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Tumor regulation of the tissue environment in the liver

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Abstract

The tumor microenvironment (TME) in the liver plays an important role in primary and metastatic liver tumor formation and tumor growth promotion. Cellular and non-cellular components of the TME significantly influence tumor development, growth, metastatic spread, anti-tumor immunity and response to tumor therapy. The cellular components of the TME in the liver not only consist of infiltrating immune cells, but also of liver-resident cells such as liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC), which promote tumor growth by negatively regulating tumor-associated immune responses. In this review, we characterize cells of the TME with pro- and anti-tumor function in primary and metastatic liver tumors. Furthermore, we summarize mechanisms that permit growth of hepatic tumors despite the occurrence of spontaneous anti-tumor immune responses and how novel therapeutic approaches targeting the TME could unleash tumor-specific immune responses to improve survival of liver cancer patients.

Keywords

Liver immunology; tumor immunology; hepatocellular carcinoma; liver metastases; immunotherapy; tumor microenvironment

1. Introduction

The TME of solid tumors has gained enormous attention in recent years, as it has become clear that its cellular and non-cellular components significantly influence tumor development and growth, metastatic spread, anti-tumor immunity and response to tumor therapy (Hanahan & Weinberg, 2011; Joyce & Fearon, 2015). The cellular component of the TME can be subdivided into immune cells and non-immune cells. Lymphocytes and myeloid cells form the immune cell compartment and endothelial cells, pericytes and

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Conflict of Interest

The authors declare that there are no conflicts of interest.

fibroblast form the non-immune cell compartment of the TME. Importantly, the liver contains specialized endothelial cells and specialized fibroblast, namely LSEC and HSC, respectively, that perform immune functions (Jenne & Kubes, 2013; Thomson & Knolle, 2010). All of these aforementioned cell types can influence the tumor and in turn can be influenced by the tumor through a variety of mechanisms. Thus, understanding the tumor-promoting, therapy-inhibiting mechanisms of the TME is instrumental for the design of successful treatment approaches.

In the liver, it is important to distinguish between the TME of primary hepatic tumors, commonly residing in inflamed liver tissue, and the TME of liver metastases of non-hepatic tumors, residing in otherwise healthy liver tissue. Hepatic immune cells play an important role in primary liver cancer formation and growth and also in 'preparing' the liver for metastatic seeding of tumors from different primary sites. In patients with liver metastasis but without underlying liver disease, the primary tumor, e.g. colon carcinoma, recruits myeloid cells to the liver to enable metastatic seeding of colon cancer cells (L. Zhao, et al., 2013). These myeloid cells also induce down-regulation of angiopoietin-like 7 protein in cancer cells to promote angiogenesis and metastasis formation (Lim, et al., 2015). Moreover, hepatic resident non-immune cells can also support metastasis development in the liver. Because of the liver's unique anatomical location and metabolic functions in the human body, the resident immune and non-immune cells of the liver are endowed with distinctive functional properties (Thomson & Knolle, 2010). For example, LSEC sample the blood, which contains nutrients and microbial antigens from the gut, and induce T cell tolerance to antigens to which no preexisting immunity exists (Schurich, et al., 2009). However, the same mechanism also leads to hepatic tolerance towards tumor-associated antigens and enables metastatic spread into the liver (Arteta, et al., 2010; Hochst, et al., 2012), illustrating one non-immune cell-mediated mechanism of tumor immune escape that is unique to the liver.

The TME of hepatocellular carcinomas (HCC), the most frequent primary liver cancer, is even more complex, because HCC usually develop in chronically inflamed livers. Indeed, chronic liver inflammation, induced by non-alcoholic steatohepatitis (NASH) or viral hepatitis, can progress to liver cirrhosis and eventually to carcinomas (El-Serag & Rudolph, 2007). Therefore, both the tumor and the underlying chronic liver inflammation shape the TME of HCC. This is particularly important for the function of myeloid cells. Macrophages, for example, can prevent outgrowth of HCC in otherwise healthy livers by clearing precancerous hepatocytes (Kang, et al., 2011), but they can also initiate HCC development in inflamed livers (Lanaya, et al., 2014). The chronic inflammation that drives liver cancer formation is also responsible for the regular occurrence of tumor neoantigens, which can trigger an anti-tumor T cell immune response (Schumacher & Schreiber, 2015). Consequently, tumor antigen-specific T cells can infiltrate HCC and several HCC-associated epitopes that are recognized by the infiltrating T cells have been identified (Flecken, et al., 2014; Mizukoshi, et al., 2011). Despite the recognition of and infiltration into the tumor by the immune system, HCC remains a cancer with a high mortality and poor 5-year survival (Njei, Rotman, Ditah, & Lim, 2015). This suggests that potent tumor-associated immunosuppressive mechanisms are in place to escape the immune response in patients with HCC. Indeed, myeloid-derived suppressor cells (MDSC) and regulatory T cells (T reg) are commonly found immune suppressive cells in HCC (Fu, et al., 2007; Hoechst, et al., 2008).

Together, these findings underscore the significance of immune cells in primary and metastatic tumor development and tumor control in the liver (Figure 1).

Thus, the tumor, immune cells and non-immune cells, like LSEC, regulate pro and anti-tumor immune responses in hepatic cancers. In this review, we will highlight the mechanisms that tumors employ to inhibit anti-tumor immune responses. Furthermore, we will demonstrate how hepatic immune cells and non-immune cells contribute to creating an immunosuppressive TME that promotes tumor immune escape and tumor growth. Strategies to therapeutically modulate hepatic immune cell functions will also be discussed (Table 1).

