Review Article

Impact of immune tolerance mechanisms on the efficacy of immunotherapy in primary and secondary liver cancers

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Abstract: The liver is a functionally unique organ with an immunosuppressive microenvironment. The liver is the sixth most common site of primary cancer in humans and is a frequent site of metastasis from other solid tumors. The development of effective therapies for primary and metastatic liver cancer has been challenging due to the complex metabolic and immune microenvironment of the liver. The liver tumor microenvironment (TME) in primary and secondary (metastatic) liver cancers is heterogenous and consists of unique immune and stromal cell populations. Crosstalk between these cell populations and tumor cells creates an immunosuppressive microenvironment within the liver which potentiates cancer progression. Immune checkpoint inhibitors (ICIs) are now clinically approved for the management of primary and secondary liver cancer and can partially overcome liver immune tolerance, but their efficacy is limited. In this review, we describe the liver microenvironment and the use of immunotherapy in primary and secondary liver cancer. We discuss emerging combination strategies utilizing locoregional and systemic therapy approaches which may enhance efficacy of immunotherapy in primary and secondary liver cancer. A deeper understanding of the immunosuppressive microenvironment of the liver will inform novel therapies and therapeutic combinations in order to improve outcomes of patients with primary and secondary liver cancer.

Keywords: Hepatocellular carcinoma (HCC); liver metastases; tumor immune microenvironment; immunotherapy in liver cancer

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Introduction

Liver cancer is the sixth most common cancer and the fourth leading cause of cancer-related death worldwide (1). Hepatocellular carcinoma (HCC) is the most common subtype, accounting for 80–90% of primary liver cancer (PLC). Chronic inflammation and cirrhosis are the strongest risk factors for HCC. Chronic inflammation may arise from viral infections [hepatitis B (HBV) and hepatitis C (HCV) viruses], alcoholic and nonalcoholic steatohepatitis (NASH), chronic toxin exposure, or other infections (e.g., liver fluke) (1). Lifestyle factors such as dietary habits, chronic alcohol consumption, and sedentary lifestyle, have led to a continued increase in the incidence of HCC (1).

The liver is also a frequent site of metastasis. Liver metastases (LM) are 20 to 40 times more common than PLC (2). LM often originate from cancers of the gastrointestinal tract (particularly colon) but may also originate from melanoma, breast, pancreatic, bladder, and lung cancers (3). It is estimated that up to 50% of patients with various cancers will either present with, or develop, LM during their disease course (4). In a Surveillance, Epidemiology, and End Results (SEER) database query from 2010–2015, among 2.4 million patients with cancer of various types, the presence of LM was associated with reduced survival (5).

Immunotherapies offer promise in the treatment of patients with liver cancer. Immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) alone or in combination with other treatments have significantly improved survival in patients with advanced malignancies including HCC (6-8). However, both preclinical and clinical data suggest that the presence of LM is associated with diminished response to ICI monotherapy (9). In this review, we provide insight into the immunosuppressive nature of the liver and reflect how this limits the response to ICI. We describe the components of the liver tumor microenvironment (TME) and highlight specific immunosuppressive mechanisms that may modulate the response to immunotherapy. Finally, we discuss the clinical efficacy of ICI in primary and secondary liver cancers and review novel therapeutic strategies that aim to improve immunotherapy efficacy by modulating the liver TME.

Liver tumor immune microenvironment

The liver is architecturally complex with distinct immune and stromal cell populations which have been documented with recent single-cell RNA (scRNAseq) studies (10-13). Recent studies exploring the interplay between myeloid and lymphoid cells in the TME have revealed the immunosuppressive nature of HCC TME (14-17). While there is substantial heterogeneity of HCC tumor cells across patients, analysis of ligand-receptor interactions between tumor and stromal cells of the TME has shown that there is consistent cross-talk between these populations—thus presenting the TME as an ideal target for immunotherapies in patients with HCC (14). Multiple groups have proposed prognostic HCC models based on gene signatures identified using scRNAseq analysis (18,19). A recent bulk RNAseq study by Gao classified HCC into different immune subclasses with different prognostic values based on TME signatures—immune desert (C1), immunogenic (C2), innate immune (C3), and mesenchymal (C4) (20). Specifically, the C1 subtype is defined by the absence of priming T cells; the C2 subtype is defined by the presence of infiltrating macrophages, $CD4^+$, and $CD8^+$ T cells, and B cell; the C3 subtype is associated with the presence of activated immunosuppressive macrophages, and the C4 subtype is associated with activated cancer associated fibroblasts (CAFs) which support epithelial-mesenchymal transition (EMT) (20-22). This section provides an overview of the various immune and stromal cellular components of the liver TME.

Immune components of hepatic TME

T cells are central to the surveillance and elimination of tumor cells. Tumor infiltrating lymphocytes (TILs) are primarily composed of $CD3^+$, $CD8^+$, $CD4^+$, and $Foxp3^+$ T lymphocytes (23-25). Tumor-infiltrating tumor-associated antigen (TAA) specific $CD8^+$ T lymphocytes infiltrate the tumor bed or peritumoral region with antigen-specific anti-tumor cytotoxicity (26). Their presence has been linked to improved disease-free survival and a higher 5-year survival rate in HCC (27,28). $CD4^+$ T lymphocytes are central to priming the $CD8^+$ T lymphocytes; however, their subsets are heterogeneous (29). Pro-inflammatory Th1 (IFN-γ, TNF-α) and anti-inflammatory Th2 (IL-4, IL-10) are two major $CD4^+$ T helper lymphocyte subsets. A Th1 to Th2 shift within the liver is associated with a poor prognosis (30-32). Regulatory T cells, Tregs (IL-10, TGFβ), another $CD4^+$ T cell subset, play a role in escaping immune surveillance by suppressing the immune response and are further classified as natural Tregs ($CD4^+CD25^+FOXP3^+$)

or inducible Tregs (FOXP3$^+$ or FOXP3$^-$) (33). Type 1 regulatory T (Tr1) cells are FOXP3$^-$ and are an important source of IL-10 (34,35).

