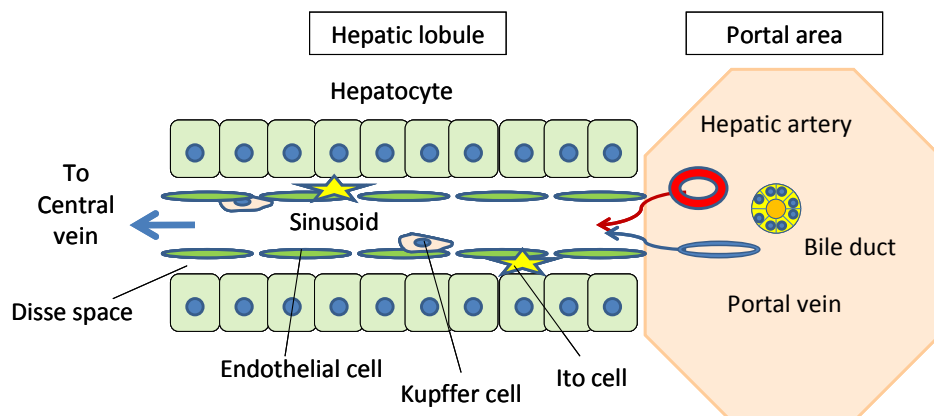


Fig. 4 Expression of TLR3 on intrahepatic biliary epithelial cells in normal and PBC livers.

TLR3 is strongly expressed on intrahepatic biliary epithelial cells in vivo, especially at sites of ductular reactions, in livers from patients with PBC (B), whereas TLR3 is very weakly expressed in normal liver (A). (ref. 47)

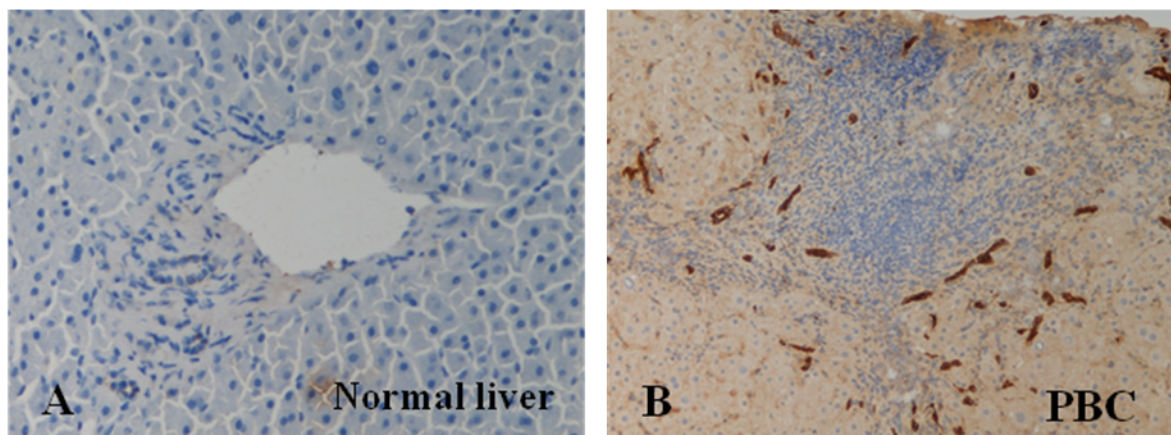


Fig. 5 Cytokines and chemokines produced by cultured BEC. BECs were studied under basal conditions for 48 hours; thence cell-free culture supernatants were analyzed by a protein array kit to evaluate 174 different proteins simultaneously. Unstimulated cells produced detectable amounts of GRO- $\alpha$ /CXCL1, ENA-78/CXCL5, GCP-2/CXCL6 and IL-8/CXCL8 (ref. 27, 60).

