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Hypoxia-inducible factors and innate immunity in liver cancer

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The liver has strong innate immunity to counteract pathogens from the gastrointestinal tract. During the development of liver cancer, which is typically driven by chronic inflammation, the composition and biological roles of the innate immune cells are extensively altered. Hypoxia is a common finding in all stages of liver cancer development. Hypoxia drives the stabilization of hypoxia-inducible factors (HIFs), which act as central regulators to dampen the innate immunity of liver cancer. HIF signaling in innate immune cells and liver cancer cells together favors the recruitment and maintenance of pro-tumorigenic immune cells and the inhibition of anti-tumorigenic immune cells, promoting immune evasion. HIFs represent attractive therapeutic targets to inhibit the formation of an immunosuppressive microenvironment and growth of liver cancer.

Introduction

Liver cancer is a deadly malignancy with very limited treatment options. The liver contains a high number of residential immune cells, particularly innate immune cells, which enable the rapid clearance of harmful substances and pathogens from the gastrointestinal system. Liver cancer is an inflammation-driven disease preceded by hepatitis virus infection and steatosis. Immune cells are therefore abundant in the microenvironment of liver cancer. Many innate immune cells such as macrophages are immunosuppressive in liver cancer, and they reduce the treatment efficacy of immune checkpoint blockade. The level of hypoxia escalates with hepatocarcinogenesis. Hypoxia is a key feature of liver cancer and has an intimate link with innate immunity. In this Review, we will discuss how hypoxia-inducible factors (HIFs) control the biological roles of different innate immune cells and cancer cells to subvert the innate immune response, creating an immunosuppressive microenvironment in liver cancer. The implications of hypoxia in innate immune responses in normal and other pathological conditions are summarized in other excellent reviews (1, 2). We will conclude by discussing the potential of targeting HIFs and the immunosuppressive innate immune cells for treatment of hepatocellular carcinoma (HCC).

Liver cancer

Liver cancer ranks the fifth most common and the third most fatal cancer worldwide (3). The incidence of liver cancer and related deaths has risen over the past 20 years in the United States (4). HCC, the malignancy derived from hepatocytes that accounts for 75%–85% of primary liver cancers, will be the focus of this Review.

HCC's high mortality rate is caused by its late symptom presentation, high resistance to conventional chemo- and targeted therapies, and frequent recurrence. Currently, the tyrosine kinase inhibitors (TKIs) sorafenib, lenvatinib, and cabozantinib are the three FDA-approved first-line targeted drugs for advanced HCC, with a modest survival benefit (5–7). Nivolumab (an immune checkpoint inhibitor targeting PD-1) was recently approved by the FDA as a second-line treatment for sorafenib-resistant HCC. A clinical trial demonstrated that nivolumab has a response rate of only 20% in HCC patients (8).

Hepatocarcinogenesis develops in a stepwise manner: from chronic liver inflammation caused by viral infections or steatosis, to liver damage, to fibrosis and cirrhosis, and finally to HCC. HBV infection remains the major cause of HCC globally, while HCV infection and nonalcoholic steatohepatitis (NASH) associated with high-fat diet represent the major causes of HCC in Western countries (4). Toxic liver damage caused by alcohol abuse also contributes to HCC (4).

Liver, liver cancer, and innate immunity

Liver has a double circulation system, receiving 20% and 80% of blood supply from the hepatic artery and the portal vein, respectively. The hepatic artery delivers oxygenated blood to the liver. The portal vein delivers oxygen-depleted blood containing pathogens, bacterial products, food antigens, and environmental toxins from the gastrointestinal tract to the liver. Pathogens and toxic agents are cleared rapidly by the innate immune cells in the liver. The liver is enriched with innate immune cells including Kupffer cells (liver macrophages), natural killer (NK) cells, natural killer T (NKT) cells, and γδ T cells (Figure 1 and refs. 9, 10). HCC development is often preceded by chronic liver inflammation that is accompanied by increases in infiltrating innate immune cells (11). The most commonly found innate immune cells in HCC include myeloid-derived suppressor cells (MDSCs), macrophages, neutrophils, and NK cells (Figure 1 and ref. 11).

