Viral Hepatitis

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Preface

Viral hepatitis remains a major public health problem throughout the world. Hepatitis A virus infects 1–90% or more of the human population, and it varies according to the socioeconomic, sanitary, and public health infrastructure of each country. Hepatitis B virus has infected one-third of the world population, with between 350 and 400 million carriers of the virus, many of whom progress to chronic liver disease and hepatocellular carcinoma. Hepatitis C virus is estimated to have infected 150–200 million people (probably a gross underestimate), with about 80% infected persistently, and this leads to serious sequelae including primary liver cancer. Infection with hepatitis D virus also occurs throughout the world and is hyperendemic in some countries, and hepatitis E is common and epidemic in a number of non-industrialized regions, with increasing evidence of zoonotic spread and sporadic infection in many countries.

Progress on all aspects of viral hepatitis is remarkably rapid, with many thousands of published accounts of original studies, and the mountain of new information is often bewildering and may be difficult to access. The pressing need for a fourth edition became clear, and the text has been revised and updated. The chapter on the history of hepatitis has been omitted (which is somewhat unfortunate because the future evolves from the past) in order to provide space for several new topics.

The fourth edition of *Viral Hepatitis* is designed to include a balanced and carefully distilled account of the more recent advances in this field written by a constellation of internationally recognized experts from many countries. We acknowledge their outstanding contributions, including those made by our two new co-editors, Professor Anna Lok and Professor Stephen Locarnini.

We hope that the book will prove useful to virologists, immunologists, specialists in infectious diseases, hepatologists, gastroenterologists, and, of course, public health and occupational health physicians and aspiring scientists. It is a book for those addressing the management and prevention of an important common infection and its associated liver diseases, which affect a large proportion of the world's population.

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and
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Introduction to Liver Biology

Chapter 1
Liver regeneration and fibrosis

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Summary

In a healthy adult liver, the rate of cell turnover is very low. Following acute liver injury, restoration of parenchymal mass is achieved by proliferation of normally mitotically quiescent hepatocytes. However, chronic liver injury results in the loss of this proliferative capacity of the hepatocytes, as increasing numbers of cells become senescent. In this situation, there is activation of hepatic progenitor cells (HPCs) from within the intrahepatic biliary tree. These bipotential cells are capable of supplying biliary cells and hepatocytes. In animal models, there is some controversy regarding the relative contribution to parenchymal regeneration from these two compartments, but human studies are compatible with the suggestion that as the severity and chronicity of the liver injury increase, immature progenitor cells contribute more to regeneration than mature hepatocytes. We are now beginning to understand the molecular signals and niche requirements that govern their cell fate. Alongside the parenchymal regeneration in chronic liver injury, there is a stereotypical wound-healing response with activation of hepatic stellate cells (HSCs) into scar-forming myofibroblasts and deposition of collagen. This change in the extracellular matrix (ECM) affects the regenerative capacity of the liver, and excess scar tissue can impair liver regeneration from either hepatocytes or HPCs.

Introduction

Normally the liver has a low level of hepatocyte turnover, but in response to modest hepatocyte loss, a rapid regenerative response occurs from all cell types in the liver to restore organ homeostasis (comprehensively reviewed in [1, 2]). More severe liver injury, particularly chronic repetitive injury (e.g., chronic viral hepatitis), is often associated with hepatocyte replicative senescence. This activates facultative stem cells of biliary origin that give rise to cords (the "ductular reaction") of bipotential transit-amplifying cells (named oval cells [OCs] in rodents and HPCs in humans) that can differentiate into either hepatocytes or cholangiocytes. Moreover, the major primary tumors of the liver (hepatocellular carcinoma [HCC] and cholangiocarcinoma [CC]) invariably arise in a setting of chronic inflammation that is accompanied by both hepatocyte regeneration and ductular reactions, and while it seems that the founder cell of CCs is a proliferating cholangiocyte, the morphological heterogeneity often observed in HCCs suggests that these tumors can arise from bipotential HPCs as well as more mature hepatocytes. HCCs also appear to possess subpopulations of cancer stem cells, which are responsible

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for continued tumor propagation and metastasis, and a number of phenotypic markers have been proposed for their identification.

Liver turnover and regeneration

Kinetic organization

The healthy liver in adults is mitotically quiescent with levels of proliferation suggesting a turnover time for hepatocytes in excess of a year. Nevertheless, there is still considerable debate as to how the liver is organized. Most studies concur that hepatic stem cells are located in the periportal region; for example, in the mouse, bromodeoxyuridine (BrdU) pulse-chase analysis following two rounds of acetaminophen intoxication has observed so-called label-retaining cells (LRCs), considered to be slowly dividing progenitor cells, as both interlobular cholangiocytes and peribiliary hepatocytes [2]. In humans, $EpCAM^{+}NCAM^{+}$ cells in the periportally located canals of Hering have been identified as putative HPCs and it is suggested that there are eight maturational lineage stages moving from the periportal (progenitor) region to the perivenous region.

An important question remains: is the liver organized like the intestine, with a unidirectional flux of cells that are "born" in the portal area and migrate along a trajectory leading to the hepatic veins? This so-called streaming liver hypothesis was first advocated by Gershom Zajicek and colleagues (reviewed in [2]); examining the location of labeled hepatocytes in intact adult rat livers over time after a single injection of tritiated thymidine, they suggested that hepatocytes moved at a speed of over 2 μm/day from the periportal region to the central vein. A recent murine study by Furuyama and colleagues [3] (reviewed in [4]) appears to support the idea that hepatocytes migrate centrifugally from portal areas (Figure 1.1). They examined the expression of the embry-

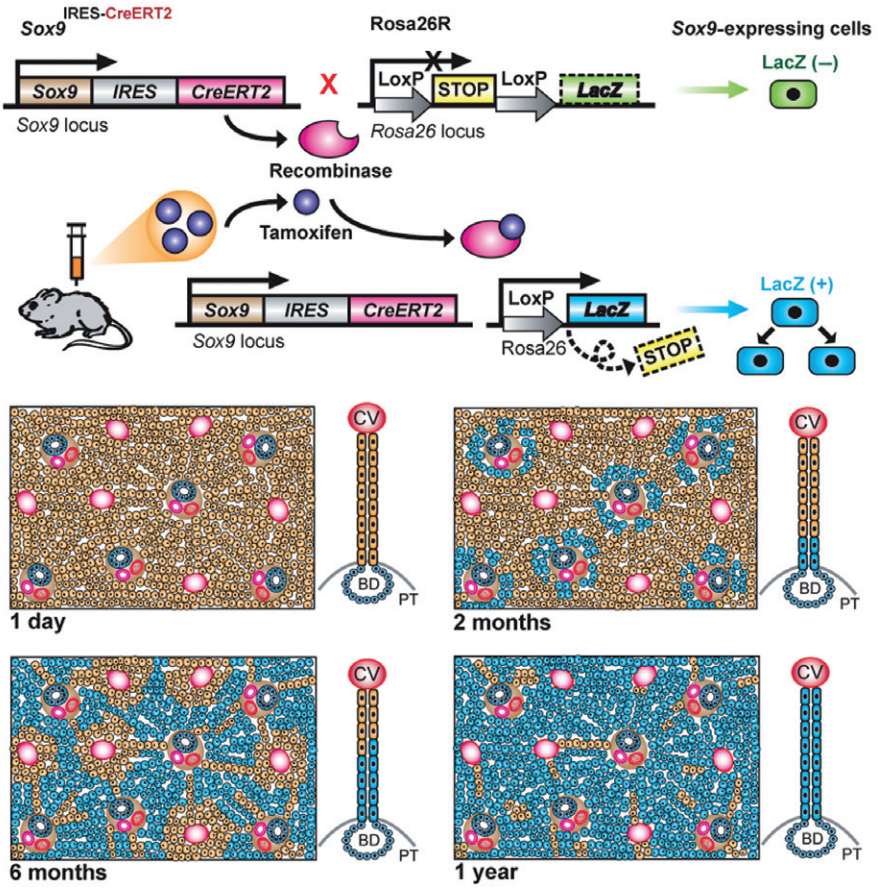

Figure 1.1 Top: Strategy of the genetic lineage-tracing study employed by Furuyama *et al.* [3] using tamoxifen-induced Cre-mediated cell tracking using Sox9IRES-CreERT2; Rosa26R mice. Bottom: Schematic illustrating the spread of X-gal staining after 8-week-old mice were injected with tamoxifen. After one day, only intrahepatic bile duct cells are labeled, but later X-gal-positive hepatocytes gradually spread from the portal tracts to the central veins, thus supporting the streaming liver hypothesis. See Alison and Lin [4] for further details. (Source: Alison and Lin. Hepatology 2011, 53: 1393–1396 [4]). (Color plate 1.1)