Natural killer (NK) cells are an innate lymphoid cell (ILC) population and play an important role in immune surveillance; deficiencies promote immune escape (36). Activation of NK cells lead to the release of lytic granules and cytotoxicity (37,38). Liver NK cells, particularly the CD56^{bright} (CXCR6$^+$CCR5$^+$CD69$^+$), play a critical role in local innate immune responses (36). Another subset of liver tumor-infiltrating NK cells expressing KLRC1 and KLRC2 genes, develop mitochondrial fragmentation, leading to deficiencies in NK-cell cytotoxicity and immunosurveillance (39). Meanwhile, natural killer T (NKT) cells express both NK and T-cell receptors (TCRs) (40) and exert both pro and anti-inflammatory effects (41,42).

Macrophages are a type of antigen-presenting cell (APC) typically responsible for innate immune response and can be classified as tissue-resident Kupffer cells (KCs) or monocyte-derived macrophages in the liver. KCs make up the largest macrophage population in the liver, originating from the yolk-sac-derived embryonic progenitors (43,44). Meanwhile, monocyte-derived macrophages are recruited from the bone marrow to infiltrate the liver in response to inflammation (43). Macrophages can promote inflammation and fibrogenesis through cell-cell signaling, which can contribute to the progression of chronic liver disease and subsequently the development of HCC over time (45,46). In the HCC TME, CD68$^+$ macrophages are associated with pro-tumor effect and poor prognosis, whereas CD68$^+$CD169$^+$ macrophages may promote CD8$^+$ T-cell activation and cytotoxic function through a potential costimulatory function of CD169 (47,48). ScRNA-seq analysis of healthy human liver identified two macrophage subpopulations, CD68$^+$MARCO$^+$ and CD68$^+$MARCO$^-$ macrophages (49). While CD68$^+$ MARCO$^+$ macrophages resembled long-lived KCs with reduced TNFα production capability, CD68$^+$ MARCO$^-$ macrophages resembled recently recruited macrophages with a pro-inflammatory phenotype (49).

Neutrophils are short-lived innate immune cells that are often the first responders to infection. Although tumor-associated neutrophils (TANs) were originally classified into two fixed phenotypes: anti-tumorigenic (N1) and pro-tumorigenic (N2). Single-cell sequencing has demonstrated that neutrophil phenotypes are highly dynamic with underlying chromatin, transcriptional, and receptor heterogeneity (10,50). Human TANs primarily express CCL2 and CCL17 chemokines, facilitating the migration and tumor infiltration of macrophages and Tregs via the CCL2-CCR2 and CCL17-CCR4 interactions, respectively (51,52). TANs are associated with poor prognosis for HCC patients with a direct impact on tumorigenesis, tumor progression, and metastasis (51).

Dendritic cells (DCs) are professional APCs originating from CD34$^+$ bone-marrow stem cells and are classified as either immature or mature based on their functional capability (53). Myeloid-derived type 2 conventional DCs (CD141$^-$CD11c$^+$CD14$^+$) are the most abundant DCs in the human liver. Other subtypes include type 1 conventional DCs (CD141$^+$CD11c$^+$CD14$^-$, lymphoid-derived plasmacytoid DCs (CD123$^+$CD11c$^-$CD303$^+$CD304$^+$), and monocyte-derived inflammatory DCs (54-56). DCs play a critical role in supporting adaptive immune response by regulating the differentiation of T cells in the liver, thus directly impacting peripheral tolerance in the liver (57,58). DCs isolated from HCC tumors express inhibitory receptor ligands including PD-1, T cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), and CTLA-4, leading to the inhibition of adaptive immunostimulatory responses (59). Additionally, DC dysfunction and immunoinhibitory DC-T cell interactions can also promote HCC growth (60-62).

B lymphocytes mediate humoral immunity by differentiating into antibody-secreting plasma cells and can serve as APCs (63). While antigen-presentation stimulates anti-tumor adaptive immune effects, B cells also secrete immunosuppressive cytokines with pro-tumor effects, and thus the overall impact of tumor-infiltrating B cells in liver tumors remains controversial (63,64). Interestingly, recent scRNA-seq analysis revealed distinct B-cell subpopulations in the HCC TME and a significantly reduced density of B cells in the HCC TME compared to the normal liver microenvironment (65). Studies have shown that increased B cell infiltration within HCC TME is associated with improved clinical outcomes (63,64).

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells generated in the bone marrow that can be classified into two primary subsets: polymorphonuclear or granulocytic MDSC (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (66). Human PMN-MDSCs (CD33$^+$CD11b$^+$CD15$^+$CD66b$^+$) share similar features with neutrophils, while human M-MDSCs (CD33$^+$CD11b$^+$CD14$^+$) express surface markers similar to monocytes, macrophages, and DCs (66). Immature MDSCs undergo differentiation into mature myeloid cells

such as DCs or macrophages in response to the presence of transcription factors, growth factors, hypoxic conditions, and pro-inflammatory cytokines within the TME (67,68). MDSCs promote tumor progression through various mechanisms such as expression of immunosuppressive cytokines and proangiogenic factors, and inhibition of T cell responses (68,69). Several studies have reported a direct association between increased MDSC density and unfavorable clinical outcomes in HCC (70-72).

ILCs are a type of innate immune cell lacking rearranged antigen-specific receptors (73). ILCs are classified into group 1, 2, or 3 based on transcription factors, phenotypic markers, and cytokine expression (73-75). Group 1 ILCs include NK cells and ILC1s (IFN-γ, TNFα), which are regulated by the transcription factor T-BET (74,76). Group 2 ILCs (IL-4, IL-5, and IL-13) are mainly regulated by GATA-3 and ROR-α transcription factors and promote hepatic fibrosis and HCC tumor progression (73,77-79). Group 3 ILCs (IL-17, IL-22) are regulated by the transcription factor RORγt (80).

