

Chapter 9

Impact of Cytokines in Hepatocellular Carcinoma Initiation and Progression

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Over the past several decades, numerous studies have shown that inflammatory diseases and infections trigger or promote the development and progression of many types of cancers [65], including hepatocellular carcinoma (HCC) which occurs in most cases at late stages of chronic liver disease associated with viral hepatitis B and C infection, metabolic disorders, and alcohol heavy consumption [41].

Chronic liver diseases, whatever their etiologies, are often associated with a sustained inflammatory response leading to repeated injury, fibrosis and at late stages, to cirrhosis. HCC arises in the setting of cirrhotic livers in 80 to 90% of cases and progresses in an inflammatory context. Despite significant progress in HCC diagnosis and improvement of the curative strategies, its incidence is still increasing in western countries and the prognosis of patients with advanced HCC remains in general very poor. Recurrence and non-response to the current anti-cancer treatment occur frequently. Diverse immune cell types associated with the release of a large spectrum of inflammatory cytokines appeared to be a key component in HCC emergence, progression and in therapeutic failures. Many factors produced by infiltrating immune cells such as chemokines, growth factors, cytokines and proangiogenic factors contribute to the promotion of cell survival, proliferation, epithelial-mesenchymal transition and genomic DNA instability [55].

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Thus, a better characterization of the underlying molecular and cellular mechanisms by which HCC development and resistance occur may open new insights for the development of more effective anticancer therapies. In addition to curative strategies for HCC, it is of interest to identify and block the immune cell types and their associated cytokines susceptible to trigger tumor initiation and progression.

9.1 Cytokines in Chronic Liver Diseases: A Risk Factor for HCC Initiation

9.1.1 *Chronic Alcohol Consumption is a Risk Factor for HCC Emergence*

Chronic alcohol consumption is associated with hepatic inflammation leading to cirrhosis development, and constitute a risk factor for HCC initiation. Chronic liver diseases are mainly due to recurrent and excessive liver inflammatory processes, as observed in alcoholic liver disease (ALD). ALD ranks among the major causes of morbidity and mortality worldwide, with a mortality rate in the USA and in Western Europe estimated to be approximately 5–6%. In USA, ALD is responsible for up to 100,000 deaths per year. ALD can present as steatosis (fatty liver, i.e. accumulation of triglycerides in hepatocytes), the prevalent lesion found in excessive drinkers that is now recognized as harbinger of worse disease to follow when liver insult is sustained. Indeed, steatosis may progress towards alcoholic steatohepatitis (ASH), when accompanied with liver inflammation and hepatocyte injury, that promotes liver fibrogenesis with a 20% risk of cirrhosis after 10–20 years. Severe alcoholic hepatitis (AH) is a specific clinical form characterized by a prolonged and intense **inflammatory reaction** despite alcohol withdrawal, and associated with a spontaneous 50% mortality rate after 6 months. Current management of steatosis and mild to moderate forms of AH relies upon abstinence. In severe AH, corticosteroids reduce the mortality rate to 15–20% after 6 months. Nevertheless, outcome after 1 year remains grim with survival rates ranging between 50 to 60%. Overall, these figures underscore the urgent need for **novel therapeutic approaches targeting inflammation** in the management of ALD.

9.1.1.1 Role of Innate Immune Cytokines

Activation of hepatic innate immune cells is the first step that triggers inflammation in alcoholic patients. Indeed, dysregulated cytokine signaling, particularly of those released by the resident macrophages of the liver (Kupffer cells), plays a pivotal role in the pathogenesis of ALD. In particular, several clinical and experimental studies have shown that overproduction of **TNF- α** by activated Kupffer cells is central to the inflammatory process associated with ALD [95, 121, 126]. The mechanism

leading to increased **TNF- α** production in response to ethanol involves enhanced intra-hepatic oxidative stress and altered gut permeability, thereby allowing enhanced translocation of endotoxin (LPS) into the portal blood, and activation of Kupffer cells following binding of LPS to its receptor Toll Like Receptor 4 (TLR4). TNF- α [141] induces hepatocyte cell death and inflammatory cell infiltration [12]. Furthermore, liver regeneration has also been shown to be impaired in ALD [60]. In addition, clinical treatments of patients with severe AH based on TNF- α neutralization with pentoxifylin (PTX) [1] or monoclonal antibodies like infliximab [104] or etanercept [13] have been shown to prevent inflammation but were associated with severe side effects including a higher susceptibility to infection leading to increased mortality rate. In addition, above and beyond its detrimental role in liver inflammation, TNF- α is not only critical for the host defence, but it also induces IL-6 synthesis to initiate hepatocyte proliferation and liver regeneration. IL-6 and TNF- α are two cytokines mainly produced by Kupffer cells in the liver and markedly induced after partial hepatectomy. TNF- α is a major regulator of the initiation of liver regeneration. It is known that IL-6 and TNF- α can stimulate hepatocyte proliferation by activating intracellular signalling pathways such as signal transducer and activator of transcription 3 (STAT-3) and CCAAT/enhancer-binding protein B (C/EBP). Altogether, these data point out the importance of controlling the balance between the inflammatory immune response required for pathogen elimination and liver regeneration, and the exacerbated inflammatory processes leading to hepatocyte cell death.