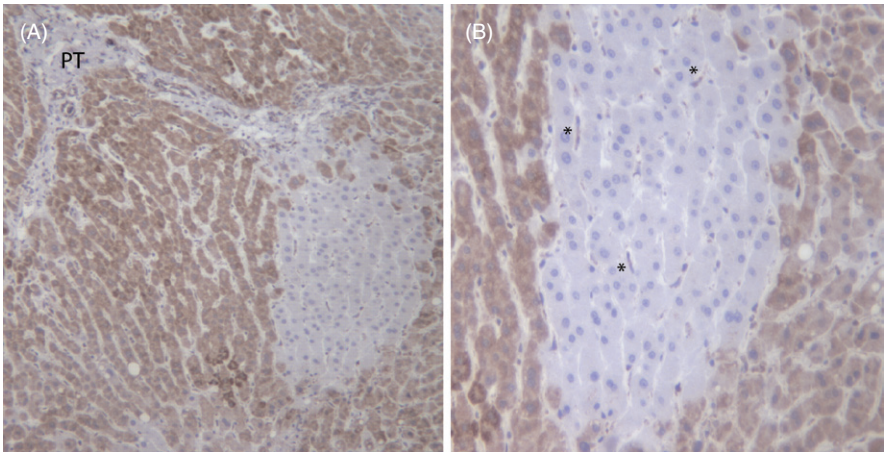

Figure 1.2 (A) A single cytochrome c oxidase (CCO)–deficient patch, appearing to emanate from the portal tract. (B) High-power magnification illustrates that within the patch there are CCO-positive sinusoid-lining cells (asterisks) indicative of different cells of origin from hepatocytes. See Fellous *et al.* [7] for further details. (Source: Fellous TG *et al.* Hepatology 2009, 49: 1655–1663 [7]). (Color plate 1.2)

onic transcription factor Sox9 in the liver. In human liver, immunohistochemistry identified interlobular bile duct cells as Sox9-expressing cells, and a similar pattern was seen in adult mice when a reporter gene, either enhanced GFP or LacZ, was knocked into the *Sox9* locus. Adopting tamoxifen-inducible genetic lineage tracing from the *Sox9* locus, detecting Sox9-lineage cells by X-gal staining, Furuyama *et al.* [3] found that X-gal positivity spread out from the portal areas toward the hepatic veins until the majority of hepatocytes were labeled within 8–12 months. Thus, the paper suggested that indeed cells "streamed," but more importantly hepatic replacement was from cytokeratin 7 (CK7)–Sox9-positive biliary cells, identifying cells within the biliary tree as drivers not only of hepatocyte replacement when regeneration from existing hepatocytes is compromised (discussed further in this chapter) but also of normal hepatocyte turnover. However, there is controversy as other studies of mice have failed to find evidence for the normal liver parenchyma being "fed" from the biliary system. Carpentier *et al.* [5] also employed lineage labeling in mice, this time from Sox9-expressing ductal plate cells in late embryonic development (E15.5), finding that these cells gave rise to interlobular bile ducts, canals of Hering, and periportal hepatocytes, and that liver homeostasis did not require a continuous supply of cells from Sox9 progenitors. Iverson *et al.* [6] have sought to quantify the dynamics of mouse liver turnover by lineage labeling following activation of an albumin–*Cre* transgene, calculating that 0.076% of hepatocytes had differentiated from albumin-naïve cells over a 4-day period.

In human liver, Fellous *et al.* [7] have identified clonal populations of hepatocytes based upon finding large patches of cells deficient in the mitochondrial DNA (mtDNA)-encoded cytochrome c oxidase (CCO) enzyme, all sharing an identical neutral mutation in the *CCO* gene indicating derivation from a single cell. Significantly, these CCO-deficient patches were all connected to portal areas and had a portal vein–to–hepatic vein orientation (Figure 1.2), suggesting a "streaming" nature but without providing information of whether they are derived from a periportal progenitor cell or an interlobular biliary cell.

Liver regeneration

The regenerative capacity of the liver is impressively demonstrated when two-thirds of the rat liver is surgically removed (a 2/3 partial hepatectomy, or 2/3 PH) and the residual liver then undergoes waves of hyperplasia and hypertrophy to restore preoperative liver mass within about 10 days [1, 2]. After a 2/3 PH in healthy adult rats, all the normally proliferatively quiescent hepatocytes leave G_0 to semisynchronously enter the cell cycle. DNA synthesis is first initiated in the periportal hepatocytes at about 15 hours after PH, with a peak in the hepatocyte DNA synthesis labeling index of ~40% at 24 hours. Midzonal and centrilobular hepatocytes enter DNA synthesis at progressively later times, but the hyperplastic response in hepatocytes is essentially complete by 96 hours, to be followed by a phase of hepatocyte hypertrophy. Elegant labeling studies have identified three groups of regenerative hepatocytes in mice, with all cells dividing at least once, but with the periportal hepatocytes that divide first dividing maybe three or more times after PH.

As might be expected, age has an adverse effect on the response; in old rats (>2 years old), a significant number of hepatocytes do not proliferate after PH,

seemingly becoming reproductively senescent. To maintain liver homeostasis, the nonparenchymal cells (cholangiocytes and endothelial cells) must also expand their numbers, and their cell cycle entry is delayed a few hours behind that of hepatocytes [2].

Molecular regulation of liver regeneration

Numerous cytokines, growth factors, and signaling pathways have been implicated in (1) the initiation (priming) of hepatocytes in order to be responsive to liver mitogens, (2) the proliferative response itself, and (3) the curtailment of the response. The "priming phase" in the first few hours after PH, which is probably instrumental in the G_0 to G_1 transition, is associated with the upregulation of many genes not expressed in the normal liver and is essentially cytokine driven [2], with activation of transcription factors such as activator protein 1 (AP1), nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), and signal transducer and activator of transcription 3 (STAT3) being particularly important. The ultimate cause of cytokine accumulation is unclear, but enteric lipopolysaccharides may be the master regulator of the innate immune response, and liver injury can be associated with a defective intestinal barrier leading to exposure to lipopolysaccharides and complement fragments. Such exposure activates the NF-κB pathway in Kupffer cells, resulting in the production and secretion of interleukin 6 (IL6) that activates the JAK/STAT pathway, leading to the initiation of DNA synthesis in hepatocytes. In mice, complement activation (in particular, C3a and C5a) leads to the recruitment of natural killer T (NKT) cells and the production of IL4 by these cells [8]. IL-4 maintains IgM levels and deposition in the liver, leading to increased C3a and C5a accumulation that in turn stimulates liver macrophages to produce IL6. The cytokine interleukin 1 receptor antagonist (IL1ra) is also important in the early phase of regeneration, reducing inflammatory stress and thus promoting proliferation [9].

The proliferative response itself appears to be driven by a number of growth factors and signaling pathways, including IL6, tumor necrosis factor alpha (TNFα), hepatocyte growth factor (HGF), amphiregulin, stem cell factor (SCF), insulin-like growth factor 1 (IGF1), T3, bone morphogenetic protein 7 (BMP7), Wnt, β-catenin, Hedgehog (Hh), and phosphoinositide-3 kinase (PI3K), although no one factor or pathway appears crucial to the process [2]. Some of these signals are autocrine, and others are paracrine; for example, in mice sinusoidal endothelial cells are involved in hepatocyte regeneration with vascular endothelial growth factor receptor (VEGFR)–dependent upregulation of the transcription factor Id1 leading to the release of hepatotrophic factors such as Wnt2 and HGF [10, 11]. Moreover, it seems that endothelial progenitor cells recruited from the bone marrow after PH provide the richest source of HGF [11]. Hepatic stellate cells (HSCs) also support liver regeneration and are activated by massive upregulation of delta-like 1 homology (Dlk1) that represses *Pparγ* in stellate cells [12]. Regenerative competence in mouse and human also appears to be maintained by activation of telomerase activity in regenerating hepatocytes [13]. Micro-RNAs (miRs) are also involved in regeneration after PH; for example, in mice there is upregulation of miR-21 in the priming phase that targets a proliferation inhibitor facilitating cyclin D1 translation, and downregulation of miR-378 that targets *odc1* messenger RNA (mRNA), ornithine decarboxylase activity being essential for DNA synthesis [14]. In rats after PH, there are also dramatic changes in miRs, with upregulation of 40% of investigated miRs in the priming phase and downregulation of 70% of miRs at 24 hours after PH, presumably facilitating maximal proliferation [15].

Equally important are the molecular mechanisms that curtail the regenerative response, ensuring the liver does not overcompensate for lost mass. Transforming growth factor beta (TGFβ) produced by stellate cells inhibits hepatocyte replication, and several mechanisms are involved in its production. In mice, serotonin acts on 5-HT_{2B} receptors in stellate cells, leading to phosphorylation of JunD via ERK, resulting in recruitment of JunD to AP1 binding sites in the promoter region of the *tgfβ1* gene [16]. The multidomain matrix glycoprotein thrombospondin-1 (Tsp1) is also involved in TGFβ1 production in mice; Tsp1 is expressed by endothelial cells in response to reactive oxygen species (ROS) shortly after PH and binds to latent TGFβ1 complexes, converting them to active TGFβ1 [17]. The IL6 response is negatively regulated through transcriptional upregulation of suppressor of cytokine signaling 3 (SOCS3), but SOCS3 is not crucial for curtailing proliferation, for although SOCS3 knockout mice have higher levels of hepatocyte proliferation after PH than wild-type mice and restore preoperative liver weight 2 days earlier, proliferation stops after 4 days and liver weight does not go above normal [18]. The Hippo pathway seems particularly important for curtailing liver size; the kinases Mst1 and Mst2 (the mammalian orthologs of *Drosophila* Hippo) are responsible for phosphorylating the Yes-associated protein (Yap) at Ser127, the mammalian ortholog of *Drosophila* Yorkie, which is a transcriptional activator of cell cycle proteins such as Ki-67 and c-Myc – phosphorylation blocks its ability to translocate to the nucleus [19]. Thus, overexpression of Yap in mice leads to massive liver weight increases (25% of body weight versus 5% normally) [20], and likewise Mst1 and Mst2 double knockouts also have massive livers and eventually develop HCC [21, 22].

A second tier of regeneration: oval cells and HPCs

Massive acute liver injury, chronic liver injury, or large-scale hepatocyte senescence results in the activation of a reserve or potential progenitor cell compartment located within the intrahepatic biliary system [1, 2]. Replicative senescence can occur in conditions such as chronic hepatitis and fatty liver disease [23]. In humans and mice, the extent of the HPC response is proportional to the degree of parenchymal damage [24, 25]. HPCs are derived from interlobular biliary cells and/or the canal of Hering, and in human liver the canal of Hering extends beyond the limiting plate, even perhaps throughout the proximate third of the lobule [26].

A number of animal models have been described to activate this progenitor response. In rats, a very effective model has been to pretreat the animals with 2-acetylaminofluorene (2-AAF) before performing a 2/3 PH (the 2-AAF/PH protocol) [27]. 2-AAF is metabolized by the hepatocyte's cytochrome P450 (CYP450) system, producing metabolites that form DNA adducts, thus preventing hepatocytes from entering the cell cycle in response to PH. Under these constraints, oval cells or HPCs are activated since they lack the CYP enzymes necessary for 2-AAF metabolism. In the mouse, dietary regimes are often employed including a choline-deficient, ethionine-supplemented diet (the CDE diet) that inflicts hepatocyte damage, or a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) regime that damages cholangiocytes [28]. An oval cell response is also seen when hepatitis B surface antigen (HBsAg-tg) mice (a model of chronic liver injury) are treated with retrorsine, a pyrrolizidine alkaloid that blocks hepatocyte regeneration. This effectively abolishes hepatocyte turnover, resulting in massive oval cell–driven regeneration [29]. The exact location of stem and progenitor cells within the biliary tree is unclear, and it is also unclear if all cells in small-caliber biliary ducts and canals of Hering are capable of giving rise to oval cells, but in the mouse a small subset (3–4%) of antigenically defined biliary cells that express Sox9 give rise to most oval cells in the DDC model [30].