Myeloid cells are integral components of the innate immune system. MDSCs are a heterogeneous population of myeloid progenitors and immature myeloid cells and are generally classified

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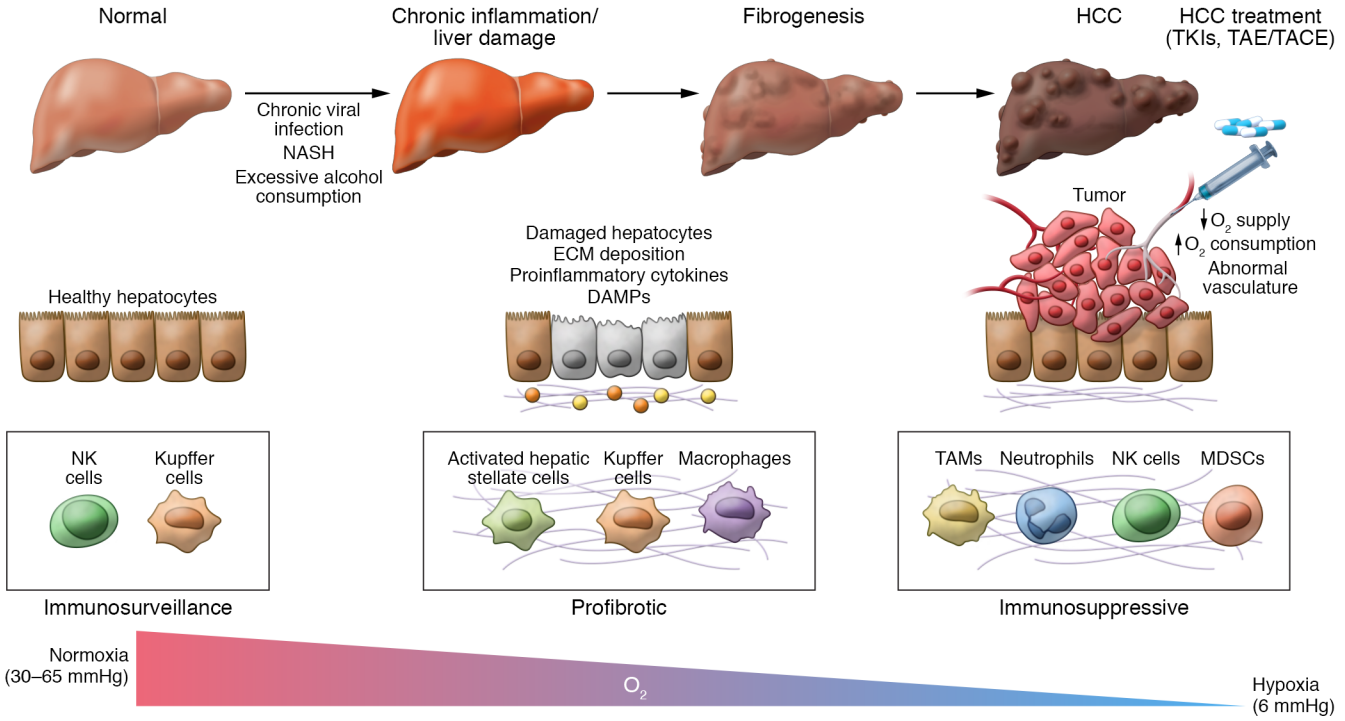

Figure 1. Hypoxia, innate immunity, and the development of HCC. Hepatocarcinogenesis is a stepwise process. Chronic hepatitis virus infection and fat-induced steatosis (NASH), the major etiological factors of HCC, drive liver inflammation and tissue damage. Excessive alcohol consumption also induces liver damage. Damage in the liver disrupts the hepatic vasculature and perturbs proper blood flow and O_2 supply, creating a hypoxic microenvironment. Hypoxic Kupffer cells, newly recruited macrophages, and hepatocytes activate hepatic stellate cells (HSCs) in the liver, which robustly deposit collagen, leading to fibrosis and then cirrhosis, which further intensifies hypoxia. When HCC is developed, hypoxia is even more severe. The growth of HCC outpaces the growth of blood vessels. Moreover, HCC cells consume all available O_2. Different current HCC treatments, such as TAE/TACE and TKIs, further induce hypoxia. Hypoxia affects the activities of different innate immune cells, such as NK cells. Hypoxia also induces the infiltration and accumulation of many different types of immunosuppressive innate immune cells, including TAMs, MDSCs, and neutrophils, in the microenvironment of HCC. The O_2 level decreases along with HCC development, driving the formation of an immunosuppressive microenvironment.

as granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (12). Myeloid progenitors normally differentiate into mature macrophages, dendritic cells, and neutrophils. However, MDSCs remain undifferentiated, rapidly expand in cancer, and are highly immunosuppressive (13). MDSCs generate high ROS levels to suppress the T cell receptor (TCR) signaling pathway (13). MDSCs also express arginase 1 (ARG1), inducible NO synthase (iNOS), TGF-β, IL-10, COX-2, and indoleamine 2,3-dioxygenase (IDO) to suppress a variety of immune cells, including macrophages, dendritic cells, T cells, and NK cells (13).