The molecular mechanisms associated with Kupffer cell activation are linked to acquisition of an **M1 phenotype** characterized by the production of a storm of inflammatory cytokines including TNF- α , IL-6, IL-12 and IFN- γ , which supports resistance to extracellular bacterial infection. However, overwhelming production of those cytokines by Kupffer cells is responsible for the development of AH. Contrastingly, **M2-polarized macrophages** are defined by production of IL-10, IL-4, and IL-13. Although those macrophages cannot control bacterial infection, they are critical for tempering the triggered inflammatory process, and in promoting tissue repair. And recently an **atypical M2 profile** has been reported that combines M1 and M2 characteristics [11] producing IL-6, TGF- β and the chemokine CXCL8 (also known as IL-8) susceptible to promote liver fibrosis.

Dendritic cells (DCs) are professional antigen presenting cells. They have the unique capacity to catch, process and load all kinds of antigens and prime effectors immune cells namely CD4⁺ and CD8⁺ T cells. Basically, two types of DCs are described. The first DC type conventional DCs respond to lipopolysaccharide and lipoteichoic acid via TLR4 and TLR2, respectively, and produce TNF- α , IL-6, IL-12. The second DC type known as plasmacitoid DCs respond to TLR7 and TLR9 activation by producing IFN- α [102]. Impaired DC functions highly contribute to tumor escape from immune-surveillance in patients with cancer [44].

9.1.1.2 Impact of Adaptive Immune Response

Indeed, in addition to the crucial role of macrophages, increasing evidence also points out the crucial role of T lymphocytes in mediating hepatitis in ALD [8, 17, 77]. The decrease in peripheral lymphocyte number is associated with an increase in the ratio of T helper cells to suppressor cells in the liver. Today, four major distinct CD4⁺ T cell subtypes have been described: the Th1, Th2, T regulatory (Treg) and more recently, the Th17 phenotype.

Th1 cells that mainly produce IFN- γ , mediate immune response against intracellular pathogens, and are also involved in some autoimmune diseases [99, 107].

Th2 cells that produce principally IL-4, IL-10 and IL-13 are involved in host defence against extracellular parasites [99, 107] and suppress Th1 cell proliferation [38].

Th17 cells defined as producer of IL-17, IL-21 and IL-22, play a major role in immune response against bacteria and fungi and participate also to the induction of several autoimmune diseases [133], but could also protect hepatocytes in acute hepatitis through IL-22 production [142]. Recently, the deleterious role of T helper lymphocytes secreting IL-17 (Th17 cells) in recruiting neutrophils has been reported in ALD [88]. In addition, persistent IL-17 production has been identified in numerous other chronic liver diseases with deleterious functions leading to cirrhosis and HCC development [52, 84]. These findings strongly support the potent role of T cells in the progression of AH. Indeed, T helper differentiation into a specific phenotype is mainly controlled by innate immune cells that in turn could respond to the variety of produced cytokines by those differentiated T cells.

Treg cells regulate immune response by maintaining the self-tolerance and are beneficial for treating autoimmune diseases [118]. First described as suppressive T cells, [118] Treg lymphocytes are involved in immunosuppression and mainly contribute to tumor immune escape. CD4⁺ CD25^{high} FoxP3⁺ Treg cells are induced by several microenvironmental factors including IL-10, TGF- β and VEGF which are overexpressed in HCC [10, 50, 140]. However, studies have reported a negative correlation between an increase in Treg infiltrating cells and clinical outcome in HCC patients [58]. In addition, it has been shown that in many types of liver disorders including chronic viral hepatitis, liver cirrhosis and HCC, Treg cell number increase favored HCC appearance and growth [74]. IL-10 mainly produced by Treg is the most studied anti-inflammatory cytokine regarding HCC. It has been shown that IL-10 is overexpressed in patients with HCC with less optimistic prognosis compared [10, 21]. Consistent with the previous studies, these reports strongly suggest that through IL-10 production, Treg lymphocytes promote HCC progression.

9.1.1.3 Inflammation-Associated Reactive Oxygen and Nitrogen Species (ROS and RNS)

Notable discoveries in mechanisms involved in ALD demonstrated the critical role of Kupffer cells in mediating AH through TNF- α overproduction, and led scientists to propose therapeutic strategies neutralizing TNF- α -mediated inflammation. In association with TNF- α , over-production of IL-6 was pointed out as driving factor of liver carcinogenesis [106]. The major mechanism that has been highlighted is the IL-6-induced reactive oxygen species (ROS) production and epigenetic changes triggering HCC development [123].

Due to ethanol oxidization by the cytochrome CYP2E1, acetaldehyde and reactive oxygen species (ROS) accumulate in the liver. This ROS accumulation promotes lipid peroxidation, DNA damage, chromosome instability and epigenetic disturbance. In an inflammatory context, epithelial and immune cell activation induce the production of reactive oxygen and nitrogen species (RONS) by NADPH oxidase, and nitric oxide synthase (NOS). Studies on chronic inflammatory diseases including inflammatory bowel diseases (IBD) and *Helicobacter pylori*-induced gastritis have reported increased level of RONS, suggesting a link between RONS production and cancer risk [111, 116]. RONS production induces cell damages including oxidative stress, lipids, proteins and DNA abnormalities through 8-oxodG and 8-nitrodeoxyguanosine accumulation [101] and therefore promote tumor initiation and malignancy. The discovery of RONS-induced DNA damages in chronic inflammatory responses including hepatitis is consistent with the involvement of RONS in diseases characterized by a higher cancer risk [101]. Furthermore, 8-oxodG and 8-nitrodeoxyguanine reactivity plays an important role in hepatitis C virus-induced chronic hepatitis [61]. All together these observations enhance the link between inflammation-induced RONS and carcinogenesis. Therapeutical use of antioxidants (e.g., *S*-adenosyl-L-methionine, polyunsaturated phosphatidylcholine) to restore alcohol-induced methionine and oxidative balance disruption, has shown promising effects but still needs to be further studied [115].