A wide range of markers have been used to identify ovals cells and HPCs (Table 1.1) [31]. Many factors, often produced by cells of a hepatic niche that intimately accompanies the reaction, can influence the oval cell–HPC response. Autocrine and paracrine Wnt signaling is clearly involved in the oval cell or HPC response in mice [28, 32], rats [33], and humans [28, 34, 35]. In the rat 2-AAF/PH model, oval cells display nuclear β-catenin and Wnt1 is essential for differentiation of oval cells to hepatocytes; exposure to Wnt1 small hairpin RNA (shRNA) blocked this differentiation, and oval cells generated an atypical ductular reaction – perhaps as the default position [35]. As oval cells and HPCs are bipotential, what regulates whether they become hepatocytes or cholangiocytes? Boulter and colleagues have described the mechanisms in mice governing these critical cell fate decisions [28]. After biliary cell damage with DDC, the intimate association of myofibroblasts with HPCs facilitated Notch signaling ensuring biliary differentiation in oval cells, in essence recapitulating ontogeny. On the other hand, after hepatocyte damage with the CDE diet, adjacent macrophages in response to engulfing hepatocyte debris were involved in Wnt signaling to HPCs that not only turned off Notch signaling but also specified hepatocytic differentiation in oval cells. On the other hand, in the rat 2-AAF/PH model, Notch1 may be important for hepatocytic differentiation since exposure to a γ-secretase inhibitor delayed the maturation process [36]. HGF signaling is also important for the oval cell response: genetic deletion of *c-met* from oval cells in the DDC model results in a diminished response with decreased hepatocytic differentiation [37]. Moreover, a failure to express stromal cell–derived factor 1 (SDF1) leads to less recruitment of macrophages and associated matrix metallopeptidase 9 (MMP9) secretion that is crucial for oval cell migration and liver remodeling (discussed further in this chapter). Hh signaling is another important pathway, and ligands acting through the receptor Patched (Ptc) on murine oval cells and human HPCs are required for progenitor cell survival [38]. Perhaps most

Table 1.1 Some of the markers used in the identification of oval cells and HPCs in the damaged mammalian liver.

A6 antigen (mouse marker)
ABCG2/BCRP1 (breast cancer resistance protein)
AFP (alpha fetoprotein)
Cadherin 22
CD24 and CD133
Chromogranin A
CK7 and CK19
c-Kit (CD117)
Claudin7
Connexin 43
Dlk1 (Delta-like protein 1)
DMBT1 (deleted in malignant brain tumor 1)
E-cadherin
EpCAM/TROP1 (epithelial cell adhesion molecule)
flt-3 ligand/flt-3
Fn14 (fibroblast-inducible factor 14-kDa protein; TWEAK receptor)
GGT (gamma-glutamyltranspeptidase)
GST-P (placental form of glutathione-S-transferase)
M2-PK (muscle type pyruvate kinase)
NCAM-1/CD56 (neural cell adhesion molecule-1)
PTHrP (parathyroid hormone related peptide)
TACSTD/TROP2 (tumor-associated calcium signal transducer)

Note: Many of these markers are also expressed on normal biliary epithelial cells.

significantly, inflammatory cells produce a range of cytokines and chemokines that initiate the response [2, 32]; SDF1 attracts $CXCR4^+$ T cells, and these cells express TWEAK (TNF-like weak inducer of apoptosis) that stimulates oval cell proliferation by engaging its receptor Fn14, a 14 kDa transmembrane receptor [39]. Tirnitz-Parker and colleagues employed the CDE diet and found that expression of Fn14 is markedly elevated [40]. Fn14 is not a receptor tyrosine kinase, but rather ligand occupancy activates NF-κB signaling as shown by the presence of active (nuclear) NF-κB in a progenitor cell line upon TWEAK stimulation. The early oval cell response to the CDE diet was delayed in Fn14 knockout mice, although interestingly there were comparable numbers of oval cells in wild-type and knockout mice after 3 weeks on the CDE diet. Significantly, recombinant human TWEAK (rhTWEAK) directly stimulated the *in vitro* proliferation of a progenitor cell line in a dose-dependent manner. Other components of the inflammatory response that can stimulate oval cells include lymphotoxin-β, interferon alpha (IFNα), TNFα, and histamine [41]. Resistance to the growth inhibitory effects of TGFβ may allow oval cells to proliferate under conditions inhibitory to hepatocytes [42].

In terms of negative regulators of the oval cell response, the neurofibromatosis type 2 (*Nf2*) gene product Merlin appears critically important [43]. Genetic deletion of *Nf2* leads to massive oval cell expansion and the development of CC and HCC; Merlin appears to control the availability of epidermal growth factor receptor (EGFR) and other growth factor receptors. Progenitor cells reside in a specialized supportive microenvironment known as a niche; not only do oval cells and HPCs have such a niche but also this niche seems to migrate hand-in-hand with the expansion of oval cells. For example, with the CDE diet the activation of stellate cells (upregulation of alpha smooth muscle actin [αSMA] expression) and deposition of collagen precede the oval cell response, suggesting that the extension of the niche is a prerequisite for oval cell expansion [44]. In fact, mouse and rat models of oval cell activation and HPC reactions in humans bear a striking similarity, in terms of both the deposition of ECM (particularly laminin) and cells (macrophages and αSMA^+ myofibroblasts) that accompany progenitor reactions suggestive of a stereotypical niche [45]. Further support for the idea that the ECM adjacent to oval cell reactions is not merely a passive bystander comes from studies of the oval cell reaction in mice that produce mutated collagen I that is highly resistant to MMP degradation [46]; here, a failure to remodel collagen stunts the reaction, seemingly through a failure to establish a laminin-rich progenitor niche. In the 2-AAF/PH model, blocking the activation of stellate cells with L-cysteine was a potent suppressor of the oval cell response, probably related to loss of cytokines such as TGFβ1 and the fibronectin matrix that, among other properties, can concentrate cytokines such as connective tissue growth factor (CTGF) for which oval cells have receptors [47].

Chronic viral hepatitis is, of course, invariably associated with cirrhosis and hepatocyte senescence [48–50], thus activation of HPCs in this setting is common. In the fibrous septae that surround regenerative nodules (RNs), differentiation of CK19-positive HPCs to form buds of intraseptal hepatocytes (ISHs) is often observed [51]. In cirrhosis we observed that RNs are invariably clonally derived (Figure 1.3), suggesting that they are not simply created by fibrotic dissection of the preexisting parenchyma; moreover, they are clonally related to the abutting HPCs (Figure 1.3), and thus have been derived from them [52]. Thus RNs may well represent the further expansion of buds of ISHs.

Stem cells and liver cancer (founders and propagators)

Whereas CCs are believed to arise from either established biliary ducts or HPCs, the origin of HCCs is more problematic. Clearly hepatocytes are the cell of origin of many HCCs in experimental models where tumor yield is directly related to hepatocyte proliferation or where oncogenic transgenes are driven by the albumin promoter. On the other hand, HPC activation is commonly seen in models of hepatocarcinogenesis and invariably accompanies chronic liver damage in humans, thus making it quite likely that HPCs are the founder cells of many HCCs [53]. An origin of HCCs from HPCs is often suggested because many HCCs contain an admixture of mature hepatocyte-like cells and cells resembling HPCs [1]. If tumors do arise from HPCs, then this indicates a block in HPC differentiation, a process that has been termed "stem cell maturation arrest" [54]. This hypothesis is supported by the fact that murine HCCs induced by a CDE diet have a mixture of neoplastic phenotypes recapitulating stages in normal development, suggesting intermediate states between bipotent oval cells and hepatocytes [55]. Likewise in humans, four prognostic HCC subtypes have been identified equating to liver cell maturational steps [56]. The poorest prognostic groups had a significant proportion of either $EpCAM^+AFP^+$ cells (hepatoblast-like) or $EpCAM^-AFP^+$ cells (HPC-like), whereas those with $EpCAM^-AFP^-$ cells (mature hepatocyte-like) or $EpCAM^+AFP^-$ cells (cholangiocyte-like) had a better prognosis. Gene expression profiling has identified a subset of HCCs with a profile consistent with an origin from HPCs, and these patients have a poor prognosis [57]; moreover, counting of CK19-positive cells in HCC can identify a poor-prognosis group [58] that may be related to an enhanced epithelial-mesenchymal transition (EMT) [59].