Stromal components of TME

Hepatic stellate cells (HSCs) are liver-specific mesenchymal cells, however, their embryonic origin remains controversial since they express marker genes of all three germ layers (81-83). Inactive HSCs exist in the quiescent state in the healthy liver but undergo transition to an activated myofibroblastic state and initiate fibrosis in response to liver injury (84,85). Activated HSCs secrete immunosuppressive cytokines and angiogenic growth factors which support tumor progression (84,86,87).

Cancer-associated fibroblasts (CAFs) are mesenchymal cells derived from HSCs or tumor cells undergoing EMT (88). CAFs secrete extracellular matrix proteins and growth factors including epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), immunomodulating chemokines and cytokines, and matrix metalloproteinase (MMP) enzymes (89). The HCC CAF phenotype primarily expresses fibroblast surface markers FSP-1 and FAP (88,90). CAFs in LM can be classified into three subtypes—myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs), and portal fibroblasts (PF)/mesothelial CAF (PF/mesCAF) (91). A proteomic and scRNA-seq analysis categorized HCC CAFs into three subtypes which share similar features as HSC, vascular smooth muscle (VSMC), and portal fibroblast cells (92).

Liver sinusoidal endothelial cells (LSECs) are the sentinels of the liver, lining the sinusoids to form a permeable interface between parenchymal liver cells and sinusoidal vasculature (93). Hepatic LSECs are uniquely fenestrated, lack a basement membrane, and promote liver stem cell quiescence (94). They are the first to interact with and eliminate circulating pathogens and tumor cells by performing endocytosis, and thus mediate immune tolerance (93-95). In the setting of malignancy, LSECs undergo morphological and phenotypic alterations which impair their immunosurveillance ability and secrete pro-metastatic cytokines and chemokines supporting the pathogenesis of LM (96-99).

Immunosuppressive mechanisms in the hepatic TME

Liver physiologically promotes immune tolerance

The healthy liver maintains a tolerogenic environment that tempers anti-tumor immune responses (*Figure 1*) (100-102). This physiologically serves to prevent unwanted reactions to antigens that are filtered from the gut through the liver (103,104). In the 1960s, studies of organ transplants in animals demonstrated that porcine liver transplant recipients survived without the help of immunosuppressive agents (105), supporting the notion that the liver has a uniquely tolerogenic environment. Recent efforts have investigated the mechanisms of immune tolerance in the liver. These studies have identified T cells to be an important mediator of inappropriate responses to self-antigens and have highlighted the mechanisms by which self-reactive T cells are suppressed from propagating autoimmunity. Specifically, murine $CD8^+$ T cells stimulated by hepatocytes presenting self-antigen undergo apoptosis after initial expansion in the liver, an important component of peripheral tolerance (106). In murine models, $CD8^+$ T cells activated in the liver have shorter lifespans and impaired cytotoxic function compared with those activated in the lymph nodes (107). Thus, the suppression of T cell function is a key mechanism of the liver tolerogenic environment. Epithelial, stromal, and immune cell interactions with T cells contribute to hepatic immunosuppression and this will be discussed further in the context of the healthy liver and primary and secondary liver cancer.

Hepatocytes make up the bulk of the liver's structure (108) and are central to the modulation of the adaptive immune response. Hepatocytes are non-professional APCs that interact with T cells in the healthy liver (109). Low levels of co-stimulatory CD80/86 and elevated levels of PD-L1 on

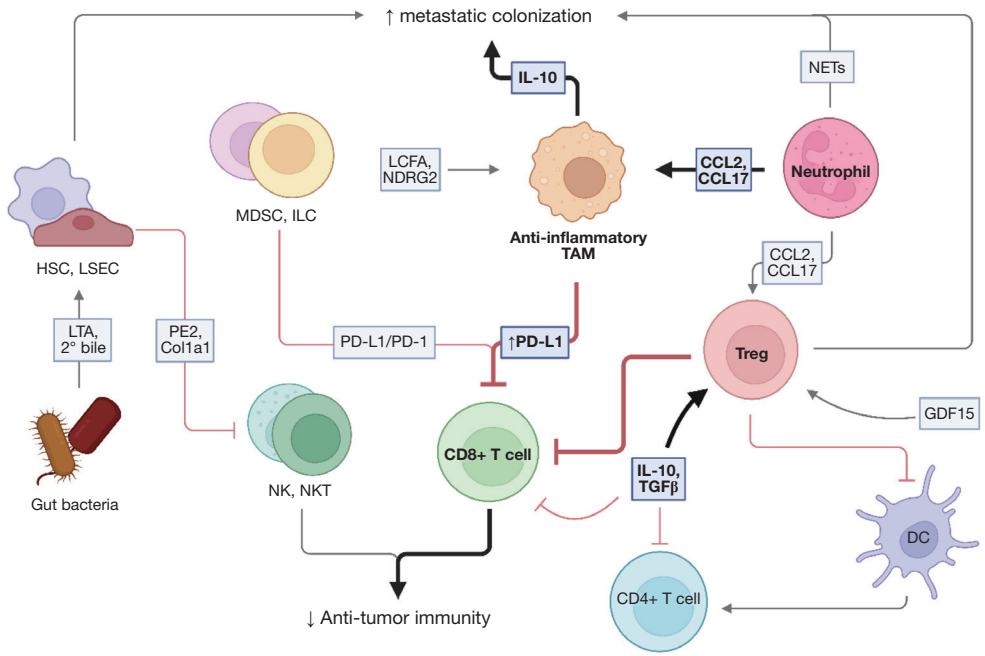

Figure 1 Immunosuppressive mechanisms in the liver immune microenvironment. The tolerogenic liver environment supports the infiltration of regulatory immune cells that limit effector CD8⁺ T cell function, resulting in the suppression of anti-tumor immunity and the formation of a cancer-permissive pre-metastatic niche. Neutrophils recruit TAMs and Tregs, which directly inhibit CD8⁺ T cells. IL-10 and TGFβ promote Treg development and directly suppress CD8⁺ T cell function. MDSCs and ILCs also modulate CD8⁺ T cell function, while signaling by HSCs and LSECs induced by bacterial metabolites inhibits NK- and NKT-mediated immunity. Macrophages contribute to metastatic colonization in the liver through multiple mechanisms. Other cells in the liver also promote the seeding of metastatic cancer, including neutrophils, Tregs, HSCs, and LSECs. LTA, lipoteichoic acid; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell; PE2, prostaglandin E2; NK, natural killer cell; NKT, natural killer T cell; MDSC, myeloid-derived suppressor cell; ILC, innate lymphoid cell; PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1; TAMs, tumor-associated macrophages; LCFA, Long-chain fatty acid; DC, dendritic cell; GDF15, growth differentiation factor 15; NETs, neutrophil extracellular traps.