9.1.2 Cytokines in Fibrosis and Increased Risk of HCC Initiation

Tissue damage due to sustained inflammation leads in most cases to fibrosis development. Liver fibrosis is caused by a large variety of chronic liver diseases and represents an important cause of mortality in the world. The immune system protects the host from foreign pathogens without disrupting tolerance toward self-antigens but during fibrogenesis, inflammation contribute to the deposition and accumulation of collagen leading to an important modification of the physiological liver architecture.

Hepatic stellate cells represent the major cell population responsible for increased deposition of extracellular matrix proteins including collagen molecules. Collagens can also be produced to a lesser extent by other cell types including progenitor cells, portal fibroblasts, and cholangiocytes. Many immune cells including Kupffer cells, natural killer cells and dendritic cells have been shown to participate to liver fibrogenesis by releasing pro-inflammatory cytokines. These cytokines such as IFN- α and IFN- β , IL-6 and IL-22, activate the JAK-STAT signaling pathways by binding to their respective receptors.

9.1.2.1 Antifibrotic Cytokines

Interferon type 1, 2 and 3 were identified as cytokines that in general inhibit liver fibrosis development. For instance, IFN- α treatment significantly reduces the hepatic fibrosis in mice by blocking collagen gene transcription via the interaction of p300 transcription factor and phosphorylated STAT1 [66]. Similarly, IFN- γ deficient mice are more susceptible to develop liver fibrosis induced by CCl₄ administration or under a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet [67, 112]. The anti-fibrotic function of IFN- γ are more likely mediated through the induction of hepatic stellate cell-growth arrest and apoptosis [112].

It has been recently demonstrated that triple knockout mice for IL-10, IL-12/23 and IL-13Ra2 are more susceptible to many pathologies related to liver including *S. mansoni*-induced liver fibrosis model. Levels of liver enzymes, hepatosplenomegaly and ascites were increased, suggesting that IL-10, IL-12p40, and IL-13Ra2 contribute cooperatively to reduce liver fibrosis in this model of *S. mansoni* infected mice [97].

In the liver, IL-6 and IL-22 are mainly responsible for the activation of STAT3.

IL-6 knock-out mice seems more susceptible to liver injury and fibrosis after CCl₄ treatment [79]. Furthermore, the lack of gp130/STAT3-mediated signaling in hepatocytes leads to worsened DDC diet feeding related chronic cholestatic liver injury and fibrosis progression [109]. Similarly, hepatocyte-specific STAT3 knock-out mice displays a higher degree of liver fibrosis compared with wild-type mice in various models of liver fibrosis induced by CCl₄ administration, feeding with a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet [109], feeding with a choline-deficient, ethionine-supplemented (CDE) diet [80].

In hepatocytes, STAT5 activation is mainly induced by growth hormone (GH). Using the hepatocyte-specific STAT5 knockout mice developed in Dr. Lothar Hennighausen's laboratory [26], STAT5 loss in hepatocytes has been shown to promote increased TGF- β levels and enhanced STAT3 activity induced by GH in the liver after CCl₄ administration [6, 62]. Moreover, STAT5 deletion in the liver promoted hepatic tumorigenesis induced by CCl₄ injection in wild-type mice [62] and was responsible for the development of spontaneous liver cancer in liver-specific glucocorticoid receptor knockout mice [100] or in GH transgenic mice [43]. Although the effects of STAT5 in hepatocytes have been widely investigated, little

is known about the potential functions of STAT5 in the fibrogenic hepatic stellate cells (HSC).

9.1.2.2 Pro-fibrotic Cytokines

It is also known that IFN- α/β and IL-12 can activate STAT4 in immune cells and promote inflammation. In several animal models, treatment with IL-12 has been shown to induce liver inflammation and reduce liver tumor growth [18, 57]. This inflammatory response is characterized by the activation of NK and NKT cells which in turn produce IFN- γ [124]. Via the activation of STAT4 in immune cells, IL-12 seems to act as a pro-inflammatory cytokine promoting fibrosis and liver injury.

Th2 cytokines such as IL-4 and IL-13 are considered to be pro-inflammatory. Studies have shown that administration of IL-13 inhibitors or IL-13 gene blockage results in a significant reduction of liver fibrosis in the *S. mansoni* infection model. In humans, a correlation has been established between elevated levels of IL-13 and liver fibrosis in patients with chronic HBV or HCV infection, suggesting that IL-13 promotes liver fibrosis in an infection dependent or independent context [135]. These pro-fibrogenic effects can be explained by the fact that IL-13 induces HSC activation and promotes the production of fibrotic proteins by HSCs in a STAT6 dependent manner. Indeed, STAT6 blockage with siRNA inhibits HSC activation in vitro [3, 122]. Moreover, STAT6-deficient mice present smaller amounts of collagen deposition in the liver compared with wild-type mice after infection with *S. mansoni* [71]. Like TGF- β , IL-4 is known to have pro-fibrotic properties as it contributes to HSC activation and collagen production [3, 69]. Furthermore, IL-4 expression levels are higher in fibrotic liver from *S. mansoni*-infected baboons [37]. Although the role of IL-13 in liver fibrosis is reported in the *S. mansoni* infection model, studies to come need to clarify to what extent STAT6 in HSC is implicated in liver fibrogenesis in patients with chronic liver diseases.