2. Cells of the hepatic tumor environment and their regulation of tumor growth

2.1 Anti-tumor immune cells in the liver

Three immune cell subsets have been shown to be capable of killing tumor cells and eradicating established tumors, namely cytotoxic T cells (CTL), Natural Killer cells (NK cells) (Figure 2) and macrophages (resident macrophages in the liver are also termed Kupffer cells) (Chao, Weissman, & Majeti, 2012; Pardoll, 2012; Vivier, Ugolini, Blaise, Chabannon, & Brossay, 2012). As discussed below, the first two mentioned subsets are considered to be exclusively anti-tumorigenic in liver cancer. However, because of their plasticity, macrophages can have both pro- and anti-tumor effector functions. After tumor formation, tumors skew macrophages towards an immunosuppressive tumor-promoting phenotype. Therefore, we discuss these cells in the section “Tumor promoting cells in the hepatic tumor environment” below. Similarly, dendritic cells can exert pro- as well as anti-tumor functions. Although dendritic cells do not directly cause tumor cell death, they initiate the anti-tumor T cell response and are therefore discussed in “anti-tumor immune cells in the liver”, in the context of CTL.

Cytotoxic T cells (CTL)—Unleashing anti-tumor T cell responses in some solid cancers, primarily melanoma, through so-called ‘checkpoint-inhibitors’, like anti-CTLA-4 and anti-PD-1 antibodies, have recently produced astounding results in a fraction of cancer patients, achieving long-term remissions (Robert, et al., 2015; Schadendorf, et al., 2015). These results uncovered the huge potential that T cell responses against tumors have. The reason as to why this treatment is only successful in some cancers has been partially attributed to the frequency of somatic mutations in tumor cells that generate neoantigens, which allow for the induction of an anti-tumor T cell response (Schumacher & Schreiber, 2015). These tumor-associated antigens enable recognition of the tumor whereas ‘Checkpoint-inhibitors’ disinhibit T cells by interfering with the inhibitory signal that would otherwise be relayed through receptors on T cells, which down-modulate the amplitude of T cell activation (Pardoll, 2012).

In HCC patients, tumor-specific T cell responses against numerous HCC-associated antigens have been reported and patients with tumor-specific T cell responses have improved progression-free survival (Flecken, et al., 2014; Mizukoshi, et al., 2011). Furthermore, local ablative therapies like radiofrequency ablation or transarterial chemoembolization, which are

primary HCC treatment modalities, enhance the anti-tumor T cell response by triggering tumor cell death (Hiroishi, et al., 2010; Mizukoshi, et al., 2013). In one study, radiofrequency ablation not only enhanced the number of tumor-specific T cells, it also induced responses against additional tumor-associated antigens, which were not detectable before ablation (Mizukoshi, et al., 2013), indicating that T cells can successfully recognize HCC. However, in patients with spontaneous tumor-specific T cell responses, the function of these T cells is impaired, but their activation could be enhanced *in vitro* by administration of anti-CTLA-4 antibodies (Mizukoshi, et al., 2011). ‘Checkpoint-inhibitors’ are therefore plausible treatment approaches in HCC patients and are currently evaluated in clinical trials using the anti-CTLA-4 antibody (Duffy AG, et al., 2015; Sangro, et al., 2013).

Anti-PD-1 antibody is another ‘checkpoint-inhibitor’ with the potential to improve patient survival. Expression of PD-L1, the ligand for PD-1, was increased on peritumoral monocytes (Kuang, et al., 2009) and intratumoral Kupffer cells (K. Wu, Kryczek, Chen, Zou, & Welling, 2009) of HCC patients. Increased expression of PD-L1 on either monocytes or Kupffer cells was also associated with poorer survival, indicating that HCC patients could benefit from anti-PD-1 antibody therapy as well. Indeed, a Phase I/II trial studying anti-PD-1 antibody in patients with advanced HCC showed promising results (El-Khoueiry AB, et al., 2015). 18% of patients had a partial response and 5% had a complete remission, which lasted 14 – 17+ months.

A third albeit less studied inhibitory ligand on Kupffer cells within HCC tumors is Galectin-9, which interacts with Tim-3 on T cell (Li, et al., 2012), causing reduced T cell activation and worsening of prognosis. However, antibodies blocking Tim-3 are still in preclinical evaluation and have not yet reached clinical trials. A similar but slightly different approach to enhance anti-tumor T cell responses is to activate surface molecules that directly stimulate T cells instead of preventing T cell inhibition. Examples are 4-1BB and OX40, which are stimulatory receptors on T cells (L. Chen & Flies, 2013). In a murine orthotopic liver tumor model, the triple combination of anti-PD-L1 antagonistic antibody and anti-4-1BB and anti-OX40 agonistic antibodies lead to significant improvement of survival, which was dependent on tumor infiltrating T cells (Morales-Kastresana, et al., 2013).

In summary, patients with HCC develop spontaneous anti-tumor T cell responses, which, however, do not lead to tumor growth control. Enhancing these T cell responses through novel cancer immunotherapy approaches might unleash their full potential, significantly improving patient survival.

Dendritic cells—In order for CTL to carry out their effector function of killing tumor cells, T cells require careful stimulation and subsequent activation to exert their tumor-specific anti-tumor properties. Dendritic cells provide these cues, i.e. antigen-specific T cell receptor engagement, co-stimulatory molecule activation and paracrine cytokine signaling, necessary for proper anti-tumor T cell function. Following phagocytosis of tumor-associated antigens, dendritic cells themselves are activated through pattern recognition receptors, such as toll-like receptors, or cytokine receptor signaling. This enables dendritic cells to induce a tumor-specific T cell response by presenting epitopes of the captured antigens to CTL. Because of their central role in initiating this response, efforts have been made to employ

dendritic cells therapeutically to improve spontaneously occurring T cell responses. In patients with HCC, one such effort is to enhance phagocytosis of tumor-associated antigens and dendritic cell activation in situ by causing tumor cell death and release of antigens through local tumor ablation, e.g. radiofrequency ablation (Ali, et al., 2005). Another approach is to expand and activate autologous dendritic cells in vitro and reinfuse these cells into the tumor following transarterial chemoembolization. This treatment yielded prolonged recurrence-free survival in HCC patients, albeit in comparison with historical controls (Nakamoto, et al., 2011). While dendritic cells capture antigens released by tumor cells in situ, it is also possible to manipulate dendritic cells in vitro to direct the ensuing T cell response against a specific antigen. Heat-shock protein 70 was reported to be overexpressed in hepatitis C virus-related HCC. Thus, a phase 1 clinical trial, in which dendritic cells transfected with heat-shock protein 70 messenger RNA were given to patients with hepatitis C virus-related HCC, showed promising clinical responses, including complete remission in two patients (Maeda, et al., 2015). Nevertheless, as of now, large clinical trials of dendritic cell-based therapies in HCC to corroborate these findings are missing.