Macrophages phagocytose pathogens. Macrophages found in tumors are collectively called tumor-associated macrophages (TAMs). In cancer, TAMs are thought to derive from circulating monocytes that originate from bone marrow–derived myeloid progenitors and are recruited to the tumor by the inflammatory factors secreted by cancer cells. At the tumor sites, tumor-secretory factors drive the differentiation of the recruited monocytes and MDSCs into TAMs. Kupffer cells also form a major population of TAMs in HCC. In cancer, macrophages are generally classified as M1 or M2 macrophages (14). M1 macrophages respond to damage-associated and pathogen-associated molecular patterns (DAMPs and PAMPs) and IFN-γ and produce proinflammatory cytokines such as IL-12 (15). These proinflammatory macrophages are responsible for an antitumor response (14). M2 macrophages do not express MHC class II and have no antigen-presenting ability (15). They produce antiinflammatory cytokines including IL-4, IL-10, and IL-13 (15) and generally support tumor angiogenesis and cancer progression (15).

Neutrophils differentiate from PMN-MDSCs and are short-lived. Macrophages draw neutrophils to the site of infection through chemokines, where they are activated by binding to DAMPs/PAMPs. Activated neutrophils phagocytose foreign particles and sequester them into phagosomes, triggering the release of granules. In the cancer context, tumor-associated neutrophils (TANs) are classified as N1 (proinflammatory) and N2 neutrophils (antiinflammatory), exhibiting anti-tumorigenic and pro-tumorigenic roles, respectively (16).

NK cells belong to the innate lymphoid cell family and are nonphagocytic with rich cytoplasmic granules containing cytotoxic granzymes and perforin (17). NK cells rapidly induce cytolytic killing of cancer cells indirectly via degranulation and directly via death receptor cell signaling (TRAIL and Fas ligand) (17).

In general, cancer cells participate in the recruitment and maintenance of immunosuppressive cells and polarization of TAMs from M1 to M2. Hypoxia and HIFs play an important