IL-6 is a major pro-regenerative factor and induces the acute phase in the liver by stimulating hepatocytes to produce acute phase proteins such as C-reactive protein, serum amyloid A and complement C3 [114]. Clinical studies showed that IL-6 hepatic expression is increased and positively correlated with the degree of liver fibrosis [32, 136]. As IL-6 receptors are widely expressed on all types of cells in the liver, it can explain how IL-6 may have distinct roles in all these types of cells by regulating positively and negatively liver fibrosis. It has been shown that IL-6 can directly promote HSC survival and proliferation resulting in enhanced liver fibrosis. Collectively, the major effect of IL-6 on liver fibrosis is the result of the balance between on the one hand the inhibitory effect through STAT3 activation in hepatocytes and the stimulatory effects enhancing HSC survival, which depend on liver fibrosis stage and etiology.

9.1.3 Influence of Cytokines in Liver Progenitor/Stem Cell Activation: A Potent Mechanism of HCC Initiation

Liver progenitor cell (LPC) proliferation is reported in ductular reaction, often observed, in cirrhotic livers, hepatitis B and C viral infections, alcoholic or non-alcoholic steatohepatitis. Their appearance is associated with increased incidence of HCC. An interest in LPC biology emerged because of their stem-cell-like capacities to promote liver regeneration and to generate liver cancer. LPCs can differentiate into mature hepatocytes and biliary cells. Their capacity to restore injured hepatic tissue has been well documented [36]. However, LPCs were also defined as precursors for HCC and described as potent Cancer Stem Cells (CSCs) when they undergo “transformation” and generate heterogeneous lineage of cancer cells [29, 73, 94, 117]. Many transcription factors such as NANOG, cMYC, KLF-4, OCT4, SOX2 are stemness markers which have been reported to be increased in cancers [131].

The signaling pathways identified in HCC are also observed in isolated liver CSCs (eg, Wnt, Notch, TGF- β , Hedgehog, and PI3K/AKT/mTOR [86, 87]. Liver CSCs can be identified based on the expression of several cell markers such as CD90, CD44, CD24, CD13, epithelial cell adhesion molecule (EpCAM), CD133 (prominin-1), and oval cell marker OV6 as well as Hoechst dye efflux or aldehyde dehydrogenase expression and activities [86, 87]. Among those markers, double positive CD133+ and EpCAM+ cells display higher expression of stem-cell related genes and appearance of drug-resistance to chemotherapeutics. CSCs can initiate tumor in xenograft transplantation experiments. Moreover, the high capacity of resistance of CSC to sorafenib therapy suggests that CSCs could contribute to the poor prognosis [54] and participate to HCC recurrence. Several lines of evidence suggest a potential role of inflammatory microenvironment in CSC-initiation and progression towards HCC.

Recently, a correlation between IGF-1R and the expression of stemness markers in HBV-related HCC has been reported and suggests that inflammatory cytokines are involved in CSC development. The hepatic microenvironment is markedly disrupted in chronic liver diseases and characterized by infiltration of lymphocytes, activation of stellate cells and expansion of hepatic progenitor cells. One of the main axis involved in liver inflammation is IL-6/STAT3 signaling that in collaboration with TGF- β potentially promotes CSC survival and proliferation in the liver [98, 125].

9.2 Mechanisms of Cytokine Contribution to Tumor Growth

9.2.1 *Role of Inflammation Related micro-RNA in HCC Development*

In addition to its ability to modulate liver immune response, alcohol can also lead to epigenetic changes in inflammatory-associated genes via the multiple mechanisms [27]. These include (i) DNA-methyl transferases increasing methylation on gene promoters, (ii) an alteration of the physiological interaction between the transcriptional proteins to the DNA due to inappropriate methylation, acetylation phosphorylation and/or ubiquitination, and (iii) more recently, a transcriptional regulation mediated by micro-RNAs (miRNA) [102]. Epigenetic regulation of DNA methylation, phosphorylation, and ubiquitination by alcohol has been extensively reviewed in many previous articles. Here we mainly discuss the role of miRNAs in pathogenesis of HCC.

MiRNA are single-stranded non-coding RNAs composed of 20 nucleotides approximately. They are mostly responsible for the post-transcriptional epigenetic regulation of targeted gene expression. The interest for the miRNA raised when evidence of aberrant expression of several miRNAs was reported in many types of cancers [15]. Many pathways such as p53, RAS/MAPK, PI3K/ AKT/mTOR, WNT/ β -catenin, and TGF- β are involved in HCC development. Abnormal expression of some miRNA has been observed in HCC compared to normal liver tissue [105]. It has been shown that miRNA-199a and miRNA-122 are highly expressed in healthy liver. Interestingly, the expression of these two miRNAs is markedly disrupted in HCC [63]. MiR-199 has been shown to stop cell cycle at G1 phase. A correlation has been reported between the downregulation of miR-199a (a member of miR-199 family) and increased recurrence rate with reduced laps before recurrence of HCC [68].