hepatocytes during antigen presentation to T cells lead to dysfunctional T cell activation (106,110,111). Hepatocytes also directly cause CD8⁺ T cell death through a process known as 'suicidal emperipolesis', in which autoreactive T cells actively invade hepatocytes for degradation in lysosomal compartments, leading to diminished CD8⁺ T cell numbers in the liver. Prevention of this process results in T cell-mediated hepatitis in preclinical models (112). Therefore, hepatocytes promote T cell tolerance in the liver.

Liver DCs also contribute to immune tolerance. Human hepatic DCs lack efficient antigen uptake, resulting in impaired CD4⁺ T cell proliferation and responsiveness. Furthermore, liver DCs secrete high levels of IL-10 to promote the generation of Treg and Th2 cells (113). Murine hepatic DCs have limited phagocytosis, express low levels of costimulatory molecules, and poorly activate T cells as compared to splenic DCs (114). This effect may be partly due to the low expression of TLR4 by liver DCs, resulting in impaired phagocytosis (115).

KCs limit autoimmune responses by presenting self-antigen, expanding Treg populations, and restricting CD4⁺ T cell priming in mice (116,117). KCs act as inefficient APCs and express low levels of MHCII to NK, NKT, and T cells, promoting tolerance by limiting the cytotoxic capabilities of these cells (118). Additionally, KCs produce immunosuppressive cytokines IL-10 and TGFβ, which suppresses T cell function and promote polarization of Tregs (119,120).

Hepatic Tregs, which are both thymically derived and peripherally induced, prevent T cell-mediated inflammation and autoimmunity by restricting immune responses to self-antigens (121). Polarization of naïve CD4⁺ T cells to

Tregs in the liver is dependent on TGFβ signaling, which is produced by KCs or LSECs (122,123). Tregs function as an IL-2 sink, restricting the ability of other T cells to sense IL-2 and proliferate (124,125). Additionally, Treg expression of CTLA-4 restrains T cell activation (126,127). Hepatic Tregs are an important source of immunosuppressive cytokines IL-10 and TGFβ (128). Importantly, loss of hepatic Tregs and thus impaired peripheral tolerance is associated with liver injury (129-131).

Hepatic anatomy supports immune tolerance

LSECs contribute to immune tolerance in the liver. LSECs are fenestrated endothelial cells that line liver sinusoids and allow efficient antigen filtration (132). They have the ability to present antigen, which supports formation of Tregs and, causes dysfunction of OT-I T cells *in vivo* (123,133). These T cells are unable to regain cytotoxic function after restimulation with a clonotypic antibody (134). This may be because LSECs can express the inhibitory molecule PD-L1 (135). Lymphocytes that home to the liver must travel through the sinusoidal channels of the liver, then bind to LSECs via atypical adhesion molecules (136). Minimal shear forces in the sinusoids means this process does not require selectins (137). LSECs can sequester activated $CD8^+$ T cells in mouse livers by binding via ICAM-1, preventing further movement through the liver, and inducing apoptosis (138). Steatohepatitis, liver fibrosis, and cirrhosis reduce tumor infiltration by T cells (139). Thus, LSECs modulate the adaptive response to self-antigens in the liver by altering the physical structure and immune signaling milieu to contribute to immune tolerance.

Immune signaling drives hepatic immunosuppression

The immune signaling milieu, including IL-10 and TGFβ, drives immunosuppression in the liver. IL-10 is central to regulating immune responses in the liver. Activating human $CD4^+$ T cells in the presence of IL-10 induces anergy (140). $IL\text{-}10^+$ B cells are elevated in patients with operational tolerance, which is a stable function of a transplanted organ without the use of immunosuppressives (141). IL-10 prevents infiltration of fibrosis-inducing neutrophils after liver injury in mice (142). Like IL-10, TGFβ is an essential modulator of liver-specific immune responses (143) and has been suggested to play a role in immunosuppression in the setting of cancer. Genes in the TGFβ pathway which display markers of T cell exhaustion are overexpressed in patients with HCC (144). Simultaneous targeting of TGFβ and PD-L1 increases immune infiltration and reduces tumor growth compared to anti-PD-L1 alone in murine breast and colorectal cancer (CRC) models (145). Together, these suppressive mechanisms prevent the induction of autoimmunity to antigens processed by the liver, as well as alloantigens after liver transplant. These mechanisms may also abate the immune system's natural ability to recognize and respond to cancer.