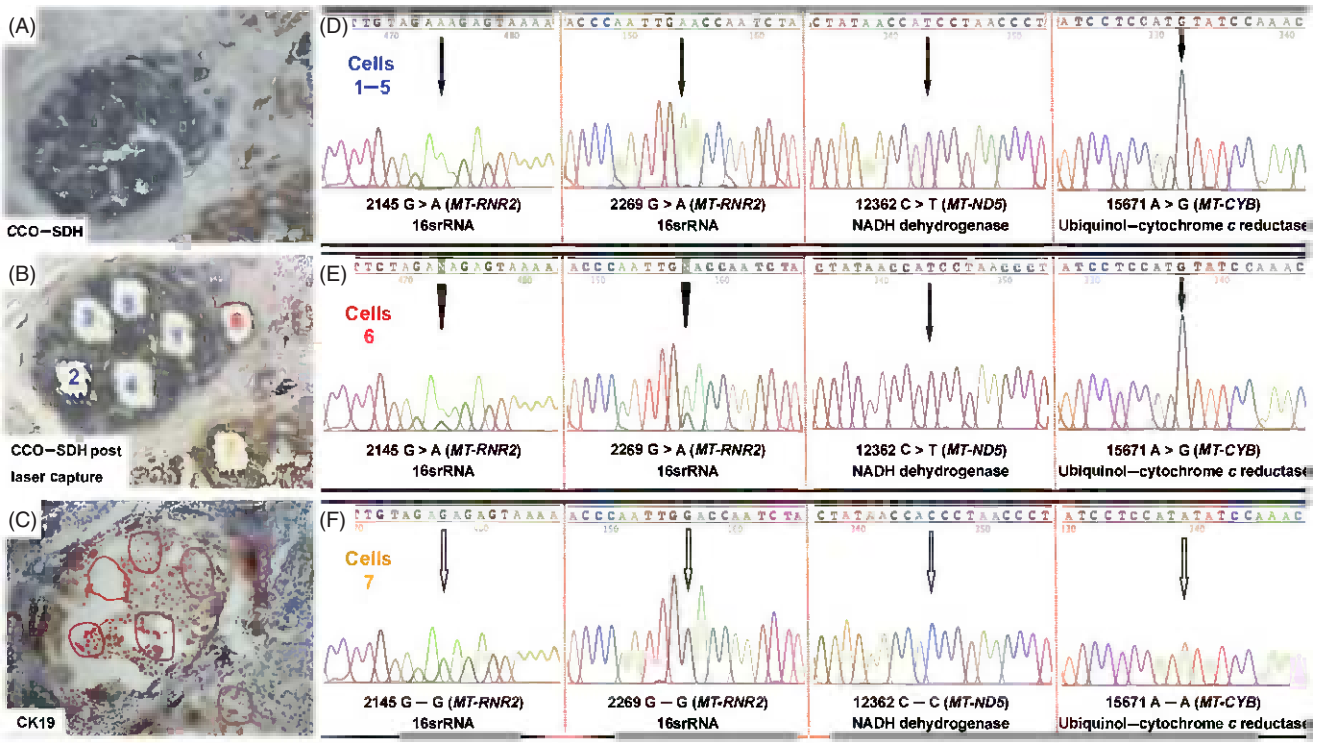

Figure 1.3 Mitochondrial DNA genotyping indicates that regenerative nodules can be derived from CK19-positive HPCs. (A) An entirely CCO-deficient nodule (stained blue for succinate dehydrogenase activity). (B) Five groups of cells (1–5) from the same CCO-deficient nodule; cells (6) from the adjacent CCO-deficient ductular reaction, confirmed by CK19 IHC on the next serial section (C, brown staining), and cells (7) from the CCO-positive nodule were laser capture-microdissected, and the entire mitochondrial genome was sequenced. (D) Cell areas 1–5 all contained four different transition mutations: 2145G>A, 2269G>A, 12362C>T, and 15671A>G (black arrows). (E) Cell area 6 from the abutting CCO-deficient ductular reaction had exactly the same mutations. Heteroplasmy was detected at locations 2145 and 2269 (arrowheads), while the mutations at locations 12362 and 15671 were homoplasmic (black arrows). (F) Cell area 7 from the CCO-positive nodule had no mutation (white arrows). See Lin *et al.* [52] for further details. (Source: Lin WR. *et al.* Hepatology 2010, 51: 1017–1026 [52]). (Color plate 1.3)

A detailed discussion of cancer stem cells (CSCs) in HCC is beyond the scope of this chapter, but a number of phenotypic markers have been proposed for their isolation including CD13, CD90, CD133, ALDH activity, and the side population [60]. As in other organs, HCC CSCs seem relatively resistant to therapy, and strategies to either reduce ABC transporter function [61–63] or induce differentiation [64] have increased CSC sensitivity. For a detailed discussion, see [65].

Liver fibrosis

Whatever the mode of chronic liver injury, a stereotypical wound-healing response occurs that results in a series of cellular and extracellular matrix changes, an increase in collagen deposition, and a disturbance in the liver architecture. In its extreme form, this results in the development of cirrhosis with gross architectural disturbance, nodule formation, heavy scarring, and vascular changes, eventually resulting in liver failure or the development of HCC.

Following liver injury there are a number of cellular responses that are key to the fibrotic response, including hepatocyte injury, and hepatic macrophages and endothelial cells are activated [66]. The cells that are primarily responsible for the deposition of ECM are the αSMA-positive myofibroblasts that are formed principally from the activation of the HSCs. Hepatic myofibroblasts are proliferative and contractile cells that directly secrete collagen matrix, and they have several important paracrine mechanisms that increase the profibrotic environment. Proliferation of hepatic myofibroblasts is stimulated by a number of mitogens, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiotensin II, VEGF, and thrombin. PDGF is a very potent mitogenic stimulus and is released by activated Kupffer cells, sinusoidal endothelial cells, platelets, and activated myofibroblasts

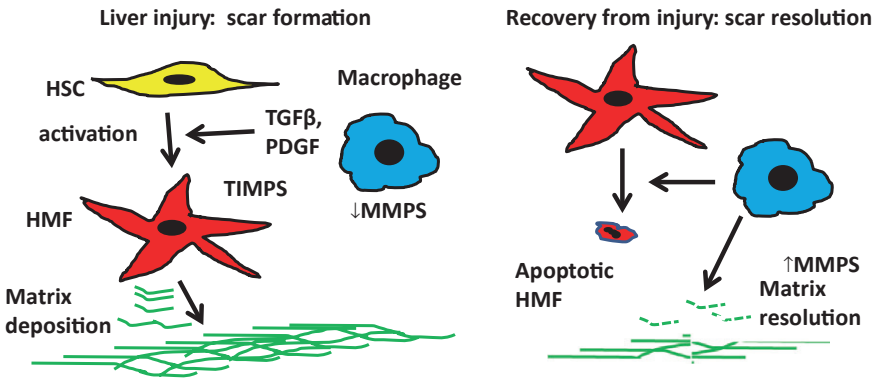

Figure 1.4 During liver injury the hepatic macrophages help, along with other factors, to stimulate quiescent fat-storing hepatic stellate cells (HSCs) into activated hepatic myofibroblasts (HMFs). The activated HMFs deposit collagen scar and secrete tissue inhibitors of metalloproteinases (TIMPS), which inhibit the degraders of scar matrix, the matrix metalloproteinases (MMPs). If the cause of liver injury is removed (e.g., viral eradication), then a recovery phase commences and, to a variable degree, the quantity of liver fibrosis lessens. In this phase, HMFs reduce their TIMP secretion, and the hepatic macrophages secrete MMPs that aid the degradation of scar and promote the apoptosis of HMFs. (Color plate 1.4)

in an autocrine manner. Importantly, myofibroblasts also secrete tissue inhibitors of metalloproteinases (TIMPs).

There has been increasing recognition that wound healing is a dynamic process involving both matrix deposition and degradation, and the balance between the factors that promote scar deposition and those that promote resolution determines the eventual degree of fibrosis within the liver. In this regard, during the formation of fibrosis there is a high level of TIMP in the liver and lower levels of MMPs. This balance reverses with the cessation of liver injury when there is active remodeling of the scar tissue in the liver. HSCs express TIMPs, which results in inhibition of matrix-degrading MMP activity. Therefore, HSCs and myofibroblasts affect the balance of matrix secretion and degradation to favor the accumulation of scar, and they are direct secretors of collagen matrix.

The hepatic macrophages are important orchestrators of the wound-healing response in the liver; they phagocytose apoptotic debris, signal to the HSCs and myofibroblasts, and secrete enzymes capable of matrix degradation. During chronic liver injury, the hepatic macrophages signal to the myofibroblasts via the secretion of TGFβ1 to promote scar deposition. Conversely, following the cessation of liver injury, when there is an active reduction in the number of activated myofibroblasts and fibrosis, the hepatic macrophages are important in promoting the degradation of scar tissue (see Figure 1.4). As mentioned in this chapter, both the myofibroblasts and hepatic macrophages are also important in the liver's regenerative response, underlining how the liver's wound response (fibrosis and its resolution) is closely linked to the epithelial regenerative response. Indeed, experimental studies have shown that the degradation of collagen scar matrix is required to enable the development of a ductular reaction. The implications are clear that strategies to minimize or even reverse liver fibrosis will likely also have an effect upon liver regeneration.

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Chapter 2
Hepatic immunology

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Summary

The immune system is an integral part of the liver as an organ. In addition to the classical roles of hepatocytes and biliary cells in metabolism and digestion, the presence of a broad range of immune cells in the liver contributes to its basic functions by sensing and reacting to external and endogenous danger signals. Several unique features characterize immune responses in the liver including the composition of immune cell types, the local tissue environment that allows close interaction between parenchymal cells and immune cells, and the gut-derived signals arriving from the portal blood. Integration of these components is pivotal for immunological homeostasis in the liver and orchestration of effective immune responses for the protection of the host.

Overview of liver immunology

The liver is a unique immunological organ due to its cellular composition and physiological function (Table 2.1). Cells of the innate and adaptive immune system actively participate in immune responses in the liver, recognizing and eliminating pathogens and other danger signals and inducing antigen-specific adaptive immune responses. Another key function of the liver is to protect the host from the presence of undesirable activated immune cells. Several factors discriminate the liver from other organs with respect to immune response. The liver is constantly exposed to gut-derived substances such as antigens, nutrients, and metabolites, as well as pathogen-derived immune activation signals from the portal circulation. The normal liver immune environment promotes immunological tolerance, which has long been recognized in the setting of liver transplantation. While the exact mechanisms for this have yet to be delineated, the presence of predominantly immature dendritic cells that induce immune tolerance instead of T cell activation and high levels of immuno-inhibitory cytokines and mediators (interleukin 10 [IL10], transforming growth factor beta [TGFß], and prostaglandin E2 [PGE2]) contribute to this phenomenon. The architecture of liver sinusoids, with slow blood flow and close proximity of liver parenchymal cells and immune cells, creates a microenvironment for prolonged interactions between these cells that may also be a factor in local immune regulation. Finally, the composition of the liver T cell and natural killer (NK)–natural killer T (NKT) cell populations is markedly different from that of the circulation and many organs, with high proportional representation of NK, NKT, and gamma delta (γδ) T cells.

Innate immunity

Innate immunity provides the first line of host defense against invading pathogens. In recent years, it was discovered that in addition to pathogens that trigger an innate immune response, the innate immune response can also recognize and respond to damaged self-molecules. A coordinated cascade of events occurs that involves recognition of exogenous or endogenous danger signals by various pattern recognition receptors. This leads to a rapid induction of intracellular signaling cascades that direct the production of pro-inflammatory

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Table 2.1 The liver is a unique immune organ.