MiR-122 is only expressed in adult normal liver and seems to be a key factor in the regulation of hepatocyte differentiation by inhibiting genes not exclusive to the liver [139] which makes it a particular miRNA in liver physiopathology. Consistent with these findings, in up to 70% of HCC, miR-122 is downregulated indicating that this miRNA should have an antitumor activity. Furthermore, miR-122 is known to promote apoptosis, block the tumor cell cycle, reduce in vivo cancer cell malignancy and increase efficacy of drugs such as Sorafenib and also doxorubicin by inhibiting p53 activity [5, 40]. Interestingly, in liver cancer patients, miR-122 loss is correlated with the development of metastasis and a reduced period before recurrence [25, 127]. Thanks to a miR-122 KO mouse model, the role of this miR is now better defined [64, 128]. MiR-122KO developed chronic liver inflammation, fibrosis and HCC like spontaneous tumors comforting the antitumor potential of miR-122. Indeed, miR122 targets CULT-1 and reduces its activity which explain the undifferentiated phenotype of HCC cells. Similar to miR-199, miR-122 inhibits cyclin G1 leading to an upregulation of p53 which is increased in HCC [51]. The link between miRNA and the inflammatory response seems very strong suggesting that

some cytokines could be involved in miRNA regulation. This mechanism by which cytokines could induce some miRNA targeting antitumor genes and thus promote tumor growth seems to be a very promising axis to explore in carcinogenesis.

In a study, up to 80% of HCC analyses showed a significant increase of miR-221 expression. This upregulation of miR-221 leads to increased tumor growth and cancer cell proliferation [47, 96]. Consistent with these observations, in transgenic mice overexpressing miR-221 in the liver, higher HCC tumorigenicity was reported and could be inhibited by administrating anti-miR-221 nucleotides, called antago-miR [16].

9.2.2 Cytokine-Induced Oncogenic Intracellular Pathways

Raf/MAPK/ERK signaling pathway is involved in cell growth and differentiation. The extracellular signal is translated from tyrosine kinase receptors including VEGFR, IGFR, PDGFR, EGFR and MET, triggering a cascade of intracellular phosphorylations [4]. RAS, a GTPase protein, and Raf, a serine/threonine kinase regulate the signal transduction in this pathway [75]. A study has shown that in HCC Raf kinase inhibitor is down-regulated leading to an over-activity of the Raf/MAPK/ERK pathway [85]. New therapeutic approaches including Sorafenib aimed to target and inhibit Raf kinase, and consequently to limit tumor growth [137].

PI3K/Akt/mTOR like the Raf/MAPK/ERK signaling pathway, controls proliferation, growth, motility and cell survival. HCC patients present an over-activation of this pathway. Indeed, it has been reported that in over 40% of HCC patients, Akt signaling and mTOR effector (p70s6k) were activated leading to increased cell survival and growth through an inhibition of TGF- β induced apoptosis [20]. These observations highlighted this signaling pathway as a potential target for therapeutic perspectives. Some strategies have been developed to block this pathway such as PI-103 inhibiting the phosphoinositide 3 Kinase (PI3K) and mTOR activation. These treatments showed efficacy in blocking Raf/MAP/ERK and PI3K/Akt/mTOR pathways leading to the reduction of EGF-induced proliferation of tumor cells [48].

Wnt/ β -catenin targets many processes including cell determination, stemness but also intercellular adhesion by interacting with E-cadherin and proliferative signal transmission through β -catenin activity [110]. Aberrant Beta-catenin activation is found in almost 40% of human HCC. B-catenin degradation is regulated by Adenomatous Polyposis Coli (APC) protein [22, 23]. Growth factors from the extracellular microenvironment bind to the Frizzled (Fzd) receptors expressed on the cell surface and activate this pathway. In murine and human HCC, an abnormal activity of this pathway has been reported [30, 56]. In absence of Wnt, the destruction complex formed by AXIN1, adenomatous polyposis coli (APC), glycogen synthase kinase-3 β (GSK-3 β), and casein kinase 1 (CK1) proteins drive the proteolysis of β -catenin through the ubiquitin/proteasome mechanism by phosphorylating the

protein. Another protein has been reported to be increased in HCC; PRC1 controls cytoskeleton organization and increase Wnt signaling by contributing to the sequestration of the complex at the membrane, promoting tumorigenesis and metastasis development [19]. The role of Wnt/ β -catenin in tumor growth is an attractive field to explore for paving the way to new therapeutic options. Indeed, antibody based therapies have already been developed. Blocking the β -catenin signaling reduced HCC tumor growth and increased apoptosis through the administration of anti-Wnt-1 antibodies [134].

NF- κ B plays a central role in liver injury, fibrosis and HCC development [91]. Its activation in macrophages lead to a large production of cytokines shaping an inflammatory tumor microenvironment of HCC [72]. In a DEN induced HCC murine model, Kupffer cell and hepatocyte specific blockade of IKK- β which is a major activator of NF- κ B, leads to decrease tumor size and reduced the production of inflammatory cytokines including TNF- α and IL-6. However, an increase of hepatotumorigenesis was reported with a deletion of IKK- β only in hepatocytes [92]. These paradoxical results highlight the double-edged sword role and complexity of NF- κ B signaling but clearly show the key role played by immune cells in shaping a favorable pro-tumor microenvironment.