Anti-tumor immunity is dysregulated in PLC

PLC restricts effective anti-tumor responses. $CD8^+$ T cells express elevated levels of PD-1 and display an exhausted phenotype in HCC patients which correlates with worse survival (146,147). Exhausted NK cells present in primary tumors of HCC patients are associated with poor clinical outcomes (148). While tumor-intrinsic mechanisms may be partly to blame for the impaired cytotoxic activity of these effectors (149,150), systemic reprogramming of myeloid and lymphoid compartments by the diseased liver also contributes to immune suppression in the TME and subsequent cancer progression. Tumor-associated macrophages (TAMs) are associated with increased tumor growth and worse survival in patients with HCC (151,152). $PD\text{-}L1^+$ KCs in the HCC stroma induce T-cell exhaustion by localizing with $CD8^+$ T cells (153). PD-L1 expression by HCC cells promotes TAM infiltration via NF-κB and STAT3 signaling (154). Additional signaling by cancer cells and TAMs increases TAM infiltration, polarizes TAMs toward an anti-inflammatory state, and predicts poor prognosis (155-157). In a mouse model of HCC, the immunosuppressive effects of an anti-inflammatory subset of TAM, $TREM\text{-}1^+$ TAMs, could not be reversed by PD-L1, suggesting a role in ICI resistance (158). Tregs also play a significant role in promoting PLC. Tregs correlated with carcinogenesis and worse survival of patients with HCC (159). The presence of growth differentiation factor 15 (GDF15) in patients with HCC promotes Treg infiltration into the tumor and drives cancer progression (160,161). Tumor-infiltrating Tregs lead to an immunosuppressive environment by suppressing MHCII expression on DCs and by interacting directly with $CD8^+$ T cells through PD-L1 interactions (162,163).

Neutrophils, MDSCs, and ILCs all contribute to the immunosuppressive environment of HCC. Neutrophils release elevated levels of extracellular traps (NETs). NETs

activate TLR4/9-COX2 signaling to induce inflammation and support the metastasis of Hepa1-6 HCC cells in mice (164). Neutrophils also recruit TAMs and Tregs, as well as inhibit T cell cytotoxicity, to promote cancer progression in patients with HCC (10,52). MDSCs are associated with poor prognosis in patients receiving anti-PD-L1 therapy. MDSC suppression improves response to anti-PD-L1 therapy by enabling $CD8^+$ T cell function (165). HCC-associated ILC2s recruit immunosuppressive neutrophils and are associated with worse survival in patients with HCC (73). Meanwhile, ILC3s induce $CD8^+$ T cell apoptosis through direct cell-to-cell interactions to support the growth of Hepa1-6 tumors in mice (166). Finally, non-immune factors in the tumor environment promote HCC growth. HSCs create physical barriers which reduce the infiltration of $CD4^+$ and $CD8^+$ T cells, Tregs, and NKT cells in fibrosis-associated HCC by depositing type I collagen (167). In the mouse gut, the secretion of lipoteichoic acid and deoxycholic acid by gram-positive bacteria induces the upregulation of COX-2 through TLR2 on HSCs, which functions to produce prostaglandin E2. Prostaglandin E2 in turn dampens the antitumor immune response by reducing IFN-γ and TNF-α production by liver immune cells (168). Furthermore, commensal gut bacteria metabolism regulates secondary bile acid concentrations in the liver, and this influences NKT-mediated tumor inhibition (169). These immune compartments contribute to immunosuppression and tumor growth in PLC.

Anti-tumor immunity is dysregulated in LM

The tolerogenic environment of the liver makes it particularly susceptible to metastasis. KCs play a key role in facilitating the colonization of circulating tumor cells in the liver. Exosomes from PAN02 pancreatic ductal adenocarcinoma (PDAC) cells induce an anti-inflammatory state in KCs that induces TGFβ and fibronectin production by HSCs. Fibronectin production leads to the recruitment of bone marrow-derived macrophages to promote metastasis in mice (170). Further, IL-10 secreted by KCs in the liver blocks the cytotoxic effect of ischemia-reperfusion injury and promotes the formation of LM by metastatic human CRC lines in nude mice (171). CXCR2 knockout and neutrophil depletion increased levels of infiltrating T cells and decreased LM, suggesting that neutrophils play a role in forming a pre-metastatic niche (172). In mice with breast cancer, these TANs facilitate the establishment of metastases by releasing NETs and chemotactically attracting cancer cells (173). Tregs also contribute to the pre-metastatic niche. Increased infiltration of Tregs in the liver of mice with PDAC after chemotherapy spurs the formation of metastases (174). Non-immune subsets, such as HSCs and LSECs, further modulate immune activity in the liver, facilitating metastasis. HSCs permit LM by inducing a quiescent state in NK cells in MDA-MB-231 metastatic breast cancer in mice (175). LSECs induce an immunosuppressive environment in B16F10 LM by binding T cells via Lyve-1, reducing the prevalence of effector T cells and leading to enhanced metastasis (176).

Anti-tumor immunity in the liver is reduced following metastatic colonization. As in PLC, the $CD8^+$ T cell mediated anti-tumor immune response is suppressed in LM and is not rescued by ICI monotherapy (177). This suppression is antigen-specific and systemic (9). Multiple immune subsets contribute to T cell dysfunction and suppression, including macrophages, neutrophils, DCs, and MDSCs, causing metastatic growth and diminished immune response at the site of the primary tumor. NDRG2-mediated NF-κB signaling and CD36-mediated internalization of long-chain fatty acids promote anti-inflammatory macrophage polarization and metastasis in CMT93 CRC and Lewis lung carcinoma cell lines, respectively (178,179). Metastasis-associated macrophages also support peripheral immune tolerance, as selective elimination of antigen-specific $CD8^+$ T cells via Fas-FasL interactions by LM-localized $FasL^+CD11b^+F4/80^+$ monocyte-derived macrophages reduces anti-tumor immunity in B16F10 melanoma and MC38 CRC murine subcutaneous tumors, contributing to anti-PD-L1 resistance (9). TANs infiltrate LM at high levels and adopt a pro-tumoral phenotype, demonstrated by the expression of genes such as arginase-1, IL-10, and TGFβ1, that supports metastatic growth and contributes to anti-PD-1 resistance in MC38 LM models. In contrast, cross-presenting DCs are strikingly absent from CRC LM in mice, depleting activated T cells and resistance to ICI (180). Finally, MDSCs correlate with CRC LM in patients (181). Accumulation of MDSCs in MC38 LM correlates with Treg cell number (182). Finally, hepatocyte CCRK signaling mediates B16F10 and MC38 LM by promoting the infiltration of PMN-MDSCs into the liver, leading to reduced levels of effector NKT cells (183). Thus, the myriad of immunosuppressive populations in the liver which enable metastatic colonization also promote ICI resistance.