The largest immune organ
Unique biological properties • Unique architecture and vascular structure that facilitate interaction between parenchymal and nonparenchymal cells and circulating immune cells • Constant exposure to gut-derived antigens and pathogen-derived substances from the portal circulation
Unusual composition of lymphocyte subsets • NK cells, NKT cells, and T cell receptor repertoire (γ and δ)
Promotes immune tolerance • IL10, PGE_2, and TGFß • Diversity of professional and nonprofessional antigen-presenting cells

cytokines and/or type I interferons that comprise innate immunity. Innate immunity is also critical in triggering and modifying adaptive immune responses [1].

Cell populations and mediators in the innate immune response

Monocytes, macrophages, and Kupffer cells

Monocytes and macrophages represent the major constituents of the innate immune cell population. These cells originate in the bone marrow and can be rapidly recruited to sites of inflammation due to their chemotactic and cell migration properties. Circulating monocytes differentiate into macrophages at sites of infection, injury, or inflammation in the tissues [2]. Kupffer cells are the resident macrophages in the liver; they contribute to elimination of gut-derived pathogens and play important roles in various liver diseases, including alcoholic and nonalcoholic liver diseases [3, 4].

Monocytes, macrophages, and Kupffer cells are the classical "phagocytic" immune cells that uptake pathogens or cell debris by phagocytosis, endocytosis, or pinocytosis. The phagocytic capacity of these cells also includes production of reactive oxygen species (ROS) that contribute to their antibacterial effector function and production of pro-inflammatory cytokines. Monocytes, macrophages, and Kupffer cells have overlapping functional repertoires where macrophages and Kupffer cells are most potent in pro-inflammatory cytokine and ROS production and relatively inefficient in antigen presentation compared to circulating blood monocytes [2].

Monocytes migrate from the circulation into the tissue, where they differentiate into tissue-specific macrophages, such as Kupffer cells in the liver. There are two main populations of monocytes, the classical and nonclassical subsets, which vary in phenotype, function, and morphology. The classical subset, which comprises approximately 90% of circulating monocytes, expresses high levels of CD14 (CD14++). The nonclassical subset is distinguished by expression of CD16 (Fcγ receptor III) and variable CD14 expression [5]. CD14+CD16+ monocytes have been identified as the main producers of tumor necrosis factor alpha (TNFα) [6] and secrete more IL10 in response to lipopolysaccharide (LPS) stimulation than CD14dimCD16+ or CD14+CD16– cells [5]. It has been reported that the pro-inflammatory CD14+CD16+ monocyte population is expanded in the circulation and liver of patients with chronic liver disease. In addition, the investigators report that CD14+CD16+ cells directly activate hepatic stellate cells, but CD14+CD16– cells do not [7]. Therefore, the CD14+CD16+ monocyte subset may contribute to an inflammatory and pro-fibrogenic intrahepatic microenvironment, which would affect the progression of liver disease. Another nonclassical monocyte subset, CD14–CD16+ cells, is more responsive to Toll-like receptor 8 (TLR8) stimulation than CD14+CD16– cells [8]. Phenotypically, monocytes of the classical lineage are larger and denser, capable of phagocytosis and production of ROS. In contrast, nonclassical monocytes are smaller, less dense cells, with better antigen presentation function [9–12].

Circulating monocytes are recruited into the target tissue by a coordinated sequence of signals. Chemokines regulate the expression of a number of integrins, which are cell surface receptors that interact with adhesion molecules on the endothelial cells' surface, enabling the monocyte to attach to and roll along the endothelium. Integrins are also involved in polarization of the monocyte, which allows it to extravasate into the tissue. Once inside the tissue, monocytes differentiate into dendritic cells or macrophages [13]. Since monocytes are a heterogeneous cell population, as described in this section, the stage at which they are recruited into the tissue may influence the final cell type [2].

Macrophages are an important component of immunological defense. Firstly, they act as an integral part of the innate immune response by engulfing pathogens and killing them via the release of ROS. Recognition of these pathogens also stimulates macrophages to release cytokines and chemokines, which recruit other cells to the site of infection. Macrophages also contribute to the adaptive immune response by processing and presenting antigens to activate T and B cells, components of the adaptive immune response [14]. Due to their extensive phagocytic capacity, macrophages also play an important part in the clearance of cellular debris that arises from necrosis and apoptosis [2]. Macrophages secrete a number of different classes of molecules depending on their activation status, including pro-inflammatory

cytokines such as IL1β, TNFα, and IL6; anti-inflammatory cytokines IL10 and TGFβ; chemokines; and proteolytic enzymes [15].

Classically activated macrophages (also referred to as M1 macrophages) are generated in response to Th1 cytokines, the most important activator being interferon gamma (IFNγ) [14, 16]. IFNγ activates IFN regulatory factor (IRF) transcription factors, including IRF1. IRF1 upregulates IFNα, IFNβ, and inducible nitric oxide synthase (iNOS), increasing the antiviral and antimicrobial properties of the affected cell [14]. This cell type secretes a number of inflammatory cytokines that amplify the Th1 immune response. Classical macrophages are able to kill intracellular pathogens by producing ROS and nitric oxide [17]. These cells are an important element in the innate immune response in addition to being potent mediators of inflammation [18].

Alternatively activated macrophages (also known as M2 macrophages) are generated in response to Th2 cytokines IL4 and IL13 [15]. Activation along this pathway enhances endocytic antigen uptake and presentation, eosinophil involvement, and granuloma formation that is required for an efficient response to parasitic infection or extracellular pathogens [14]. This cell type is distinct from classical macrophages in that they do not produce nitric oxide [17].

Macrophages play an important role in liver fibrosis. Macrophages produce the pro-fibrotic cytokine TGFβ [15, 17]. In addition, alternatively activated macrophages may be involved in production of the extracellular matrix [17]. However, current evidence suggests that liver macrophages act as regulators of fibrosis and fibrogenesis [19].

Kupffer cells are liver resident macrophages. They account for approximately 80% of the body's macrophage population [20], and constitute approximately 20% of the nonparenchymal cells in the liver [21]. Kupffer cells are localized to the sinusoidal vascular space in the periportal area. In this location, they are able to clear endotoxins, microorganisms, and cellular debris from the portal circulation entering the liver [20, 21]. Kupffer cells act cooperatively with neutrophils to eliminate pathogens from the blood [20, 22]. In addition, Kupffer cells are important producers of cytokines and chemokines in the liver following injury or endotoxemia [23].

Dendritic cells and antigen presentation

Dendritic cells (DCs) are the main type of antigen-presenting cells in the immune system that uptake antigens, induce antigen-specific T cell activation, and produce inflammatory and immunomodulatory cytokines. DCs are 10 times more potent at antigen presentation and T cell activation compared to monocytes and macrophages. Dendritic cells efficiently uptake and process antigens due to their rich subcellular endosomal compartments. The processed antigenic peptides are presented in the context of major histocompatibility complex class (MHC) II molecule and co-stimulatory signals to initiate activation of naïve $CD4^+$ T lymphocytes [24].

Dendritic cells can be separated into various subtypes based on their origin, cell surface marker expression, and functional capacity. Myeloid DCs are derived from the bone marrow and are present in both murine and human livers. Blood monocytes can differentiate into monocyte-derived myeloid dendritic cells upon *ex vivo* stimulation with IL4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Differentiation of circulating monocytes into dendritic cells is triggered *in vivo* by the tissue environment. Both conventional and monocyte-derived DCs (mDCs) produce the immunomodulatory cytokines IL12 and IL10 that contribute to the efficiency of their T cell activation and antigen-presenting capacity. The mDC1 (myeloid CD1c+ DC) represents the largest population of myeloid DCs (also known as conventional DCs) in the blood, which produce inflammatory cytokines and chemokines upon stimulation [25]. The mDC2 (myeloid CD141+ DC or myeloid BDCA3+ DC) represents a minor subset of blood leukocytes that have recently been identified as the human homologue of the mouse CD8+ DC subset [26, 27]. mDC2s are major producers of IL12 and cross-present antigen for CD8 class 1–restricted cytotoxic T lymphocyte (CTL) responses under TLR3 ligation [28, 29]. Plasmacytoid DCs (pDCs) represent a small population in the peripheral blood but are enriched in the liver; they are the most potent producers of IFNα in viral infections [30].

Both mDCs and pDCs are present in the liver in an immature phenotype that is characterized by a high capacity to uptake antigens but relatively low T cell activation potential. Compared to other tissues, the majority of DCs in the liver possess an immature phenotype. This phenomenon has been attributed to the state of "immune tolerance" in the liver. Pathogen-derived signals, in the presence of inflammation, rapidly induce maturation of immature DCs in the tissue. During the maturation progress, DCs change their phenotype and increase surface expression of T cell co-stimulatory molecules, resulting in their superior antigen presentation and T cell activation capacity [30, 31].

NK and NKT cells

NK and NKT cells are lymphocytes that, unlike B and T cells, do not express an antigen receptor with somatic diversification [32]. Human NK cells express CD56 and CD16 but lack CD3. NK cells constitute up to 50% of the

hepatic lymphocyte population in humans, compared to 5–20% in the peripheral circulation [31]. In addition to their increased numbers, liver-derived NK cells also exhibit enhanced cytotoxic capacity against tumor cells compared to splenic and peripheral blood NK cells derived from rodents or humans [32].

NK cell function is regulated by a balance between stimulatory and inhibitory receptors that are constitutively expressed on the cell surface. NK cells are inactivated when inhibitory receptors bind to MHC I receptors on the target cell [32]. NK cells become activated when a cell with abnormal expression of MHC I or stress-related proteins is detected. Upon activation, NK cells release granules that lyse the target cells, or they induce apoptosis via engagement of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) [31, 32].

Hepatic NK cells are important mediators of the innate immune response against tumors, viruses, intracellular bacteria, and parasites. Decreases in the number of NK cells are associated with progression of hepatocellular carcinoma [32] and chronic hepatitis C virus (HCV) infection [31]. Activated NK cells also play a role in liver injury and repair by controlling the balance between pro-inflammatory and anti-inflammatory cytokines in the liver microenvironment [21].