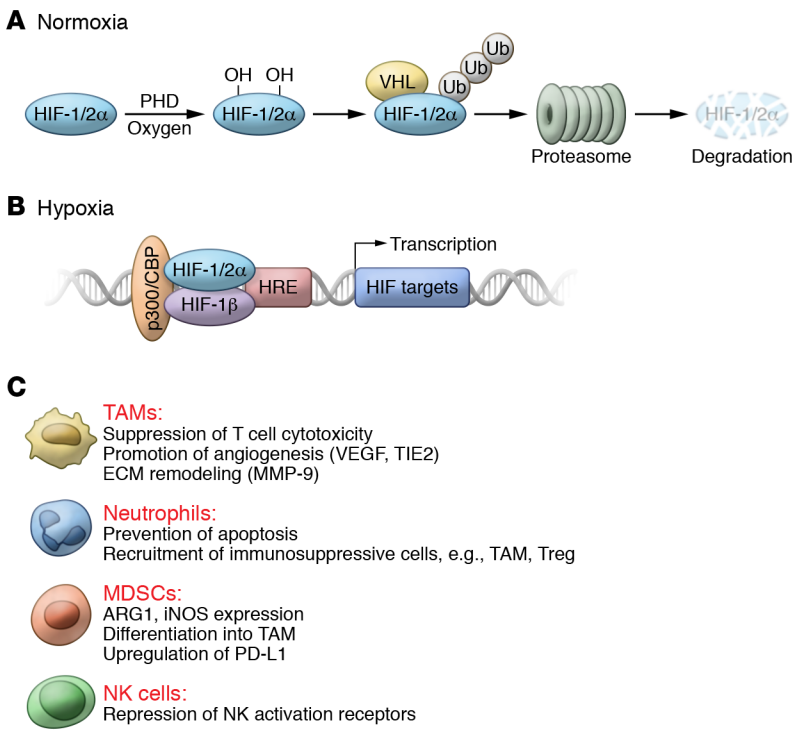

Figure 2. Roles of the hypoxia/HIF signaling pathway in innate immune cells in HCC. (**A**) In the presence of O_2, the HIF-1/2α subunit is hydroxylated by the PHD enzymes at two specific proline residues, enabling the binding of VHL. VHL targets the hydroxylated HIF-1/2α subunit for ubiquitin-mediated proteasomal degradation. (**B**) In the absence of O_2, the HIF-1/2α subunit is stabilized and dimerizes with HIF-1β, together with cotranscriptional factors p300 and CBP, to drive the transcription of genes encompassing hypoxia-responsive elements (HREs). (**C**) The hypoxia/HIF signaling pathway in the innate immune cells directly affects their properties in HCC.

role in accentuating the immunosuppressive characteristics of innate immune cells in the microenvironment of HCC.

Establishment of hypoxic niche in liver cancer

The O_2 tensions in the periportal region and perivenous regions of normal liver are about 60–65 mmHg and 30–35 mmHg, respectively (18). O_2 tension drops to 6 mmHg in liver cancer tissues (19). Hypoxia starts to develop in inflamed liver tissues as the result of viral infection or steatosis. The general mechanisms by which inflammation drives hypoxia are summarized in an excellent review (1). Hypoxia further accumulates at the time of liver injury and intensifies during fibrosis, cirrhosis, and HCC progression (Figure 1). Liver damage disrupts the hepatic vasculature, affecting blood flow and O_2 delivery into areas of injured tissues (20). Cirrhosis further obstructs blood flow into the liver. In later stages of hepatocarcinogenesis, a number of causes further intensify hypoxia: HCC cells consume and deplete O_2 quickly, but HCC is not supported by functional vasculature. HCC palliative therapy, such as transarterial embolization/chemoembolization (TAE/TACE), which aims to obstruct the blood supply to restrict tumor growth, inadvertently induces hypoxia (21). TKIs block multiple kinases responsible for angiogenesis, thereby further inducing intratumoral hypoxia (22).

Mechanisms of HIF stabilization

The major molecular mechanisms induced by hypoxia are mediated through HIFs, heterodimers composed of the O_2-sensitive HIF-1/2α subunit and the constitutively expressed HIF-1β subunit (refs. 23, 24, and Figure 2A). In the presence of O_2, HIF-1/2α is hydroxylated at specific proline residues by prolyl hydroxylases (PHDs) (25). The von Hippel-Lindau (VHL) ubiquitin ligase complex recognizes the prolyl-hydroxylated HIF-1/2α and conjugates HIF-1/2α with ubiquitin, directing HIF-1/2α for proteasomal degradation (26). In the absence of O_2, HIF-1/2α is stabilized and dimerizes with HIF-1β, together with the coactivator p300/CBP, to drive transcription of genes encompassing hypoxia-responsive elements, which are the conserved core sequence 5′-RCGTG-3′, in different cell types that experience hypoxia (27).

Hypoxia and HIFs in early stages of hepatocarcinogenesis

HIF protein induction can be detected in early diseased liver tissues and contributes to early steps of hepatocarcinogenesis (20). In the fibrotic liver, hypoxic Kupffer cells and newly recruited macrophages function as M1 macrophages (Figure 1). These proinflammatory cells and hepatocytes generate a high level of secretory growth factors, including PDGF-β, in a HIF-dependent manner (28–30). PDGF-β promotes proliferation of hepatic stellate cells (HSCs) in the liver (31). HSCs differentiate into myofibroblasts, which actively produce collagen, causing fibrosis and cirrhosis (32). Hypoxia also induces VEGF secretion in Kupffer cells and HSCs (28, 33). VEGF activates HSCs, promotes fibrogenesis, and recruits monocytes (32, 34, 35). Apart from effects on PDGF-β and VEGF, HIFs drive the expression of a list of proinflammatory cytokines and fibrogenic genes. Mice with hepatocyte-specific *Vhl* knockout demonstrated increased HIF-1/2α expression and an induction of fibrotic genes, including collagen synthesizing and modification enzymes (*Plod2*, *Tgm2*) and α-SMA (a marker for activated myofibroblasts) (36). Deleting *Hif1a* in HSCs reduces collagen synthesis and impedes macrophage-mediated clearance of necrotic cells in damaged liver (37). All these studies unequivocally demonstrated that HIF signaling in all liver cell types is critical in driving early hepatocarcinogenesis.