NK cells—Although novel cancer immunotherapies targeting T cell have been in the limelight recently, preclinical data suggest that NK cells are also capable of controlling HCC growth and therefore constitute another anti-tumor immune cell with promising implications for HCC immunotherapy. In a study using 3 independent HCC patient cohorts, gene expression analysis and immunohistochemistry of tumors revealed a superior survival of patients with high intratumoral NK cell accumulation (Chew, et al., 2012). These results were confirmed in another independent study, which in addition showed that NK cell frequency and function decreased in advanced disease stages (Y. Wu, et al., 2013). In contrast to T cells, NK cells do not recognize tumors through tumor-associated antigen expression on the tumor cell surface. Rather, NK cells possess a fine-tuned equilibrium of activating and inhibiting receptors on their surface. Depending on ligand expression by target cells, signaling through combinations of these NK cell receptors either leads to activation or control of NK cell cytotoxicity (Long, Kim, Liu, Peterson, & Rajagopalan, 2013). Accordingly, in patients with HCC, reduced expression of the ligand for NKG2D, an activating NK cell receptor, is associated with tumor progression and early tumor recurrence (Kamimura, et al., 2012). These data illustrate the significance of NK cells in controlling liver cancer growth.

However, given the high mortality and poor survival of primary liver cancer patients, these tumors ultimately also evade NK cell cytotoxicity. Accordingly, many NK cell-inhibiting mechanisms in HCC patients have been uncovered. In addition to the cited mechanism of NKG2D ligand down-regulation on cancer cells, HCC cell can also shed these ligands, causing elevated NKG2D ligand levels in blood of HCC patients and subsequent down-regulation of NKG2D expression on NK cells (Jinushi, et al., 2005). Not only the cancer cell itself, but also tumor-infiltrating MDSC and monocytes can inhibit NK cell activation by blocking activating-receptors Nkp30 and 2B4 on NK cells (Hoechst, et al., 2009; Y. Wu, et al., 2013). Pharmacologically, the NKG2D ligand down-regulation and the ligand shedding can be reversed *in vitro* by a proteasome inhibitor and sorafenib, respectively (Kamimura, et

al., 2012; Kohga, et al., 2010). However, the biological *in vivo* significance of these findings has not yet been investigated.

Similarly to ‘Checkpoint-inhibitors’ for T cell activation, antibodies that block inhibitory receptors on NK cells have entered clinical trials. One such antibody, lirilumab, targets the killer cell immunoglobulin-like receptor, KIR, and is currently being evaluated in a phase I trial recruiting, among others, HCC patients (Segal NH, et al., 2014). Therefore, NK cells, like T cells, can recognize and infiltrate HCC, affecting tumor growth. However, pharmacological activation is necessary to tap their full potential to overcome the tumor-mediated resistance mechanisms in order to improve survival of HCC patients.

2.2 Tumor promoting cells in the hepatic tumor environment

Immune cells as well as non-immune cells play an important role in tumor-induced immune evasion in patients with liver cancer. As described above, T cells and NK cells have the potential to control growth of HCC or even eradicate established tumors. Despite their potential, survival of HCC patients is poor, indicating that vigorous immunosuppressive mechanisms are in place to protect the tumor from immune attack. Indeed, liver cancers induce a plethora of cellular and non-cellular mechanisms to evade the immune system. Here, we will highlight the tumor-elicited cellular mechanisms that aid growth and progression of liver cancers (Figure 3).

Myeloid cells/MDSC—MDSC have been shown to accumulate in cancer patients of virtually all tumor types, in which they can suppress innate and adaptive anti-tumor immune responses (Poschke & Kiessling, 2012). Likewise in patients with HCC, MDSC suppress T cell proliferation, inhibit NK cell activity and induce tumor-promoting T reg (Hoechst, et al., 2008; Hoechst, et al., 2009). To suppress T cell proliferation, MDSC express the enzyme arginase to deprive T cells of the amino acid arginine, which is necessary for T cell proliferation. Consequently, low levels of arginine induce cell cycle arrest of T cells (Rodriguez, Quiceno, & Ochoa, 2007). As noted above, NK cell activity is inhibited by engagement or blockade of inhibitory or activating NK cell receptors, respectively (Hoechst, et al., 2009). The poor overall survival of HCC patients despite spontaneous anti-tumor T cell and NK cell responses against HCC suggests that potent immunosuppressive cells, like MDSC, are significant contributors to the tumor immune escape. Accordingly, MDSC accumulate in peripheral blood and within tumors of HCC patients (Hoechst, et al., 2008). Furthermore, with disease progression, the frequency of MDSC in peripheral blood increases and elevated numbers of MDSC correlate with shorter recurrence-free survival of HCC patients after local ablative therapies (Arihara, et al., 2013), confirming a negative association of MDSC accumulation and tumor control; illustrating their significance for tumor immune escape in patients with liver cancer.

MDSC, or myeloid cells in general, not only promote growth of primary liver cancer by inhibiting anti-tumor immune responses, but they also play an important role in “paving” the way for the development of metastases in the liver. It has been well established, that before metastatic seeding occurs in organs like lungs and livers, tumors “prepare” these organs by expanding metastasis-enabling myeloid cells (Kaplan, et al., 2005). In mice with pancreatic

intraepithelial neoplasia, which commonly develop liver metastases upon progression to full-blown pancreatic cancer, accumulation of hepatic immunosuppressive myeloid cells, i.e. MDSC, precedes and promotes metastatic spread into the liver (Connolly, et al., 2010). In summary, tumor-induced expansion of myeloid cells/MDSC in the liver is crucial for escaping anti-tumor immune responses and facilitating metastatic seeding. Therefore, pharmacological inhibition of MDSC/myeloid cell accumulation could improve spontaneous or therapeutically induced anti-tumor immune responses, prevent hepatic metastasis formation and lead to improved patient survival.