Therapeutic strategies to surmount ICI resistance in primary and secondary malignancies

The role of ICI in the management of PLCs

The PD-1 inhibitors, nivolumab and pembrolizumab, were initially studied as single arm phase II trials after sorafenib failure and showed similar overall response rates (17–20%) (184,185). Pembrolizumab was then studied in the second line setting in a placebo-controlled randomized phase III trial (KEYNOTE-224), but statistically significant improvements in overall survival (OS) and progression-free survival (PFS) were not observed. However, post-progression therapies approved during the time of the trial (e.g., nivolumab, regorafenib) may have impacted OS. Based on these data, pembrolizumab was approved as a second-line therapy after sorafenib in advanced HCC. Nivolumab was compared to sorafenib (CheckMate 459) in patients with advanced treatment-naïve HCC, and statistical significance for OS was also not met [median OS: 16.4 *vs.* 14.7 months; hazard ratio (HR) =0.85; P=0.075]. Nivolumab yielded a higher disease control rate (median: 7.5 *vs.* 5.7 months) and better safety profile (grade 3 or 4 treatment-related adverse event rate 22% *vs.* 49%) (186). However, due to not meeting the primary endpoint of OS, the FDA's approval of nivolumab in the treatment of advanced HCC was overturned in 2021.

While the role of single-agent ICI in the management of HCC is uncertain, these initial studies spurred several studies evaluating various ICI combination strategies. The combination of PD-L1 inhibitor atezolizumab with the vascular endothelial growth factor (VEGF) inhibitor bevacizumab became the frontline standard of care in 2020 for HCC, based on data IMbrave150 trial, where treatment-naïve patients with advanced HCC were randomized to receive atezolizumab/bevacizumab or sorafenib. Patients who received the combination treatment had prolongation of PFS (median PFS: 6.8 *vs.* 4.3 months; HR =0.59; P<0.001) and OS (median OS: 19.2 *vs.* 13.4 months; HR =0.58; P=0.0006) (187). In addition to the reduction of tumor vascularization, VEGF blockade impacts the immune infiltration within the TME. VEGF signaling directly upregulates the proliferation of suppressive immune cells, inhibits DC maturation, increases the number of Treg cells, and promotes MDSCs. Bevacizumab was shown to reduce MDSCs in patients with lung and colon cancer (188,189), and the combination of anti-PD-1 and anti-VEGFR2 treatment is associated with decreased TAMs and increased $CD8^+$ T cells within the liver TME in preclinical HCC models (190).

Another treatment option for HCC is dual CTLA-4 and PD-1/PD-L1 blockade. In patients with advanced HCC after progression on sorafenib, the combination of CTLA-4 inhibitor ipilimumab with nivolumab yielded an objective response rate of 32% and a median OS of 22.8 months (186). In the phase 3 HIMALAYA trial, treatment-naive patients with advanced HCC received CTLA-4 inhibitor tremelimumab plus the PD-L1 inhibitor durvalumab, durvalumab alone, or sorafenib. Patients on the combination arm had an objective response rate of 20.1% and median OS of 13.6 months. The 36-month OS rate was improved in the combination arm [30.7% *vs.* 24.7% (durvalumab alone) *vs.* 20.2%], leading to the approval of combination durvalumab and tremelimumab in the first-line setting for patients with advanced HCC in 2022.

Although there is a strong rationale to study combining anti-angiogenic tyrosine kinase inhibitors with ICI in HCC, the combination of lenvatinib and pembrolizumab compared with lenvatinib alone, as well as cabozantinib and atezolizumab compared with sorafenib alone were not shown to be superior regimens (191,192).

The role of ICI in the management of LM

The initial observation of diminished response to PD-1 blockade in patients with LM was reported by Tumeh *et al.* In a cohort of patients with metastatic melanoma who received pembrolizumab, presence LM was associated with a lower response rate and shorter PFS [overall response rate (ORR): 30.6% *vs.* 56.3%, median PFS: 5.1 *vs.* 20.1 months, P<0.0001] (193). A similar observation was noted in patients with advanced non-small cell lung cancer (NSCLC) treated with PD-1 blockade (193). Several studies have since reported similar findings in other solid tumors.

In a pan-cancer analysis evaluating clinical data of a published cohort of 1,661 patients who received ICI therapy, patients with LM had significantly shorter OS than those without LM (10 *vs.* 20 months; HR =1.66; P<0.0001) in multivariate analysis (194). This cohort included patients with breast, colorectal, esophagogastric, head and neck, melanoma, NSCLC, renal, and non-melanoma skin cancers. A subgroup analysis showed that the presence of LM was associated with shortening of OS in the ICI monotherapy group (P<0.0001) but did not reach statistical significance in the ICI-based combination therapy group (P=0.0815) (194). Further, a meta-analysis of patients with

Table 1 Use of locoregional therapy to overcome ICI resistance in patients with PLC and LM

Locoregional technique	Patient population	N	Intervention	Outcome	Reference
Stereotactic body radiotherapy	Metastatic NSCLC after progression on ≥1 systemic therapy	39	Ipilimumab (anti-CTLA-4 antibody) in combination with radiation therapy to one metastatic site (6 Gy ×5 or 9 Gy ×3)	ORR 18%; disease control 31%; median OS 13 months	(195)
	Unresectable HCC	5	SBRT followed by anti-PD-1 antibody	2 of 5 patients with CR, 3 of 5 patients with PR, median PFS 14.9 months	(196)
	Unresectable or recurrent HCC	64	SBRT-ICI *vs.* TACE (propensity score matching analysis)	12-month PFS improved in SBRT-ICI group (93.3% *vs.* 16.7%; P<0.001); 24-month OS improved in SBRT-ICI group (80.4% *vs.* 8.3%; P<0.001)	(197)
Hepatic ablation	Colorectal cancer with liver metastases	38	RFA treatment followed by primary tumor resection	Radiofrequency ablation increased T-cell infiltration and PD-L1 expression in human colorectal tumors	(198)
	HCC	32	Tremelimumab (anti-CTLA-4 antibody) in combination with ablation	26% achieved confirmed partial response; 12-month PFS rate 33.1%; median OS 12.3 months	(199)
TACE and transarterial radioembolization	Unresectable HCC	34	TACE plus camrelizumab (anti-PD-L1 antibody)	Objective response rate was 35.3%; median PFS 6.1 months; median OS 13.3 months	(200)
	BCLC stage C HCC	1	Y-90 radioembolization in combination with nivolumab (anti-PD-1 antibody)	>50% reduction in size of primary tumor	(199)