9.2.3 *Cytokine Gene Polymorphisms Contribute to Altered Immune Response*

In addition to the environmental factors responsible for cytokine release during chronic liver diseases, alteration in cytokine or cytokine receptor gene expressions can occur and chronically dysregulate the inflammatory response leading to cancer development. Such alteration results from single nucleotide polymorphisms (SNPs) in coding or non-coding regions of the genes. Several cytokine gene polymorphisms have been recently reported, including IL-1 β [34], resulting in increased cytokine release. Furthermore, genetic polymorphisms of IL-6 are associated with fibrosis progression in chronic HCV infected patients [28]. Polymorphisms were identified in virtually all other cytokines such as TNF- α , IL-6, IL-10, TGF- β 1 and IFN- γ [33].

9.3 Cytokines in HCC Progression with Metastasis

Tumor microenvironment is shaped by myeloid and lymphoid cells including tumor infiltrating lymphocytes (TILs) responsible for the control of tumor growth (Fig. 9.1). According to their phenotypes myeloid and lymphoid cells will inhibit or promote tumor growth. Myeloid cells basically play a major role in the immune response against tumor by recognizing these tumor antigens and generating humoral and cellular specific immune responses. However, their ability in tumor-associated

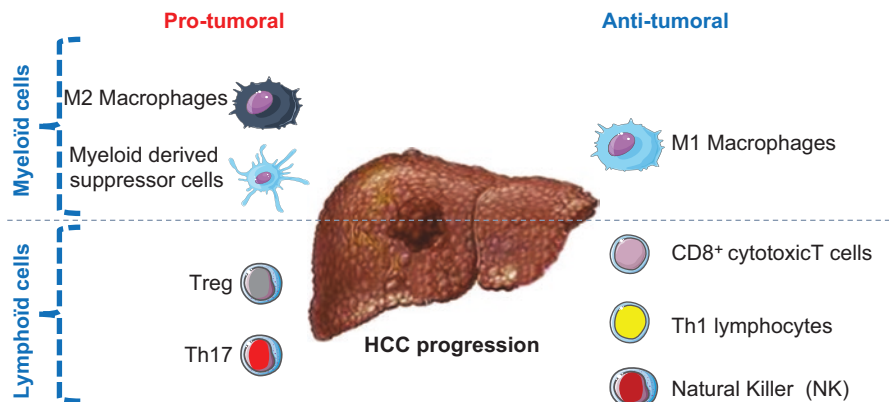


Fig. 9.1 Immune cell microenvironment of HCC. The HCC microenvironment is composed of pro-tumoral immune cells including M2 macrophages, myeloid derived suppressor cells, Treg and Th17 lymphocytes, and anti-tumoral cells gathering M1 macrophages, cytotoxic CD8 T cells, Th1 and NK cells

antigen recognition is often altered and lead to pro-tumoral properties of those cells. Among lymphoid cells, two types of TILs can be distinguished: anti-tumoral effector cells such as CD8⁺ lymphocytes which are associated with a better prognosis when they are highly present in the tumor, that are opposed to pro-tumoral cells such as regulatory T cells (Treg) which are associated with a poor prognosis.

9.3.1 Myeloid Immune Response in HCC Progression

Tumor Associated Macrophages (TAMs) also play a key regulatory role in tumor-related inflammation and angiogenesis. TAMs are also involved in tumor relapse by facilitating tumor regrowth, revascularization, and spread after anti-cancer therapies. TAMs are associated to tumor growth through the production of growth factors such as EGF, VEGF and bFGF. They contribute to the invasiveness of tumor cells and metastasis by favoring extracellular matrix remodeling via the release of metalloproteases 2 and 9 (MMP2 and MMP9). They also contribute to vascularize the tumor by increasing angiogenesis and lymphangiogenesis via MMP9, VEGF and PDGF synthesis [45].

Tumor-associated neutrophils (TAN) similarly to TAMs, have been described [93] CXCL8 chemokine production by tumoral cells in HCC are responsible for the chemotaxis of neutrophils in the stroma surrounding the nodules [83]. More recently, an analysis of 919 HCC identified an overexpression of the CXCL5 chemokine that correlated with an increase in neutrophil-infiltrating cells in the liver and a poor prognosis of the diseases [144]. Those recruited neutrophils favors tumoral progression in part by increase in ROS production (as mentioned earlier in this chapter)

[55]. Interestingly, neutrophil-derived ROS was associated with mutations and DNA damage [53] and activation of proteolytic enzymes including MMP-2, 7, 8, 9 and inactivation of the Tissue inhibitor metalloprotease-1 (TIMP-1) which consequently favour tumor invasiveness [31].

Myeloid Derived-Suppressor Cells (MDSCs) represent a heterogeneous population of cells sharing similarities with TAMs and TANs. They are often observed in HCC and their presence is associated with a poor prognosis. This population of cells is basically composed of main sub-types: monocytic MDSCs and granulocytic MDSCs. Under certain conditions they can adopt several phenotypes that control their ability to promote or restrain tumor progression. For instance, in hypoxic conditions or in presence of tumor -derived factions, MDSC can differentiate into immunosuppressive TAMs [24]. Furthermore studies reported in vitro their capacity to differentiate in a macrophage, DC or granulocyte phenotype [103]. However, in general, MDSCs are described as suppressor of T cell activation and therefore can alter T-cell mediated anti-tumor function and favour tumor progression [130].