The accumulation of immunosuppressive MDSC is mediated by tumor-secreted cytokines and/or chemokines (Gabrilovich & Nagaraj, 2009). G-CSF, GM-CSF, IL-6, CCL2 and VEGF are commonly found in tumor-bearing hosts and also cause MDSC expansion in HCC-bearing mice (Eggert, et al., 2014; Kapanadze, et al., 2013) and humans (Arihara, et al., 2013). These cytokines and chemokines induce proliferation of immature myeloid cells in the bone marrow and subsequent migration to the tumor. In patients with hepatic metastases from colorectal cancers, the expansion of metastasis-enabling myeloid cells in the liver is triggered by tumor-produced chemokines like CCL2 or CCL15 (Itatani, et al., 2013; L. Zhao, et al., 2013), suggesting that interfering with any of these pathways could reduce MDSC accumulation. Indeed, blocking MDSC-inducing cytokine or chemokine receptors has been shown to decrease their numbers in different animal tumor models and enhances efficacy of T cell immunotherapy (Highfill, et al., 2014; Mok, et al., 2014). For example, in a murine model of rhabdomyosarcoma, MDSC express the chemokine receptor CXCR2 and blocking this receptor by anti-CXCR2-monoclonal antibody therapy enhanced anti-PD-1 antibody treatment success (Highfill, et al., 2014). Another factor frequently involved in MDSC expansion is GM-CSF. Accordingly, neutralizing GM-CSF via antibody administration in a murine liver metastases model improved efficacy of chimeric-antigen receptor (CAR) T cell therapy (Burga, et al., 2015).

It is important to note however, that other mechanisms inducing MDSC accumulation and conferring immunosuppressive function, especially within the tumor, have been reported and could potentially limit the efficacy of the aforementioned treatment approaches to decrease MDSC numbers. For example, endoplasmic reticulum stress (ER-stress) in combination with hypernutrition causes NASH and subsequent HCC development, indicating that ER-stress contributes to primary liver cancer initiation. On the other hand, ER-stress also triggers MDSC expansion and enhances immunosuppressive function (Condamine, et al., 2014; Thevenot, et al., 2014). Additional tumor-associated mechanisms altering MDSC function or triggering accumulation, independent of cytokine receptor signaling, include tumor-associated hypoxia and tumor-derived lactate (Corzo, et al., 2010; Husain, Huang, Seth, & Sukhatme, 2013). Hypoxia within tumors can skew tumor-infiltrating MDSC into more potently immunosuppressing tumor-associated macrophages (TAM). In contrast, tumor-derived lactate enhances MDSC expansion and NK cell dysfunction. In summary, numerous pathways exist how tumors regulate MDSC expansion and function, either directly by secreting cytokines or indirectly by factors that are characteristic for the tumor microenvironment, like hypoxia, high lactate levels or ER-stress. Additional approaches to alleviate MDSC-mediated immunosuppression could therefore further enhance the efficacy of cytokine neutralization or cytokine receptor blockade. Approaches include depletion of

MDSC or restraining their immunosuppressive phenotype. Multiple molecules have been studied for their therapeutic effect on MDSC. These have been recently summarized in comprehensive reviews discussing MDSC biology in the liver and approaches to target MDSC in general (Marvel & Gabrilovich, 2015; Medina-Echeverz, Eggert, Han, & Greten, 2015). Approaches to deplete MDSC include low-dose gemcitabine or 5-fluorouracil treatment and approaches to inhibit their immunosuppressive function include molecules that interfere with ROS and NO production by MDSC, such as PDE-5 inhibitors or nitric oxide-releasing aspirin (De Santo, et al., 2005; Serafini, et al., 2006; Suzuki, Kapoor, Jassar, Kaiser, & Albelda, 2005; Vincent, et al., 2010).

Macrophages—In contrast to MDSC, macrophages naturally reside in healthy livers, where they are termed Kupffer cells. Upon liver injury, these liver resident macrophages proliferate and accumulate. Nevertheless, circulating monocytes also migrate to the liver and proliferate in response to inflammatory triggers; thereby contributing to the overall hepatic macrophage pool, i.e. resident macrophages plus monocyte-derived macrophages (Lavin, Mortha, Rahman, & Merad, 2015). It is still unclear, whether macrophage origin (resident vs. monocyte-derived) in the TME shapes their function and if this causes different macrophage responses against tumors. Therefore, we collectively summarize findings herein on macrophages in the hepatic TME independent of cell origin.

Because of their plasticity, i.e. polarization between a pro-inflammatory (M1) and anti-inflammatory (M2) phenotype, macrophages can have both pro- and anti-tumor effects (Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). Precancerous hepatocytes overexpressing oncogenic Nras in an otherwise healthy liver are cleared by macrophages, preventing outgrowth of aggressive HCC (Kang, et al., 2011); exemplifying one mechanism of macrophage-mediated anti-tumor function. Macrophages can also recognize and phagocytose tumor cells upon down-regulation of CD47, a so-called “don’t eat me”-signal, on the tumor cell surface. However, many tumors up-regulate CD47 as an immune escape mechanism, including HCC (Xiao, et al., 2015). Accordingly, a study found that antibody-mediated blockade of CD47 enhances macrophage killing of HCC tumor cells. However, this study did not take into account the inflammatory environment of primary liver cancers, because tumor cells were injected into healthy livers. Macrophage polarization is strongly affected by the tumor-surrounding milieu and liver diseases that precede HCC development, like fibrosis, are associated with the tumor-promoting M2 macrophage phenotype (Sica, Invernizzi, & Mantovani, 2014). Hence, several lines of evidence suggest that macrophages in inflamed livers in mice and humans actually support growth of liver cancers, for example, by secreting the pro-inflammatory cytokine interleukin-6, which induces proliferation of hepatocytes (Lanaya, et al., 2014; Zhu, et al., 2008). If antibody-mediated targeting of CD47 also enhances cancer killing in inflamed livers, remains to be investigated.