ICI, immune checkpoint inhibitor; PLC, primary liver cancer; LM, liver metastases; NSCLC, non-small cell lung cancer; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ORR, objective response rate; OS, overall survival; HCC, hepatocellular carcinoma; SBRT, stereotactic body radiotherapy; PD-L1, programmed death-ligand 1; CR, complete response; PR, partial response; PFS, progression-free survival; TACE, trans-arterial chemoembolization; RFA, radiofrequency ablation; BCLC, Barcelona Clinic Liver Cancer.

NSCLC treated with anti-PD-1/PD-L1 ICI was conducted assessing 6,274 patients across 11 publications. The pooled results showed that anti-PD-1/PD-L1 treatments correlated with better OS (HR =0.73; 95% CI: 0.64–0.83; P<0.05) and PFS (HR =0.77; 95% CI: 0.6–0.94; P<0.05) compared with standard chemotherapy in both patients with and without LM (194). However, subgroup analysis showed that while ICI monotherapy could not prolong PFS in patients with LM, ICI-based combination therapy could. Conversely, in patients without LM, both ICI monotherapy and combination therapy prolonged both PFS and OS. Together, these findings suggest that the presence of LM diminished tumor response in patients who received ICI monotherapy, especially in NSCLC. While combination treatments may overcome hepatic resistance mechanisms to ICI, the optimal strategy remains under investigation.

Use of locoregional therapies to the liver to overcome ICI resistance

It is hypothesized that the elimination of LM by surgical resection, radiation, or other locoregional treatments can restore ICI efficacy in patients with LM (*Table 1*).

Stereotactic body radiation therapy (SBRT) is an effective treatment modality for non-surgical candidates in patients with primary and oligometastatic liver tumors with adequate liver function. In pre-clinical models, radiation improves ICI efficacy. For example, in mice models with melanoma, the combination of anti-CTLA-4, anti-PD-L1/PD-1, and radiation produced major tumor responses (201). While anti-CTLA-4 can suppress Treg cell number and function, thereby increasing the $CD8^+$ T cell to Treg ($CD8^+$/Treg) ratio, radiation enhances the diversity of the T cell receptor repertoire of intratumoral T cells. The addition of

PD-L1 blockade reverses T cell exhaustion to mitigate the depression of $CD8^+$/Treg ratio and further activates T cell expansion (201). Even in patients with advanced NSCLC, where anti-CTLA-4 antibodies have failed to demonstrate significant monotherapy activity, the addition of radiation to anti-CTLA-4 therapy achieved disease control in 31% of patients (195). Increased serum interferon-β and early dynamic changes in blood T cell clones after radiation were predictors of response. These data suggest that the combination of liver SBRT and ICI may act synergistically to improve tumor response rate and outcomes of patients with LM.

Preclinical HCC models have also shown the combination of radiation and ICI to exhibit therapeutic synergism and improved OS (202,203). Small series have shown promising signs of clinical activity in patients with HCC (196,204). A propensity score matching analysis of patients with HCC who received SBRT-ICI versus trans-arterial chemoembolization (TACE) showed a significantly improved response rate (87.5% *vs.* 16.7%), 24-month PFS (77.8% *vs.* 2.1%), and 24-month OS (80.4% *vs.* 8.3%) in the SBRT-ICI arm (197). Studies are ongoing evaluating the efficacy of SBRT-ICI in early and advanced stage HCC (NCT05488522, NCT04857684).

Further, in the context of LM, liver-directed radiotherapy may modulate systemic ICI response. In mice bearing subcutaneous and liver tumors treated with radiotherapy, anti-PD-L1, or the combination, it was shown that liver-directed radiotherapy did not modulate T cell number in the subcutaneous tumor on its own, but along with PD-L1 blockade significantly increased T cell infiltration into the subcutaneous tumor (9). Mice which received the combination therapy had regression of both the subcutaneous and liver tumors, suggesting that liver-directed radiotherapy improves systemic efficacy of ICI by restoring peripheral $CD8^+$ T cells (9). Prospective clinical trials are ongoing to understand the clinical efficacy of combining hepatic SBRT with ICI in advanced malignancies with LM (NCT05169957, NCT04923776).

Local ablation increases liver immunogenicity and activation of DCs in HCC (205). In preclinical models, radiofrequency ablation increases T cell infiltration and expression of immune checkpoints (PD-L1, LAG-3) within the treatment zone and distant sites, via activation of serum and intra-tumoral cytokines (198,206,207). Thus, the addition of ICI to ablation may result in more effective antitumor immunity. This was studied in a small retrospective cohort of patients with CRC with LM, where the combination of radiofrequency ablation and PD-1 blockade significantly enhanced T cell immune responses, higher response rates and prolonged survival (198). Similarly, the combination of tremelimumab (CTLA-4 inhibitor) with tumor ablation led to 26.3% response rate with a median time to tumor progression of 7.4 months in patients with HCC (199).