NKT cells express T cell markers as well as NK cell markers. Classical NKT cells (also known as invariant NKT or iNKT cells), which are CD1d dependent, are capable of producing type I and type II cytokines. Similar to NK cells, iNKT cells can induce cell lysis via perforin or the Fas ligand. Nonclassical NKT cells, which are CD1d independent, produce only type I cytokines. The number of NKT cells is enriched in the liver, comprising up to 10% of the liver lymphocyte population [32]. NKT cells recognize nonpeptide antigens such as lipid and glycolipid and, when stimulated, are able to rapidly secrete large amounts of IFNγ and IL4, influencing the balance between a pro- and anti-inflammatory microenvironment in the liver [21]. These characteristics suggest that NKT cells are involved in connecting the innate and adaptive immune responses in the liver [32]. NKT-mediated cytotoxicity has been identified as a key factor in experimental hepatitis models induced by concanavalin A and endotoxin. NKT cells are also important in protecting against liver infection. NKT- or CD1-deficient mice are more susceptible to certain viral infections, and NKT cells activated by the CD1d ligand downregulate HBV replication via induction IFNγ secretion [21].

γδ T cells are an alternative T cell type that express a γδ T cell receptor instead of the more common αβ T cell receptor [31]. These cells recognize stress proteins and nonprotein antigens. Although their number is limited in the circulation, they comprise between 15 and 25% of the liver T cell population [21]. It has been shown that γδ T cell numbers increase in patients with viral hepatitis but not in those with nonviral hepatitis [32]. It has also been reported that hepatic γδ T cells are elevated in mice with viral infection or liver tumors [31, 32], indicating that this cell type plays a role in immune surveillance in the liver.

Neutrophil leukocytes

Neutrophil leukocytes are the first line of defense in most bacterial infections and in tissue damage. Regulated expression of a cadre of adhesion molecules permits leukocytes to rapidly invade inflamed tissue from the microvessels of the circulation [33]. Neutrophils are highly chemotactic and exert direct antibacterial effects through their expression of elastase, myeloperoxidases, and ROS. Although neutrophils are primarily involved in the clearance of infection, they have also been implicated in the initiation of tissue damage in alcoholic liver disease and sepsis [23].

Cytokines, interferons, and chemokines

Cytokines, interferons, and chemokines are soluble protein "messengers" and executors of innate and adaptive immune responses. Cytokines are soluble signaling molecules that provide communication between different cells in the tissue, in the systemic circulation, and in distant organs; they have paracrine and autocrine effects. The interleukin family of cytokines can be divided into various categories of cytokines that promote inflammation including IL1α, IL1ß, IL6, and several other types of cytokines such as IL17. IL10 is an anti-inflammatory cytokine that also has negative effects on antigen-presenting functions of innate immune cells and directly inhibits T cell proliferation. Other cytokines such as TNFα, IL12, IL18, IL33, IL21, and IL22 have immunoregulatory functions [34].

The pro-inflammatory cytokine TNFα is primarily synthesized by monocytic cells, including macrophages, Kupffer cells, and microglia. There are two receptors for TNFα, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 is expressed on most tissues, while TNFR2 is expressed mainly on immune cells. TNFα is released during infection or trauma, and is able to generate a cytokine cascade [35]. Several studies have noted that TNFα is an important mediator in the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) in both humans and animals [36].

The IL1 family of cytokines includes IL1α, IL1β, IL18, IL33, and the IL1 receptor antagonist (IL1RA). These cytokines have important roles in the innate and adap-

tive immune response. The IL1 family members signal through related receptors that include an extracellular immunoglobulin domain and a cytoplasmic Toll–IL1 receptor (TIR) domain. When the ligand binds to the receptor, a second subunit is recruited; assembly of the receptor heterodimer induces signaling [37]. IL1 and IL18 require cleavage by the inflammasome complex (see section below on inflammation) to produce the biologically active form for secretion. IL1β increases the expression of adhesion markers on endothelial cells, which work in conjunction with chemokines to induce the migration of immune cells from the circulation into the target tissue [38]. Members of the IL1 family play an important role as co-stimulators of T cells. For example, IL33 enhances Th2 responses; IL18, in the presence of IL12, enhances Th1 response by producing IFNγ; however, in the absence of IL12, it enhances the Th2 response by producing IL4 [38].

Interferons are the first line of defense against viral infections in innate immunity and are produced by immune cells as well as some parenchymal cells, including hepatocytes in the liver. Type I IFNs include IFNα and IFNß; Type II IFN, IFNγ, is a major immunoregulator; and the recently discovered Type III IFNs, also called IFNλs, include IL28a, IL28b, and IL29. Type I and Type III interferons have direct antiviral effects, while IFNγ is an immunomodulator that activates and amplifies innate and adaptive immune responses [39]. Recent clinical data identified that several single-nucleotide polymorphisms (SNPs) near the *IL28* gene are strongly associated with HCV clearance during natural viral clearance as well as in response to therapy with IFNα plus ribavirin [40, 41].

The family of chemokines includes a large array of mediators that direct immune cell trafficking, recruitment, and homing to various tissues in a cell-specific manner [42]. Chemokines are separated into four families based on the pattern of cysteine residues. The CC chemokine family includes RANTES, which attracts T cells, and monocyte chemoattractant protein-1, which provides the signal for monocytes to migrate from the bloodstream into tissue to differentiate into macrophages. The CXC family includes IL8, which targets neutrophils, monocytes, and mast cells. Fractalkine is the only member of the CX3C family. The final family, XC, includes lymphotactin and SCM-2β. Chemokines exert their effects by binding to G protein–coupled receptors (GPCRs). The biological effect of the chemokine–receptor binding depends on the coupling of the different G proteins within the receptor itself [43]. Chemokines are produced by both parenchymal and immune cells in the liver; their cell-specific effect is provided by the cellular expression of the respective chemokine receptors [42].

Complement

The complement system is composed of soluble and membrane-bound proteins that associate in the form of a "cascade." Activation of the complement cascade leads to the assembly of a pore-forming structure known as the membrane attack complex (MAC) on the surface of the target cell. There are three separate pathways in the complement cascade, each of which is specific for different targets: the classical pathway, the lectin pathway, and the alternative pathway. The classical pathway recognizes antibody-bound targets, such as immune complexes and dead cells. The lectin pathway recognizes mannose-binding lectin (MBL) bound to carbohydrate molecules on bacterial cells. The alternative pathway is activated by foreign agents such as bacteria, viruses, and fungi [32, 44].

Complement proteins are involved in maintaining homeostasis through tissue repair and regeneration as well as through inflammation. Dysregulation of the complement cascade is implicated in a number of autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis. Complement proteins are involved in clearance of debris, thereby reducing exposure to potential auto-antigens. Complement also functions to maintain B cell tolerance, which reduces the production of auto-antibodies [45]. Activation of the complement cascade has been found in alcoholic liver disease; mice deficient in complement C3a or the complement receptor are partially protected in the early phase of alcoholic liver disease [46].

The complement cascade is a critical element in the immune system, and the liver plays a vital role in maintaining that system. The liver is the primary site of complement protein synthesis. Pro-inflammatory cytokines secreted during an inflammatory response such as IL6, IFNγ, and TNFα stimulate hepatocytes to produce complement proteins. Hepatocytes also synthesize the complement regulatory proteins C1 inhibitor, factor H, and factor I. C3a and C5a are critical for liver regeneration after injury. Complement can also contribute to the pathogenesis of liver disorders such as liver fibrosis and alcoholic liver disease [32, 45]. HCV has been shown to decrease C3 levels both *in vivo* and *in vitro* [47].

Inflammation in liver diseases

Inflammation is the response of the innate immune system to danger signals. Inflammation is triggered by recognition of danger signals, which induces an inflammatory response; this process is normally self-limited with resolution of the inflammation. In all chronic liver diseases, whether induced by viral hepatitis, metabolic factors, or alcohol abuse, persistent insult prevents

resolution, resulting in chronic inflammation that leads to chronic liver disease, fibrosis, and cirrhosis (Figure 2.1). The three determining stages of inflammation are recognition, response, and resolution.

Immune recognition of danger signals occurs with the help of pattern recognition receptors (PRRs) (Figure 2.2). The major families of PRRs are Toll-like receptors (TLRs); the RIG-I-like receptors (RLRs), including RIG-I and MDA5; the NOD-like receptors (NLRs), such as NALP and IPAF; as well as other intracellular sensors (Figure 2.3) [1, 48–51]. While initial discovery of TLRs was made in innate immune cells, TLRs are expressed and functionally active in virtually all cell types in the liver [52].

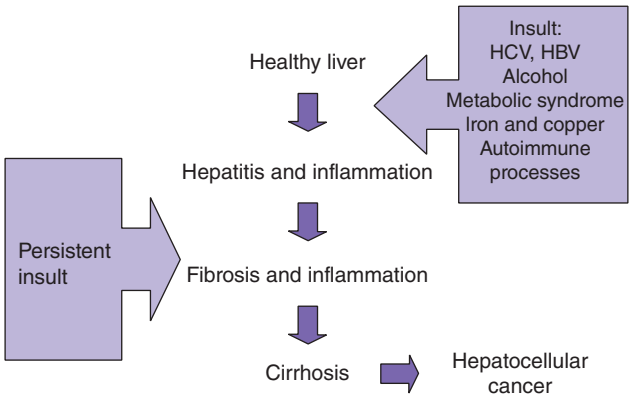

Figure 2.1 Progression of chronic liver disease. HBV: hepatitis B virus; HCV: hepatitis C virus.

Pathogen-associated molecular patterns (PAMPs) represent foreign danger signals that the host recognizes as "exogenous danger." In "sterile" inflammation associated with tissue injury, damaged host cells release damage-associated molecular patterns (DAMPs) that are recognized by the same repertoire of TLRs and PRRs as exogenous danger signals. In certain pathologies, PAMPs and DAMPs can both be involved in and amplify inflammatory responses.