Like MDSC or myeloid cells in general, TAM can inhibit both T cell and NK cell responses against HCC through expression of inhibitory receptors on their cell surface (Li, et al., 2012; Y. Wu, et al., 2013; Yan, et al., 2015). As discussed in the section on CTL, these receptors are possible targets for immunotherapeutic agents. Furthermore, macrophage numbers can increase in livers harboring HCC through mechanisms that induce proliferation and recruitment analogous to MDSC, leading to poorer survival in HCC patients (Zhu, et al.,

2008). These mechanisms involve cytokines and chemokines like M-CSF and, as in MDSC, can be targeted pharmacologically. Accordingly, interfering with these pathways could dampen tumor-promoting effects of myeloid cells, i.e. TAM and MDSC.

In addition to their immunosuppressive properties, macrophages can promote tumor growth through a direct effect on the tumor cells themselves. It is well established that they induce epithelial-mesenchymal transition of tumor cells and thereby enhance aggressiveness and metastatic spreading of cancers, including HCC (Fan, et al., 2014; Xia, et al., 2014; Yeung, et al., 2015). Thus, macrophages can promote tumor growth through inducing proliferation and/or aggressiveness of cancer cells and through restraining anti-tumor immune responses.

Regulatory T cells (T reg)—In contrast to MDSC, which are believed to accumulate only in pathological settings, T reg possess important physiological functions in healthy humans, because dysfunctional T reg can be a cause of autoimmune diseases, allergy and other immunopathologies (Sakaguchi, Miyara, Costantino, & Hafler, 2010). However, tumors can take advantage of their strong immunosuppressive abilities to evade immune attack. Like with MDSC, T reg accumulate in HCC tumors and accumulation increases with disease progression. T reg also suppress anti-tumor CTL and an increase of the CTL to T reg ratio is an independent prognostic factor for improved survival (Gao, et al., 2007). Moreover, elevated levels in peripheral blood of HCC patients are associated with poorer overall survival (Fu, et al., 2007; X. H. Yang, et al., 2006). Analogous to MDSC, T reg are associated with suppression of T cell responses and aiding liver metastases development in colorectal cancers patients (Pedroza-Gonzalez, et al., 2013). Furthermore, T reg can curb the efficacy of tumor immunotherapy. In an orthotopic murine model of primary liver cancer, non-responders to interleukin-12 therapy had increased intratumoral T reg numbers and interleukin-10 levels (Zabala, et al., 2007). T reg inhibit T cells either in a contact dependent manner, through various membrane-bound molecules like TGF- β (Nakamura, Kitani, & Strober, 2001) or CTLA-4 (Peggs, Quezada, Chambers, Korman, & Allison, 2009; Wing, et al., 2008) or by secreting immunosuppressive cytokines like interleukin-10 and interleukin-35 (Collison, Pillai, Chaturvedi, & Vignali, 2009; Collison, et al., 2007). In summary, despite being of different cellular origin (lymphocytic vs. myeloid), T reg and MDSC share similarities in that they both dampen anti-tumor immune responses and promote growth of primary and metastatic liver cancer, although their effector mechanisms of immunosuppression may differ.

Two mechanisms responsible for T reg accumulation within tumors have been proposed (Bilate & Lafaille, 2012). Either, pre-existing Treg can be recruited by the tumor and proliferate locally (Curiel, et al., 2004; Ghiringhelli, et al., 2005), or tumors induce the conversion of conventional CD4⁺ T cells to T reg (Liu, et al., 2007; Valzasina, Piconese, Guiducci, & Colombo, 2006). In support of the latter concept, tumor-induced MDSC from HCC patients can also prompt a phenotypic switch from conventional CD4⁺ T cells to immunosuppressive T reg (Hoechst, et al., 2008). However, it is likely that both mechanisms exist in parallel and contribute to the overall expansion of tumor-associated T reg (Zhou & Levitsky, 2007). In any case, TGF- β has been identified to be a key cytokine for the induction of T reg in cancer bearing hosts (Ghiringhelli, et al., 2005; Liu, et al., 2007; Zhou

& Levitsky, 2007). Thus, several mechanisms of intratumoral T reg induction exist, ultimately promoting tumor immune escape and facilitating cancer growth.

Efforts have been made to pharmacologically reduce T reg numbers or modulate their function. Because of its central role in not only inducing T reg but also mediating their immunosuppressive function (see above), targeting TGF- β is a promising approach to ease the detrimental effects of intratumoral T reg accumulation and several molecules and antibodies are in different stages of preclinical and clinical evaluation (Flavell, Sanjabi, Wrzesinski, & Licona-Limon, 2010). Another strategy to minimize T reg-mediated immunosuppression is anti-CTLA-4 antibody therapy. Anti-CTLA-4 antibodies do not only disinhibit CTL, but also restrain the suppressive abilities of CTLA-4 expressing T reg. Blockade of CTLA-4 on both T cell subsets contributes to the anti-tumor activity (Peggs, et al., 2009) and could potentially lead to enhanced anti-tumor T cell responses in patients with HCC (Duffy AG, et al., 2015). Other approaches to target T reg, of which only a few are mentioned here, include low-dose cyclophosphamide administration (Greten, et al., 2010) and toll-like receptor stimulation (Nishikawa, et al., 2006; Y. Yang, Huang, Huang, & Pardoll, 2004).

Liver sinusoidal endothelial cells (LSEC)—LSEC are specialized endothelial cells that line the hepatic sinusoids. Fenestration of LSEC allows for substrate transfer from the sinusoidal blood to hepatocytes and vice versa. LSEC have a high endocytosis rate and take up macromolecules from the sinusoidal blood by scavenger receptors (Smedsrod, et al., 2009). Moreover, LSEC have also important immunological functions. LSEC can efficiently cross-present antigens to CD8 T cells. Cross-presentation, the ability of antigen-presenting cells to present extrinsic peptides on their MHC-I molecule for interaction with CD8 T cells, is usually attributed to macrophages and dendritic cells only. Using this pathway, LSEC induce tolerance to CD8 T cells towards antigens from sinusoidal blood (Limmer, et al., 2000). However, virally infected LSEC can sufficiently induce T cell immunity, even in the absence of dendritic cells (Kern, et al., 2010).