Table 1. HIFs' roles in innate immune cells and their crosstalk in HCC and other disease models

Innate immune cells	Role of hypoxia/HIF in crosstalk	Mechanism of crosstalk with other immune cell types	Contributions of crosstalk to cancer	Disease model	References
TREM-1$^+$ TAMs	Transcriptional activation of TREM-1 in TAMs	Recruit Tregs to suppress CD8$^+$ T cells	Immunosuppression, cancer promoting, PD-L1 blockade resistance	HCC	48
Kupffer cells	Induction of CCL5 in Kupffer cells	Recruit Ly6G$^+$ neutrophils	Immunosuppression, cancer promoting	HCC	51
MDSCs	Induction of ARG/iNOS in MDSCs	Suppress antigen-specific and non–antigen-specific T cells	Immunosuppression, cancer promoting	Multiple cancers	58
MDSCs	Induction of miR-210 in MDSCs to reduce IL-16 and CXCL12	Suppress T cells	Immunosuppression, cancer promoting	Multiple cancers	59
MDSCs	Transcriptional activation of PD-L1 in MDSCs	Drive T cell exhaustion	Immunosuppression, cancer promoting	Multiple cancers	60
TANs	Promotion of TAN survival	Recruit macrophages and Tregs	Immunosuppression, cancer promoting, sorafenib resistance	HCC	63, 71
NK cells	Repression of NK cell activity	Polarize macrophages toward M1 phenotype	Fibrosis repressing	NASH fibrosis	77
MDSCs	Not available	Suppress NKp30$^+$ NK cells	Immunosuppression, cancer promoting	HCC	78

Hypoxia and HIFs in later stages of hepatocarcinogenesis

HIF-1/2α protein is highly expressed in human HCC tissues (38–40), and expression correlates with poor clinical outcome in HCC patients (41, 42). A hypoxic score based on seven hypoxia-related genes displayed strong prognostic values in HCC (43). Interestingly, a recent single-cell RNA sequencing study demonstrated that liver cancers infiltrated with higher amounts of nonmalignant cells, including TAMs, have stronger hypoxic signaling (44). Another single-cell RNA sequencing study on tumor-infiltrated immune cells confirmed that HCC contains a variety of innate immune cells, including conventional NK cells, liver-resident NK cells, MDSCs, and TAMs (45). Notably, it was found that TAMs coexpress M1 and M2 gene signatures, consolidating the recent perception on the complicated biology of TAMs beyond the M1 and M2 classifications (45). TAMs may exist in a continuum of functional states (14), and coexistence of proinflammatory and antiinflammatory macrophages is possible in human HCC.

HIFs' roles in innate immune cells in HCC

HIFs in TAMs. HIF-1α and HIF-2α have distinct roles in myeloid cells. HIF-1α supports glycolysis and ATP production in myeloid cells, enabling them to infiltrate and adhere at inflammatory tissues (46). HIF-2α drives the expression of receptors such as CXCR4, M-CSFR, and fibronectin 1 (FN1) in myeloid cells, enabling them to migrate to and infiltrate inflammatory and cancer tissues (47). In a diethylnitrosamine-induced mouse HCC model, deletion of HIF-2α in myeloid cells suppressed TAM infiltration into HCC (47). HIF-1α also transcriptionally activates the receptor TREM-1 in TAMs (48). TREM-1$^+$ TAMs recruit immunosuppressive Tregs to HCC in a hypoxia-dependent manner, leading to reduced infiltration of CD8$^+$ T cells and poor survival in human HCC patients (Figure 2B and ref. 48). NF-κB was shown to induce *Hif1a* mRNA in macrophages (49). HIF-1α protein was reported to be degraded by hypoxia-associated factor (HAF; encoded by *SART1*), an E3 ubiquitin ligase, in an oxygen-independent manner (50). The Kupffer cells in *Sart1* heterozygous KO mice constitutively express HIF-1α, which induces RANTES/CCL5 to recruit Ly6G$^+$ neutrophils into the liver, driving HCC (51).