TLRs are evolutionarily conserved sensors of PAMPs. Of the 13 TLRs, most are functionally active in humans. TLRs expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) recognize extracellular PAMPs, while intracellularly localized TLRs (TLR3, TLR7, TLR8, and TLR9) sense nucleic acid sequences (Figure 2.4) [1]. The cytoplasmic TIR domain of TLRs interacts with the TIR domain of adapter molecules such as the common adapter MyD88 utilized by all TLRs except for TLR3. MyD88 recruitment triggers downstream signaling via IRAK1/4 kinases and IKK kinase activation to culminate in NF-κB activation and induction of pro-inflammatory cytokine genes. TLR3 and TLR4 utilize the TRIF adapter that activates IKKε/TBK, leading to IRF3 or IRF7 phosphorylation and, after their nuclear translocation, induction of Type I IFNs [48, 53]. TLR4 recognizes endotoxin derived from Gram-negative bacteria, TLR2 senses microbial lipopeptides, while TLR1

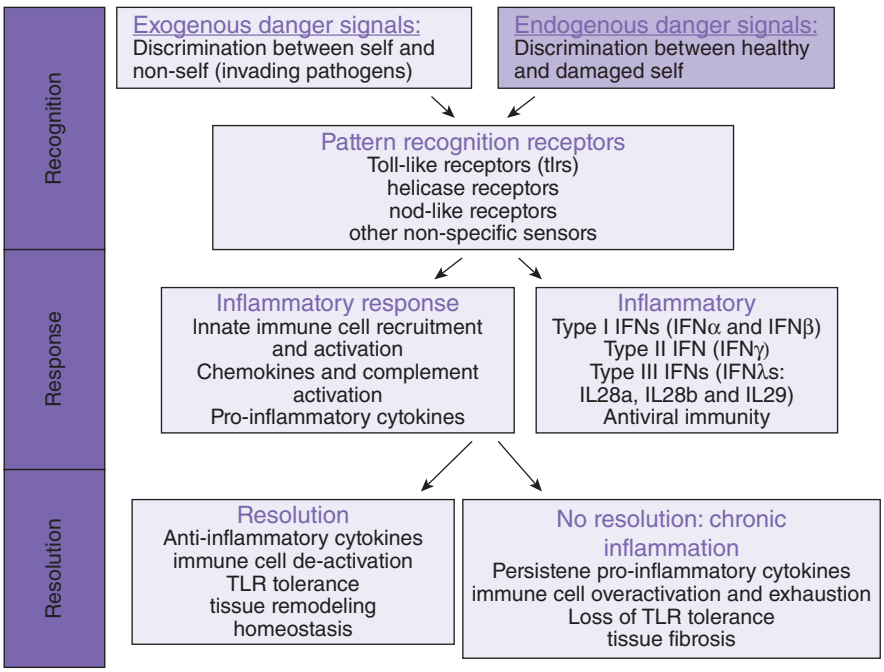

Figure 2.2 Danger signals and their recognition.

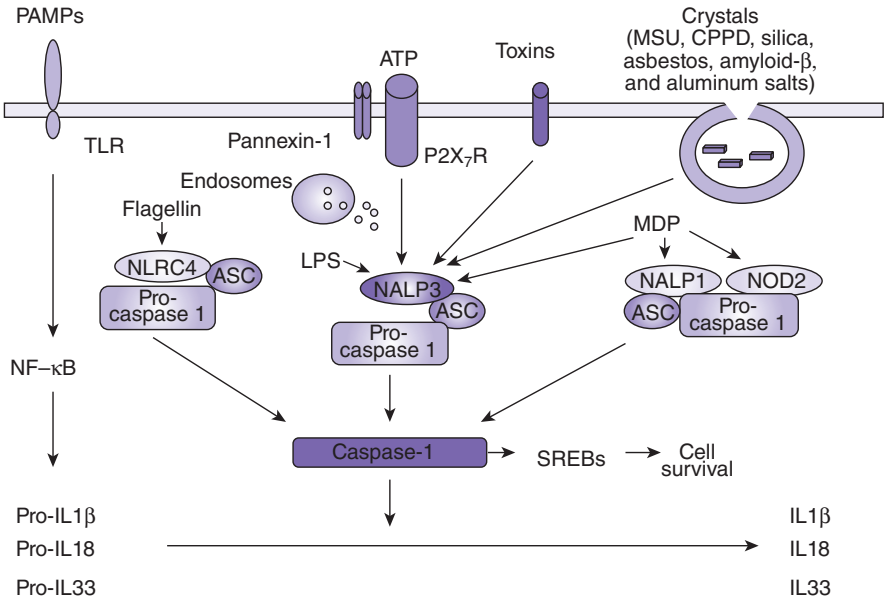

Figure 2.3 Activation of the inflammasome by PAMPs and DAMPs. PAMPs: pathogen-associated molecular patterns; ATP: adenosine triphosphate; MSU: monosodium urate; CPPD: calcium pyrophosphate dihydrate; TLR: Toll-like receptor; LPS: lipopolysaccharide; MDP: muramyl dipeptide; ASC: apoptosis-associated speck-like CARD domain-containing protein; NALP: NACHT-LRR-PYD-containing protein; NOD: nucleotide-binding oligomerization domain-containing protein; SREB: sterol regulatory element-binding protein. (Color plate 2.1)

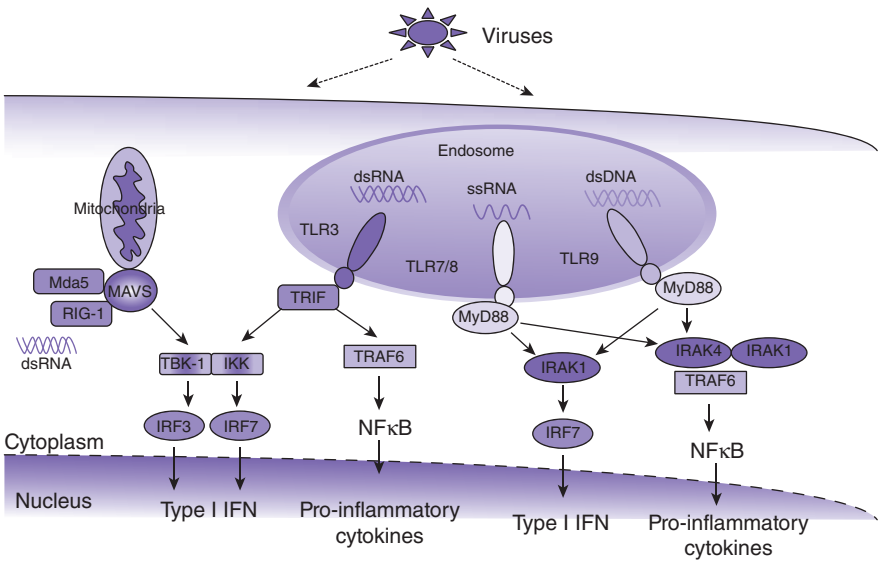

Figure 2.4 Intracellular sensors of viral infection. dsRNA: double-stranded RNA: ssRNA: single-stranded RNA; dsDNA: double-stranded DNA; MAVS: mitochondrial antiviral-signaling protein; TRIF: TIR domain-containing adapter-inducing interferon-β; MDA5: melanoma differentiation-associated protein 5; IRAK: interleukin-1 receptor-associated kinase; TRAF: TNF receptor-associated factor; IRF: interferon regulatory transcription factor; TBK: TANK binding kinase; IKK: IkappaB kinase. (Color plate 2.2)

and TLR6 combined with TLR2 distinguish between triacyl- and diacyl-lipopeptides [54]. TLR3 recognizes viral double-stranded RNA, and bacterial flagellin stimulates TLR5 [55, 56]. TLR7 and TLR8 are triggered by viral single-stranded RNA [57], and TLR9 recognizes prokaryotic CpG-rich DNA [58].

All TLRs are broadly expressed in the liver in diverse cell populations. Kupffer cells express TLR4, TLR2, TLR3, and TLR9 [59–61], and stellate cells can be activated via TLR2, TLR4, and TLR9 [62, 63]. Liver sinusoidal endothelial cells express TLR4 [64, 65], and primary cultured hepatocytes express mRNA for all Toll-like receptors of which TLR2, TLR3, TLR4, and TLR5 are expressed at low levels and show weak responses *in vivo* [66, 67]. LPS, a component of Gram-negative bacteria, is a strong activator of innate immune responses via the TLR4 complex because of its lipid A portion [68]. TLR4 cannot directly bind LPS, and the binding of LPS to the co-receptors CD14 and MD2 facilitates activation of TLR4. CD14, a GPI-anchored protein, facilitates the transfer of LPS to the TLR4–MD2 receptor complex that modulates LPS recognition [69]. MD2 associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs [70]. The association between LPS and CD14 can be further facilitated by LPS-binding protein (LBP) [71].

NLRs are sensors of the inflammasome complex that, upon activation, lead to caspase-1 cleavage. The activated caspase-1 cleaves pro-IL1 to the biologically active 18 kD IL1ß [49, 51, 72, 73]. Inflammasomes are multiprotein complexes that sense intracellular danger signals via the sensor (NLR) that forms a complex with the effector molecule, pro-caspase-1, with or without the contribution of an adapter molecule, such as apoptosis-associated speck-like CARD domain-containing protein (ASC) [73–76] . Inflammasome activation leads to autoactivation of inactive pro-caspase-1 precursor into p20 and p10 subunits that form the active caspase-1 [67, 69–71] resulting in cleavage of pro-IL1β and pro-IL18 into mature forms and inactivation of IL33 [73–77]. As a pro-inflammatory cytokine, IL1ß regulates inflammation and binds to the IL1 receptor (IL1R) to exert its broad biological effects. The IL1R also recognizes IL1α and binds IL1R antagonist (IL1ra). IL1ra is a soluble protein induced by the same danger signals as pro-inflammatory cytokines, and it is a naturally occurring inhibitor of inflammation by occupying the IL1R without inducing activation [38]. IL18 activates NK cells to produce IFNγ [38, 78, 79], and IL33 is a chromatin-associated cytokine of the IL1 family that drives Th2 responses [80, 81]. The full-length active IL33 is cleaved and inactivated by caspase-1 [77].