In tumor-bearing mice, LSEC can induce CD8 T cell tolerance towards circulating antigen from apoptotic tumors cells (Berg, et al., 2006). Tolerance towards common antigens associated with colorectal cancer, like carcinoembryonic antigen, does also occur in human patients, representing a liver-specific tumor immune escape mechanism (Hochst, et al., 2012). The endocytotic uptake of tumor-associated antigens by LSEC is, at least partially, mediated by mannose receptors on LSEC. Pharmacological blockade of the mannose receptor abrogates tumor-induced and LSEC-mediated immunosuppression (Arteta, et al., 2010) and could serve as a potential target in therapy of hepatic metastases. Furthermore, LSEC can contribute to an immunosuppressive environment in the liver by PD-L1 expression or by inducing T reg through membrane-bound TGF- β (Carambia, et al., 2014). In summary, the ability of LSEC to confer T cell tolerance towards cancer-associated antigens and to create an immunosuppressive environment has been established. However, the significance of these pathways for HCC development and progression in patients and ultimately for patient survival has yet to be confirmed.

Hepatic stellate cells (HSC)—HSC reside in the space of Disse, between LSEC and hepatocytes, and are best known for their central role in liver fibrosis development, which ultimately can lead to HCC (Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014). However, they are also capable of shaping hepatic immune responses (Thomson & Knolle, 2010). In patients with HCC, activated HSC have been reported to induce an immunosuppressive environment and are associated poor clinical outcome (Ji, et al., 2015; W. Zhao, et al., 2014). Upon activation, HSC skew monocytes from an inflammatory to an immunosuppressive phenotype and induce features of aggressive growth in HCC cells. This HSC-monocyte interaction results in early tumor recurrence and poor survival of liver cancer patients (Ji, et al., 2015). Moreover, they can induce the contact-dependent accumulation of MDSC, either through CD44 on HSC or through hydrogen peroxide depletion by catalase (Hochst, et al., 2013; Resheq, et al., 2015). HSC can also directly trigger T cell dysfunction through pathways analogous to other immunosuppressive cells. They are capable of inducing T cell apoptosis through PD-L1 expression (also known as B7-H1) (Yu, et al., 2004) and inhibit T cell proliferation and function through TGF- β (Y. Li, et al., 2015). Therapeutically, these immunosuppressive pathways are targetable using the approaches described above.

Under physiological conditions, HSC are quiescent. Upon inflammation-induced activation, HSC differentiate to myofibroblast-like cells and exert their fibrogenic and immunological functions (Pellicoro, et al., 2014; Thomson & Knolle, 2010). Inhibition of the epidermal growth factor receptor on HSC by the tyrosine kinase inhibitor Erlotinib has been shown to reduce activation of HSC in different mouse models of liver fibrosis (Fuchs, et al., 2014). Furthermore, Erlotinib prevent progression of fibrosis and ultimately protected from HCC development. Unfortunately, however, the effect of Erlotinib-mediated inhibition of HSC activation on immunological functions of HSC and its effect on HCC growth remains to be investigated. In summary, activated HSC promote HCC initiation and growth through immunological and non-immunological mechanisms.

3. Effect of the tumor microenvironment on primary liver cancer growth

In the vast majority of cases, HCC develop in livers with an underlying liver disease such as viral hepatitis or NASH. These underlying liver diseases not only fuel HCC initiation through the production of inflammatory, proliferation-inducing cytokines (He & Karin, 2011; Luedde & Schwabe, 2011; Sun & Karin, 2012), but they are also associated with strong negative regulation of spontaneous anti-tumor immune responses. Hypoxia, for example, is known to be an early phenomenon in liver cirrhosis development (Corpechot, et al., 2002) and skews myeloid cells in the TME towards a potent immunosuppressive phenotype (Corzo, et al., 2010). In HCC, hypoxia induces secretion of CCL20 by tumor cells, which in turn causes inhibition of T cell function through IDO up-regulation in macrophages (Ye, et al., 2016). Lipid accumulation in patients with NASH, preceding HCC development, is another metabolic factor, which negatively influences anti-tumor immune responses. A recent study showed that accumulation of a specific fatty acid, linoleic acid, in hepatocytes causes a selective loss of CD4 T cells through mitochondrial ROS-mediated apoptosis (Ma, et al., 2016). Decreased numbers of CD4 T cells in NASH livers accelerated growth of genetically and carcinogen-induced liver cancers. Thus, metabolic factors like

hypoxia or lipid accumulation that occur before HCC initiation and persist during HCC growth restrain anti-tumor immune responses (Figure 1).

In addition to the aforementioned metabolic factors, an oncogene-induced intracellular anti-proliferative response program, i.e. cellular senescence, that has primarily been linked to cancer suppression, can surprisingly also promote growth of neighboring tumor cells in the liver (Eggert, et al., 2016). Senescent hepatocytes accumulate in inflamed livers through activation of oncogenic pathways or DNA damage (Jurk, et al., 2014; Mudbhary, et al., 2014). In livers without proliferating tumor cells, these senescent hepatocytes are cleared by macrophages, which have developed from infiltrating monocyte precursors, and thus prevent HCC initiation (Kang, et al., 2011). However, if full-blown tumor cells arise in livers with senescent hepatocytes, e.g. through bypassing the senescence program or additional senescence-inhibiting oncogenic mutations (Mudbhary, et al., 2014), the nascent tumors secrete factors to impede maturation of senescence-induced monocyte precursors, causing accumulation of immunosuppressive immature myeloid cells that inhibit anti-tumor NK cell responses (Eggert, et al., 2016). The accumulation of these senescence-induced immature myeloid cells is mediated by CCR2. Other studies have recently also implicated HCC-secreted CCL2 in murine and human tumor progression and blocking the CCL2-CCR2 axis led to improved mouse survival by disinhibiting anti-tumor T cells (X. Li, et al., 2015). Thus antagonists of CCR2 constitute an interesting target to inhibit tumor- and TME-induced immunosuppressive myeloid cell accumulation. In summary, in patients with HCC, the tumor by itself and in combination with the underlying liver disease shapes anti-tumor immune responses towards a favorable milieu for tumor growth.