TACE is a widely accepted treatment modality for patients with unresectable intermediate stage HCC. Transarterial radioembolization (TARE) using Yttrium-90 (Y90) is also an emerging and now adopted option for treating unresectable HCC (208). TACE may promote immunogenic cell death. In a cohort of patients with HCC treated with TACE, the proportion of circulating Th17 $CD4^+$ T cells increased and was associated with improved outcomes (209,210). In another cohort of patients with HCC treated with TACE, PD-L1 and PD-1 expression on tumor cells significantly increased following treatment (200). Similarly, in patients treated with TARE, the hepatic TME after treatment suggested signs of local immune activation with higher expression of granzyme B, infiltration of $CD8^+$ T cells, NK cells, and NKT cells. These studies indicate that the combination of TACE/TARE with ICI should be further investigated in the treatment of HCC.

Histotripsy is an investigational ultrasound ablation technique that uses short high-amplitude pulses to create inertial acoustic damage to tissues (211,212). The rapid expansion and collapse of cavitation microbubbles leads to cellular destruction of the target tissue (213) in a precise manner at the histologic level with real-time visualization by diagnostic ultrasound. In immunocompetent rat HCC models, partial histotripsy of local tumor resulted in complete response and prolonged disease-free survival compared to untreated controls (214,215). In murine tumor models, histotripsy demonstrated local and systemic anti-tumor immune responses with and without concurrent CTLA-4 blockade (211,216,217). A multi-center phase I study evaluated hepatic histotripsy in eight patients with unresectable multifocal liver tumors including HCC, CRC with LM, cholangiocarcinoma (CCA), and breast cancer with LM, in which no device-related adverse events were noted (218). A patient with CRC LM had sustained reduction of non-treated tumors in the liver following histotripsy (219). At a cellular level, histotripsy disrupts cellular structures to release tumor-specific antigens and damage-associated molecular patterns, which can stimulate innate and adaptive immune responses, and subsequently modulate the TME and diminish cancer progression

(215,216). Further research is required to identify the potential role of histotripsy in clinical practice and to consider combination strategies using hepatic histotripsy and ICI to overcome immune resistance.

Use of combination systemic therapies overcome ICI resistance

The addition of VEGF blockade may restore ICI efficacy in patients with LM. Enhancement of $CD8^+$ T cell function with anti-angiogenic agents has been demonstrated in solid malignancies including HCC (187,220-222). In patients with NSCLC and LM combination of VEGF blockade, chemotherapy, and ICI significantly prolonged PFS compared to chemotherapy and ICI alone (222). VEGF signaling has been implicated in diminished anti-tumoral immunity by several mechanisms, including reducing cytotoxic activity of peripheral $CD8^+$ T cells (223), enhancing Treg cell activation (224-226), and inducing immunosuppressive effects of MDSCs (68). VEGF-A also directly induces FASL expression leading to apoptosis of $CD8^+$ T cells (227). Thus, blocking the VEGF pathway in combination with PD-1/PD-L1 blockade may synergistically restore ICI efficacy in patients with LM by reducing $CD8^+$ T cell apoptosis within the liver, and enhance T cell activity and function systemically.

Adoptive cell transfer of chimeric antigen receptor-modified T cells (CAR-T) has been successful in treating hematologic malignancies but has had a modest impact on the treatment of solid tumors. In HCC, glypican-3 (GPC-3) provides a novel prognostic therapeutic target for CAR-T in HCC (228). *In vitro* and xenograft models of HCC have shown early signs of activity of GPC-3 targeted CAR-T cells in treating $GPC3^+$ HCC (229). Other potential targets for CAR-T cell therapy in HCC may include AFP (NC03132792), mutant *TP53*, and HBV antigens (NCT03899415). The polarization of immune cells within the liver TME may be shaped by CAR-T cell therapy, as well as cell-based therapy using TILs, which may induce the release of TAAs generating an antitumor immune-response. The identification of unique antigens on aberrant cells or tumor-associated cell death pathways may emerge as a new therapeutic strategy to overcome anti-inflammatory microenvironments and reactivate tumor immunosurveillance within the liver (230).

Immune checkpoint molecules beyond PD-1, PD-L1, and CTLA-4 may be targeted to enhance an anti-tumor immune response in primary and secondary liver cancers. In HCC, increased number of $PD\text{-}1^+CD8^+$ T cells and $PD\text{-}L1^+$ tumor cells are associated with poorer prognosis (231). TIM-3 expression on $CD4^+$ and $CD8^+$ TILs and TAMs inhibits T cell function in HCC, while TIM-3 expression on Treg cells further enhances T cell suppression (232). LAG-3 represents another targetable immune checkpoint, which normally is upregulated upon activation of T cells and can provide a de-activating signal to T cells. LAG-3 is preferentially expressed on tumor-specific $CD4^+$ and $CD8^+$ TILs as compared to other immune compartments (233). These preclinical data support further evaluation of the combination of LAG-3 and TIM-3 with PD-1 and PD-L1 blockade in the treatment of HCC.

In other solid malignancies, clinical trials are underway evaluating LAG-3 modulators along with PD-1 or CTLA-4 inhibitors. For example, LAG-3 blockade was shown to enhance TILs in preclinical models of CRC with LM (234). Relatlimab, the first commercially developed anti-LAG-3 antibody, was the first LAG-3 inhibitor to be approved along with nivolumab to treat unresectable or metastatic melanoma (235). Prospective clinical trials are needed to understand whether blockade of TIM-3, LAG-3 and/or other immune checkpoints (e.g., TIGIT, PVRIG, KIR-L, NKG2A, CD47) can enhance the efficacy of ICI in advanced malignancies with LM (236).

Conclusions

The hepatic TME of primary and secondary liver tumors is characterized by immune cells, suppressive cells, and complex pro-inflammatory and immunomodulatory signaling. The magnitude of the innate and adaptive immune response depends on the interactions between tumor cells and components of the liver TME. ICI monotherapy and along with other systemic therapies play a fundamental role in managing both primary and secondary liver cancer. However, further characterization of the underlying cellular and molecular mechanisms leading to immune evasion is needed to inform effective novel combination strategies and improve ICI efficacy in both PLC and LM.

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Footnote

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