Inflammasome activation is a multistep process where the initial signal results in upregulation of inflammasome expression; this step is mostly initiated by TLR activation. The second signal triggers functional inflammasome activation by an inflammasome ligand [75, 76]. Inflammasome ligands include both pathogen-associated (PAMPs) and endogenous danger molecules (DAMPs) [73–76]. DAMPS are released from activated, damaged, or dying cells and represent a broad range of molecules including HMGB1, fibronectin, and heat shock proteins, among others [82]. The four main prototypes of inflammasomes are NLRP1 (NALP1), NLRP3 (NALP3, or cryopyrin), NLRC4 (IPAF), and AIM2 [76]; while each of these has different ligand recognition sites and utilization of adapter molecules, all lead to caspase-1 activation.

There is increasing evidence for the involvement of the inflammasome complex in different types of liver diseases, and their potential as targets for disease modification is an emerging field in hepatitis research [83]. For example, inflammasome activation was found in acetaminophen-induced liver disease as well as in NASH [84–86].

RNA helicases such as RIG-I and MDA5 are another important intracellular pattern recognition receptor family. These receptors sense double-stranded RNA and induce Type I IFNs via the mitochondrial antiviral-signaling protein (MAVS) (also known as IPS) adaptor [49]. Translational research elegantly identified RIG-I as a target of the HCV serine protease NS3/4 [87], and now successful therapies are entering clinical practice to cure disease (Figure 2.4). Decreased expression and function of MAVS were also found in NASH, linking decreased Type I IFN production with fatty liver disease [88].

Depending on the expression profile of TLRs, other pattern recognition receptors and the components of the intracellular signaling pathways determines the response of individual cells to danger signals. Expression of pattern recognition receptors is not limited to immune cells in the liver. Essentially any of the cell types in the liver have some form of pattern recognition system that enables them to sense danger signals [32].

Resolution of inflammation is determined by the balance of pro- and anti-inflammatory cytokines and mediators elicited by the initial danger signal. The same TLR ligands that induce pro-inflammatory cytokines in the early phase of inflammation also trigger anti-inflammatory mediators such as IL10 and TGFß that downregulate the initial inflammation to establish homeostasis [34].

Adaptive immunity

$CD4^+$ T cells

Activation of T lymphocytes is largely dependent on their interactions with antigen-presenting cells (APCs)

such as dendritic cells, monocytes, and macrophages. Classically, APCs that are exposed to pathogens or other antigens will interact with naïve $CD4^+$ T cells, and the type of interaction may determine the development of the T cells into a Th1, Th2, T regulatory (Treg), or Th17 phenotype. Th1 $CD4^+$ T cells are potent producers of IFNγ and TNFα, while Th2 $CD4^+$ T cells produce IL4, IL10, and IL13. $CD4^+$ Tregs produce IL10 and TGFß, while Th17 cells secrete IL17 and IL22. Induction of the Th1 phenotype requires mature DC1 type interaction with native $CD4^+$ T cells and the presence of IL12 and IL18 as co-stimulatory molecules. Th2 cells were shown to be induced by interaction with DC2 in the presence of antigen and IL4 [89].

$CD8^+$ T cells

The majority of hepatic T cells are $CD8^+$, comprising 70% of the liver T cell population compared to 35% in peripheral blood [90]. Functions of $CD8^+$ T cells include induction of apoptosis via the Fas ligand, secretion of pro-inflammatory cytokines, and cytolysis. Activated $CD8^+$ T cells are recruited to the liver independent of their antigen specificity; however, proliferation occurs only when the specific antigen is encountered [21]. In chronic HCV infection, $CD8^+$ T cells commonly display an exhausted phenotype that includes higher expression of the inhibitory receptor PD1 [91].

Regulatory T cells

The immune system has sophisticated mechanisms in place to control overt immune activation in response to pathogens and/or antigens. Regulatory T cells (Tregs) play a central role in immune balance as mediators of peripheral immune tolerance. Tregs have a pivotal role in preventing autoimmune processes such as primary biliary cirrhosis [92], controlling rejection in liver transplantation [93], and limiting chronic immune activation and inflammation (e.g., in viral hepatitis) [94, 95]. Natural Tregs arise in the thymus while induced Tregs are generated from CD4+ T cells in the periphery in the presence of cytokines and an immunosuppressive tissue environment. Naturally occurring forkhead box P3 (Fox P3/Cd4+/CD25+ Treg) cells display a diverse T cell repertoire that is specific for self-antigens; however, Tregs are also induced or converted from activated CD25+ T cells during inflammatory processes in the peripheral tissue [96]. T regulatory 1 (T_R1) cells mediate their immunosuppressive activity via IL10, while T helper 3 (T_H3) cells produce the immunoinhibitory cytokine TGFß [97]. IL35 is a recently recgonized cytokine that is suggested to regulate Treg functions [98]. In addition to suppression by inhibitory cytokines, the basic mechanisms of Treg cell function include suppression of dendritic cells. For example, Tregs that inhibit DCs via CTLA4 could condition DCs to express indoleamine 2,3 dioxygenase (IDO), a potent regulatory molecule [99, 100].

Induction of regulatory T cells (Tregs) is typically initiated by their interaction with immature DCs in a cytokine environment enriched for TGFß or IL2. Tregs have been identified as major modulators in the immune response against HCV infection, and several studies suggest that increased number and increased activity of the different regulatory T cell populations contribute to impaired HCV clearance in chronic HCV infection [94].

Table 2.2 The balance between T helper type17 (Th17) cells and T regulatory (Treg) cells is critical in immune homeostasis.

CD4+ Th17 cells	CD4+ Treg cells
Autoimmune diseases	Prevent autoimmunity
Rheumatoid arthritis	
Colitis	Maintain tolerance
Experimental autoimmune encephalitis (EAE)	CD25+ CD4+ FoxP3+ Treg mediate spontaneous liver transplant tolerance
Liver diseases	
Acute liver injury	
Liver granulomas	
Ischemia–reperfusion	

CD17

The inflammatory Th17 T cell phenotype is induced by mature DCs with co-stimulation by IL1ß, IL6, IL23, and TGFß. The balance between Th17 and Tregs is critical in immune homeostasis (Table 2.2) [101–106]. In autoimmune diseases, as well as in certain liver disease such as acute liver injury, liver granulomas, and ischemia–reperfusion liver injury, predominance of Th17 cells contributes to disease pathology [103]. Studies suggest that administration of Tregs can improve these conditions. Consistent with this, Tregs prevent autoimmunity and maintain immune tolerance in the form of spontaneous liver transplant tolerance by CD25+CD4+FoxP3 Tregs [105].

B cells

The role of B lymphocytes is relatively poorly characterized in liver diseases compared to other immune cells. B cells comprise less than 10% of the human hepatic lymphocyte population. The majority of these cells are CD5+, a negative regulator of B cell receptor signaling. CD5+ B cells are significantly increased in the blood and liver of individuals with HCV [31].

Table 2.3 Hepatic antigen-presenting cells (APCs).

Professional APCs	Nonprofessional APCs
Monocytes	Macrophages
Dendritic cells (DCs)	• Kupffer cells
• Myeloid DCs	Liver sinusoidal endothelial
• Plasmacytoid DCs	cells (LSECs)
	Stellate cells
	Cholangiocytes
	Hepatocytes

The liver is a unique immune organ

There are several aspects to the liver that contribute to its unique status in immune tolerance. Hepatic immune tolerance has been noted in allogeneic transplantation of liver or organs together with liver as they had reduced requirement for immunosuppression post transplant. APCs play a key role in recognition of nonself and in activation of T cell responses. In the liver, there are different types of APC populations. Professional APCs include dendritic cells of both the myeloid and plasmacytoid DC lineage (Table 2.3). Liver macrophages and Kupffer cells can also perform as APCs, although their function is less superior compared to that to DCs. The majority of DCs present in the liver have a predominantly "immature" phenotype that is suboptimal in inducing potent antigenic responses by T lymphocytes. Several of the liver parenchymal and nonparenchymal cells that are not of immune origin have been shown to act as nonprofessional APCs, including liver sinusoidal endothelial cells (LSECs), stellate cells, cholangiocytes, and hepatocytes [20]. However, poor APCs promote T cell tolerance instead of antigen-specific T cell activation. Professional APCs provide coordinated signals to T cells for their full activation, including MHC II and TCR, CD80/86, and CD28 interactions, and in the absence of such signals T cell tolerance is induced instead. Nonprofessional APCs in the liver generally lack sufficient expression of some of these signals (MHC II or co-stimulatory molecules CD80/86), thereby inducing T cell tolerance instead of sufficient activation [107].

The anatomical position of the liver results in its blood supply from the portal circulation that is the collection site of all of the gut-derived venous blood. The portal blood is enriched in nutrients and in gut-derived substances including pathogen-derived compounds. The unique architecture and the vascular structure of the liver sinusoid, where blood flow slows down, facilitate interactions between parenchymal and nonparenchymal cells and circulating immune cells. A significant amount of research has focused on the role of the gut–liver axis in contributing to liver homeostasis and liver inflammation. Under normal homeostasis and with intact gut barrier and hepatocyte functions, the liver "detoxifies" the portal blood and avoids activation of the innate immune system [89, 108]. It has been shown that different types of liver diseases, including alcoholic and nonalcoholic liver disease as well as cirrhosis from any etiology, result in increased portal levels of LPS (which, as mentioned, is a component of Gram-negative bacteria), and this has been suggested to result in Kupffer cell activation and pro-inflammatory cascade activation in the liver. Sensitization of Kupffer cells to gut-derived LPS was found in animal models of alcoholic and nonalcoholic steatohepatitis [109, 110].

Additional contributors have also been proposed to promote the immunotolerant liver environment, such as the unusual composition of T cell populations characterized by the large proportion of NK, NKT cells, and γδ T cells [32, 90]. Additional factors include the relatively high expression of IL10, TGFß, and PGE2 in the liver; all are anti-inflammatory mediators that also inhibit APC function and antigen-specific T cell activation [20].

Several studies described that activated T cells tend to home to the liver, where they die by apoptosis [111, 112]. Peripheral T cell activation occurs in most infections, particularly in viral infections. For example, in cytomegalovirus (CMV), Epstein–Barr virus (EBV), and herpes simplex virus (HSV) infections, activated T cells home to the liver, where hepatocytes may suffer from "bystander" damage. Clinically, these viral infections cause hepatocyte damage and lead to increased serum transaminases that are thought to be predominantly a result of this "bystander" damage from activated T cells.

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