9.3.2 *Lymphoid Immune Response in HCC Progression*

9.3.2.1 **Infiltrating CD8⁺ T Cells and Their Associated Cytokines**

CD8⁺ T cells are major actors in antitumor immunity through their antigen specific cytotoxicity capacities targeting tumor antigens. These latter are ingested by host antigen presenting cell such as dendritic cells and processed into peptides which are presented via class I and class II MHC molecules respectively to CD8⁺ and CD4⁺T cells. In many cancer including colorectal and ovarian cancers, an increased number of tumor infiltrating CD8⁺ T lymphocytes (TIL) predicts a favorable prognosis. Regarding HCC, a correlation has also been reported between the presence of TIL and patient prognosis [46]. Indeed, the penetration of CD8⁺ T cells is correlated to an improved recurrence-free survival after liver resection [59]. These beneficial effects are explained by the inflammatory microenvironment generated by CD8⁺ effector T cells within the tumor leading to the establishment of an anti-tumor response. Studies have shown in murine models that through IL-12 stimulation, CD8⁺ T cells were activated which induce IFN- γ release leading to increased hepatoma cell apoptosis [76].

Recent findings [39] have highlighted CD8⁺ T specific responses targeting tumor-associated antigens (TAA) in HCC. It has been shown that TAA-specific CD8⁺ T cell immune response was visible in more than 1 out of 2 HCC patients and already detectable in early stages of the disease. Consistent with the correlation between improved progression-free survival TAA-specific CD8⁺ T responses these results comfort the major role played by these cells in anti-tumor immunity.

However, in some patients with HCC, impaired functions of CD8⁺ T cells have been reported [49]. Indeed, tumors develop mechanisms to escape to immune

surveillance; one of them is the up-regulation of the ligand for PD-1 (PD-L1) responsible for addressing an inhibitory signal to PD-1 expressing cells namely CD8⁺ and CD4⁺ T cells [42]. This PD1/PD-L1 interaction leads to T cell inactivation and consequently, to the inhibition of their anti-tumor function and ultimately to the promotion of tumor aggressiveness. PD-L1 expression in HCC has recently been characterized and its crucial role in HCC progression has been strongly suggested [14].

Interestingly, it has been observed in HCC patient cohort that even with increased peripheral and intratumor PD-1 expression on CD8⁺ T cells, tumor cells were also rich in PD-L1 expression. These findings thus showed a correlation between a high PD-L1 expression within the tumor and a poorer outcome with early HCC recurrence after liver resection because of the induction of CD8⁺ T cell apoptosis [120]. The challenges of next studies will be to determine the mechanisms by which tumors promote PD-L1 expression on tumor cells and to find strategies to bypass the inhibitory signal delivered to PD-1 expressing cells including CD8⁺ T lymphocytes.

9.3.2.2 IL-17-producing Cells

IL-17 is in majority produced by Th17 lymphocytes and targets a large variety of cells through its ubiquitously expressed receptor IL-17RA. However, other IL-17-producing cell types have been identified including $\gamma\delta$ T cells or neutrophils [113]. It has been reported that in HCC IL-17 levels were increased compared to non-tumor tissues [89]. Furthermore, a positive correlation has been established between high expression of IL-17 and microvessel density in tissues and poor survival in patients with HCC [143] suggesting that IL-17 may promote HCC growth by promoting angiogenesis.

In addition, neutrophils detected inside HCC tumors are associated with a poor recurrence-free survival for patients with HCC after liver resection. Peritumoral neutrophils promote angiogenesis leading therefore to stimulate tumor growth [81, 82]. More surprisingly, a study has shown that IL-17 can recruit neutrophils. Peritumoral tissue was also found enriched in Th17 lymphocytes which number is correlated with tumor activated monocytes that have been reported to induce IL-17 producing cells proliferation [81, 82]. Despite the positive correlation between IL-17 producing cells and poor survival in HCC patients, the underlying mechanisms by which these cells lead to HCC progression remain poorly defined.

9.3.2.3 Inflammation-Induced Epithelial Mesenchymal Transition (EMT)

Physiologically, EMT is a key step during embryogenesis, pathological events, inflammation but it can also trigger metastasis development in cancer context [9, 70]. During this process, morphological modifications occurs in epithelial cells

which adopt a fibroblast-like phenotype. Through a complex cytoskeleton reorganization, many intercellular junctions characterizing epithelial cells are lost such as desmosomes, adherent junctions, tight junctions and gap junctions. EMT thus promotes epithelial markers loss including E-cadherin in favor of an induction of fibroblast markers such as fibronectin, matrix metalloproteinase. Such transformation allows those transformed cells to leave the original tissue and colonize other tissue through the blood circulation. TGF- β is one of the most relevant inflammatory mediator involved in EMT. It is considered as a key factor in embryogenesis but also in fibrosis and cancer development in many models [35, 138] through SMAD2, SMAD3 and SMAD4 [119, 129]. Studies have brought evidence that inflammation promotes EMT via the induction of pro-inflammatory cytokines. It has been shown that together TNF- α and IL-6 enhance TGF- β signaling pathways which stimulate EMT [7]. These two cytokines are known to trigger NF- κ B which induces many factors implicated in EMT. Lastly, ROS synthesis has also been shown to induce EMT [131]. Exploring more deeply the involvement of cytokines in EMT in a context of CHC could open new therapeutic options.