In many cancer settings, studies are now underway investigating the effect of combining immunotherapy with targeted-therapies, e.g. kinase inhibitors, to improve patient survival. Because a plethora of clinical trials using targeted-therapies alone have failed to show an improvement of survival in HCC patients (Llovet, Villanueva, Lachenmayer, & Finn, 2015) and because immune responses are shaped by the tumor and the underlying liver inflammation, it is important to explore the efficacy of combination treatments in preclinical models that closely resemble the situation in humans, i.e. HCC growth in inflamed or cirrhotic livers. New murine HCC models take these two components into account (Reiberger, et al., 2015). After cirrhosis induction through carbon tetrachloride administration, tumor cells are either implanted orthotopically into the liver or initiated in genetically engineered models using viral expression of Cre recombinase. Employing this model, the authors showed that Sorafenib treatment alone led to hypoxia-mediated immunosuppression in liver tumors. However, combining Sorafenib with inhibition of the stromal cell-derived 1 alpha receptor (CXCR4) and ant-PD-1 antibody therapy enhanced anti-tumor immunity and reduced tumor growth (Y. Chen, et al., 2015), demonstrating that unleashing powerful anti-tumor immune responses against HCC is possible in cirrhotic livers. However, in addition to pharmacological T cell activation, it is necessary to block the potent immunosuppressive mechanisms associated with HCC and the underlying liver disease. With the wealth of new targeted-therapy and immunotherapy options, the number of combinations seems unlimited. Preclinical studies, such as the aforementioned, will aid in selecting mechanistically synergistic and promising combinations for further investigation in humans.

In summary, several lines of evidence have validated the importance of the immune system in HCC development and progression, but also its ability to control HCC growth. This suggests that employing the immune system in HCC by enhancing anti-tumor immune cells or interfering with pro-tumor immunosuppression constitutes a promising strategy to improve the survival of patients with HCC. Nevertheless, potent liver tumor-associated immunosuppression within the TME needs to be overcome for successful therapy.

4. Regulation of metastatic seeding into the liver by immune cells

Development of liver metastases from non-hepatic tumors differs from the development of HCC in that they usually form in an otherwise healthy liver without underlying liver inflammation or cirrhosis. Furthermore, metastatic tumors originate from various tissues, like lung, pancreas, colon, stomach or breast and are therefore heterogeneous in their phenotype compared to primary liver cancers (Goodman, 2007). Recently, it has also become clear that the primary tumor expands different myeloid cell populations in the liver through secretion of various factors before metastatic seeding; thereby “priming” the target organ for successful settlement of circulating tumor cells and causing metastatic organotropism (Hoshino, et al., 2015; Kaplan, Psaila, & Lyden, 2006).

Like with primary liver cancers, the immune system has the ability to not only support metastatic tumor growth, but also to suppress metastatic tumor development. The ability of the immune system to recognize and infiltrate primary liver cancers is established, but studies comprehensively investigating the immunogenicity of liver metastases from different primary tumors are missing. However, it has been shown that metastasis-infiltrating lymphocytes from patients with gastrointestinal cancers can recognize cancer cell lines generated from the same tumor, indicating that metastases are possibly also amenable to lymphocyte recognition and killing (Turcotte, et al., 2014). Further evidence for metastasis surveillance in the liver by lymphocytes is provided by studies in mice. In the liver, NK cells suppress metastasis through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression (Takeda, et al., 2001). These NK cell responses towards colon cancer metastases can be triggered through inflammasome-induced IL-18 expression in Kupffer cells (Dupaul-Chicoine, et al., 2015). These findings establish surveillance of non-hepatic cancer cells by lymphocytes in the liver as a means to prevent metastasis formation. Nevertheless, liver metastasis in advanced stages of gastrointestinal and non-gastrointestinal malignancies is common, illustrating that mechanisms, facilitating the development of these tumors distant from the primary site, must be in place.

In recent years, it has been shown that metastasis formation is merely a process of settlement of cancer cells, detached from the primary tumor, in a distant organ. Rather, the primary tumor “primes” the target organs through tumor produced and secreted factors that induce myeloid cell expansion and migration to the target organ (Hoshino, et al., 2015; Kaplan, et al., 2006). Haematopoietic bone marrow progenitor cells accumulate in organs like lungs and livers distant from the primary tumor before tumor cell arrival to “prepare” these tissues for metastatic seeding by expression of matrix metalloproteinase 9 (MMP 9) (Kaplan, et al., 2005). This process is called “pre-metastatic niche” formation. Pancreatic cancer secreted exosomes induce pre-metastatic niche formation by macrophage migration inhibitory factor

(MIF) mediated macrophage recruitment (Costa-Silva, et al., 2015). Accordingly, patients with early stage pancreatic cancer with high MIF expression in exosomes were more likely to develop liver metastases.

In addition to initiating metastatic niche formation, myeloid cells in the liver of pre-invasive abdominal tumor-bearing mice can also enable metastatic tumor growth by inhibiting CTL (Connolly, et al., 2010). Analogous to primary liver tumors, these myeloid cells are recruited to the liver through chemokine expression in cancer cells. For example, colorectal cancer cells recruit CCR1+ myeloid cells through CCL15 or CCR2+ myeloid cells through CCL2 to facilitate liver metastases development (Itatani, et al., 2013; L. Zhao, et al., 2013). Metastasis promoting myeloid cells can also be recruited through tumor-necrosis factor receptor-2 on myeloid cells (Ham, et al., 2015). Therapeutically, interfering with early myeloid cell accumulation by blocking chemokines or cytokines before metastatic seeding occurs poses therefore an interesting strategy to prevent or reduce metastatic burden in patients at risk. Once liver metastases have formed, approaches to break tumor-induced immune tolerance similar to those employed in primary hepatic tumors, like radiofrequency ablation in combination with PD-1 blockade, can be used (Shi, et al., 2016). In summary, myeloid cells play a significant role not only in primary liver cancer development, but also in aiding successful metastatic seeding into the liver. Furthermore, as in HCC, they can confer tumor tolerance through inhibition of anti-tumor lymphocytes and can be targeted therapeutically.