In addition to the primordial functions of TAMs in T cell suppression and immune evasion, TAMs also promote angiogenesis. Hypoxia induces an array of angiogenic factors (VEGF, PDGF-β, FGF, angiopoietins) in primary human macrophages to promote angiogenesis (ref. 52 and Figure 2B). The induction of VEGF in macrophages was directly shown to be mediated through HIF-1α (53). Apart from directly secreting angiogenic factors, hypoxic TAMs release MMP-9, which cleaves the extracellular matrix to liberate the sequestered VEGF (ref. 54 and Figure 2B). Moreover, hypoxia induces TIE2, a monocyte receptor. TIE2-expressing monocytes (TEMs) represent a strong proangiogenic myeloid subset and are attracted by endothelial cell–derived angiopoietin-2 in hypoxic tumors (55). Interestingly, TAM/TEM infiltration is frequently found in HCC tissues and is positively correlated with microvessel density, with prognostic value in HCC (56, 57).

HIFs in MDSCs. An interesting study comparing the difference of splenic MDSCs and tumor-associated MDSCs in tumor-bearing mice has highlighted the roles of hypoxia in MDSCs (58). The concentration of O_2 in the spleen is higher than that in tumors. Splenic MDSCs in tumor-bearing mice suppress antigen-specific T cells through ROS induction (58). Hypoxia in the tumor activates HIF-1α in tumor-associated MDSCs, thereby driving ARG1 and iNOS expressions to suppress both antigen-specific and non–antigen-specific T cells (ref. 58 and Figure 2B). Along these lines, an interesting study showed that a well-known HIF-1α–induced microRNA, miR-210, is highly expressed in tumor-associated MDSCs as compared with splenic MDSCs (59). MiR-210 in tumor-associated MDSCs increases ARG1 and reduces IL-16 and CXCL12 to suppress T cells (59). HIF-1α drives the differentiation of tumor-associated MDSCs into TAMs to further promote tumorigenesis (ref. 58 and Figure 2B). It is of great interest that HIF-1α transcriptionally activates PD-L1 in MDSCs and other myeloid cells (ref. 60 and Figure 2B). PD-L1 is a well-known immune checkpoint surface protein that drives T cell exhaustion