9.4 HCC Therapeutic Failure and Cytokine-Based Therapy

At late stage, patients are not eligible for surgical resection of the tumor or for liver transplant, and the efficacy of classical radiotherapy and chemotherapy is very poor. Since 2008, the SHARP (Sorafenib HCC Assessment Randomised Protocol) trials combining multikinase inhibitory and anti-angiogenic properties is considered as a standard for advanced HCC and showed an improved overall survival in Child-Pugh class A patients with advanced HCC upon treatment. HCC-patients given Sorafenib have a longer progression-free survival (PFS) with a median overall survival reaching 10.7 months in sorafenib treated patients vs 7.9 months in the placebo patients [90]. Moreover, the high capacity of resistance of CSC to sorafenib therapy suggests that CSCs may contribute to the poor prognosis.

In HCC many cytokine levels are deregulated leading to promote or inhibit carcinogenesis. The development of combined therapy like IFN- α with ribavirin has markedly reduced HCC incidence. However antiviral therapies only help to delay the development of HCC. More and more anti-tumor therapeutic options use strategies to regulate cytokines levels or modulate immune cell activity. Loco-regional immune-chemotherapy based on lymphokine-activated killer cells (LAK) is a relevant approach in HCC treatment. LAKs release many cytokines including IFN- γ , IL-2 and IL-12 promoting cytolytic activity against tumor cells [78]. Many studies also proposed to enhance cytokine responses and more specifically to the liver. A murine HCC model was developed with adenoviral vector carrying IL-12 leading to reduce tumor growth and induce immune infiltration potentially responsible for the inhibition of angiogenesis [2]. Consistently, IL-12 intrahepatic administration in BALB/c has shown early infiltration of lymphocytes and macrophages resulting in a reduction of tumor progression [108]. Furthermore, combined immunotherapy in

a murine model of HCC based on IL-12 and GM-CSF triggered a powerful antitumor response and avoid the side-effect of IL-12 treatment alone [132].

9.5 Conclusion

Although liver inflammation is critical for protection against infections and for triggering liver regeneration mechanisms, it must be finely tuned and “turned off” right after the clearance of the pathogens and the achievement of tissue repair. Indeed, excessive and recurrent liver inflammation is a common process observed in livers of alcoholic patients and in non-alcoholic steatohepatitis, in drug and chemical intoxication, during viral and bacterial infection, as well as in certain idiopathic liver pathologies such as autoimmune hepatitis. A high variety of immune cells can infiltrate the liver tissue (Fig. 9.2). Their quantity and their activity defined by their ability to produce a large spectrum of cytokines, depend on the underlying chronic liver disease. One of the major challenges in the liver field is to understand the cellular and molecular mechanisms underlying the chronic inflammatory processes associated with acute and chronic injury. The recent advances in immunology field

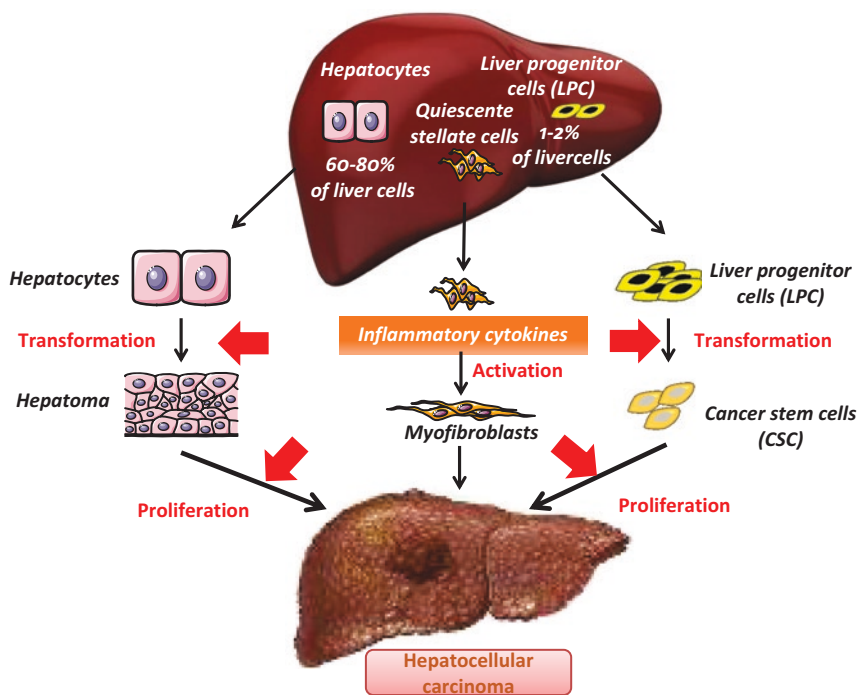


Fig. 9.2 Influence of cytokines in HCC initiation and progression. Inflammatory cytokines are responsible for stellate cell activation and fibrosis development (IL-6, TGF- β , IL-1 β), liver progenitor cell expansion (IL-6, TNF- α , IL-22) and DNA alteration via ROS production

sheds some light on the important role of cytokine milieu in which tumoral cells can emerge and proliferate. This also demonstrates how complex and heterogeneous is the liver inflammatory response according to the etiology leading to HCC. This strongly suggests that a better characterization of the inflammatory process would allow developing a personalized medicine for patients, and would constitute a promising strategy in HCC prevention and cure.

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