5. Conclusions

The TME in the liver plays an important role in primary and metastatic liver tumor formation and tumor growth promotion. Like in malignancies of other tissues, immune cells can enhance malignant transformation, facilitate metastatic seeding or induce or restrain anti-tumor immune responses. In addition, non-immune cells unique to the liver, namely LSEC and HSC, can alter tumor-associated immune responses. In both, primary or metastatic tumors, cytotoxic lymphocytes are capable of killing cancer cells and spontaneous anti-tumor immune responses are readily detectable. However, several clinical trials aiming at enhancing anti-tumor immune responses via different means, e.g. vaccines, cytokines or adoptive cell therapy, in liver cancer patients have been conducted, but clinical responses overall were disappointing after single agent immunotherapeutic approaches (Makarova-Rusher, Medina-Echeverz, Duffy, & Greten, 2015), most likely, because tumors evaded immune responses through many potent tumor- and TME-induced immunosuppressive mechanisms. Thus, new approaches like ‘checkpoint-blockade’, especially in combination with targeted molecular therapies or local ablative therapies, seem promising in ‘unleashing’ the host anti-tumor immune response. It is noteworthy that recent research has shown that targeted molecular therapies, originally designed to hinder tumor cell proliferation, can also positively influence anti-tumor immune cell responses by alleviating myeloid cell-mediated resistance to ‘checkpoint-blockade’ (De Henau, et al., 2016). Treatment with a selective inhibitor of the gamma isoform of phosphoinositide 3-kinase can reshape the tumor immune microenvironment by blocking this enzyme in myeloid cells, ultimately promoting anti-tumor T cell responses. The ideal combination of immunotherapy and targeted molecular therapy would therefore, based on the aforementioned observations, achieve the following: i)

enhance anti-tumor immune responses, ii) alleviate tumor-associated immunosuppression and iii) hinder tumor cell proliferation. In summary, harnessing immune responses by enhancing anti-tumor immune cells or interfering with pro-tumor immunosuppression has the potential to significantly improve patient survival.

Abbreviations

CTL	cytotoxic T cells
HCC	hepatocellular carcinomas
HSC	hepatic stellate cells
LSEC	liver sinusoidal endothelial cells
MDSC	myeloid-derived suppressor cells
NASH	non-alcoholic steatohepatitis
NK cells	Natural Killer cells
TAM	tumor-associated macrophages
TME	tumor microenvironment
T reg	regulatory T cells

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Fig. 1.

Components of the tumor microenvironment restraining anti-tumor immune cells. Following activation, T cells and NK cells target tumors for tumor cell killing. However, liver resident cells and/or liver infiltrating immune cells such as immunosuppressive myeloid cells or regulatory T cells inhibit their anti-tumor function. Furthermore, the underlying liver disease also negatively regulates T cell and NK cell function either directly by inducing of CD4 T cell death or indirectly by recruiting immunosuppressive myeloid cells.

Fig. 2.

Anti-tumor immune cells and mechanisms restraining their anti-tumor function. Molecular mechanisms inhibiting anti-tumor T cells (left) and anti-tumor NK cells (right) are shown. Therapeutic strategies to reverse this inhibition in order to unleash T cell and NK cell function are provided at the bottom of each box. Question marks indicate proposed, not yet studied approaches.

Fig. 3.

Pro-tumor effector mechanisms of immune and non-immune cells in HCC. Molecular mechanisms of regulatory T cells (upper left), myeloid-derived suppressor cells (upper right), liver sinusoidal endothelial cells (lower left), tumor-associated macrophages (bottom center) and hepatic stellate cells (lower right) that support tumor growth are shown. Therapeutic strategies to counter these mechanisms are provided at the bottom of each box. Question marks indicate proposed, not yet studied approaches.

Table 1

Selected potential treatment approaches for HCC or liver metastases (with the exception of radiofrequency ablation, these therapies have not been approved for the treatment of patients with HCC thus far).

Targeted immune cell/therapeutic strategy	Therapeutic agent/intervention	Mechanism of action
Anti-tumor immune cells		
Cytotoxic T cells enhancing T cell tumoricidal activity	Nivolumab (anti-PD-1 antibody), Ipilimumab (anti-CTLA-4 antibody)	blocking T cell inhibitory signals, so called 'checkpoints'
	anti-4-1BB agonistic antibody, anti- OX40 agonistic antibody	activating T cell stimulatory receptors
Dendritic cells inducing tumor-specific T cell responses	radiofrequency ablation of HCC	tumor cell death causes release of tumor-associated antigens → presentation to reactive T cells
	in vitro-transfection of autologous dendritic cells with heat shock protein 70 mRNA	activation/expansion of T cells targeting heat shock protein 70-expressing HCC
NK cells enhancing NK cell tumoricidal activity	Lirilumab (anti-KIR antibody)	blocking NK cell inhibitory signals
Pro-tumor immune cells		
MDSC reducing MDSC-mediated immunosuppression	anti-GM-CSF antibody, anti-CXCR2 antibody	inhibition of MDSC expansion and recruitment
	Atezolizumab (anti-PD-L1 antibody)	blockade of T cell inhibition through PD-L1 on MDSC
	Tadalafil (PDE-5 inhibitor)	blockade of MDSC-mediated T cell inhibition
Macrophages reducing macrophage-mediated immunosuppression	CSF-1R inhibitors	inhibition of macrophage accumulation
Regulatory T cells reducing T reg-mediated immunosuppression	anti-TGFβ antibody	inhibition of T reg expansion and blockade of T reg-mediated T cell inhibition