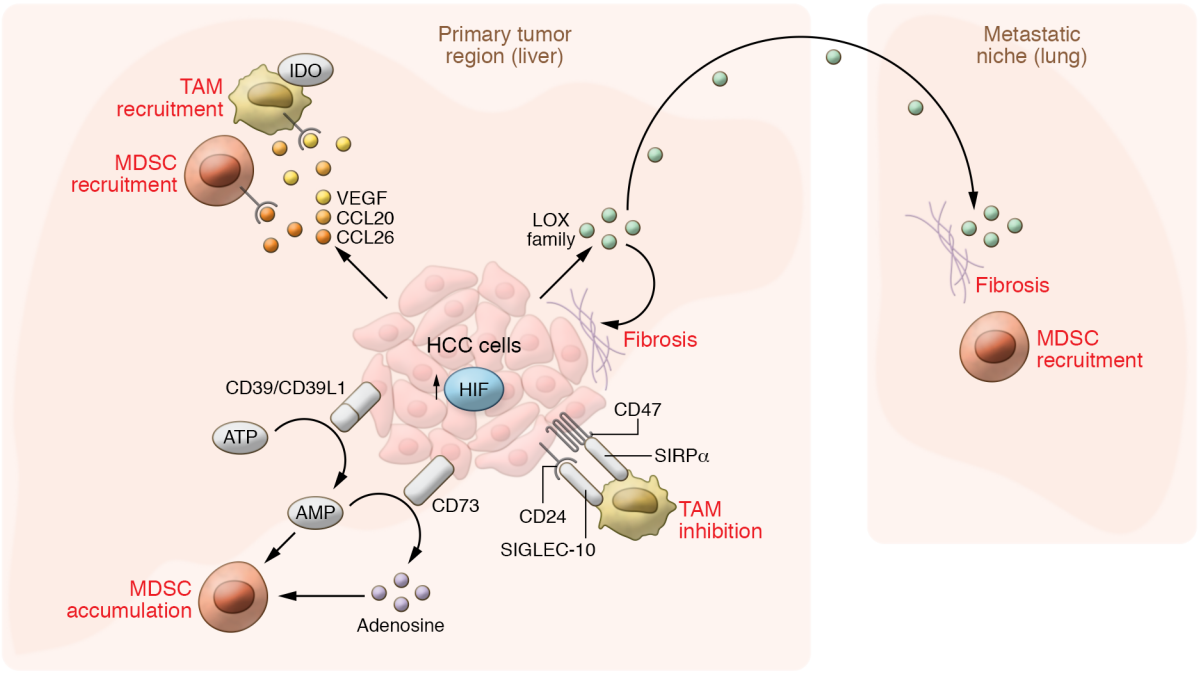

Figure 3. Roles of the hypoxia/HIF signaling pathway in HCC cells that affect innate immune cells in the tumor microenvironment. HIFs activate the transcription and secretion of the chemokines CCL20, VEGF, and CCL26 in HCC cells. These chemoattractants recruit immunosuppressive TAMs and MDSCs to HCC. HIFs activate the transcription of the don't-eat-me signal surface markers (CD47, CD24) in cancer cells including HCC cells. CD47 and CD24 are well-characterized liver cancer stem cell markers. CD47 and CD24 prevent cancer cells from being phagocytosed by the macrophages. HIFs activate the transcription of members of the purinergic signaling pathway (CD39, CD39L1, CD73) in HCC cells to create an adenosine- and AMP-rich microenvironment that favors the accumulation of MDSCs. HIFs activate the transcription of members of the LOX family in HCC cells. The LOX family cross-links collagen in the primary cancer and metastatic niches. At the primary liver cancer niche, the LOX family increases tissue stiffening and promotes local invasion of cancer cells. At the metastatic niche in the lung, cross-linking of collagen mediated by the LOX family helps to recruit MDSCs, creating a favorable niche for HCC cell colonization.

through binding to PD-1, an inhibitory receptor on T cells (61). PD-L1 expression on monocytes in tumors is associated with poor survival in HCC patients, and blockade of PD-L1 reinstates T cell immunity against HCC (62).

HIFs in neutrophils. Hypoxia promotes survival and prevents apoptosis of neutrophils through HIF-1–mediated transcription of NF-κB (ref. 63 and Figure 2B). Cancer cells secrete high levels of G-CSF and other chemokines such as CXCL1, CXCL2, CXCL5, and CXCL8 to recruit and activate neutrophils (64, 65). TGF-β then polarizes TANs into immunosuppressive N2 phenotype with high ARG1, CCL2, and CCL5 expression (66). Depleting neutrophils activates $CD8^+$ T cells and represses HCC growth (66, 67). Interestingly, TGF-β and HIF-1 could be reciprocally induced (68, 69) and activate the same genes in HCC (70). Thus, hypoxia/HIF-1 might also contribute to N2 polarization of neutrophils. HCC cells activate the PI3K/AKT and p38/MAPK pathways in TANs to induce their expression of CCL2 and CCL17 (71). TANs, through CCL2 and CCL17, recruit macrophages and Tregs to HCC through CCR2 and CCR4 receptors (71). Apart from attracting immunosuppressive cells, TANs also promote angiogenesis in tumors. Interestingly, increased TAN infiltration was found in tumors from HCC patients treated with sorafenib (71). Sorafenib treatment, through inducing hypoxia and HIF-1 stabilization, activates the NF-κB pathway in HCC cells to drive CXCL5 secretion, which further attracts TANs to HCC (71). Depletion of TANs sensitizes HCC to sorafenib treatment in mice (71). In a large cohort of 452 HCC patients, immunohistochemical study confirmed that the number of TANs significantly correlated with the number of M2 macrophages and Tregs as well as poor prognosis (71), indicating the immunosuppressive roles of TANs in HCC.

Hypoxia, HIFs, and NK cells. NK activity is balanced between signals from NK activation receptors and NK inhibitory receptors. NK activation receptors bind to ligands expressed by infected and cancer cells. NK inhibitory receptors bind to the classical MHC class I molecule, which is only expressed by host cells to prevent them from being mistakenly attacked by NK cells. The NK activation receptors, including NKp46, NKp44, NKp30, and NKG2D, are repressed by hypoxia (ref. 72 and Figure 2B). Meanwhile, ligands that bind to NK activation receptors are repressed in cancer, thereby preventing NK cell–mediated cancer cell killing. In HCC, expression of NKG2D ligand was reduced in more aggressive HCC (73). Nonclassical MHC class Ib molecules (HLA-E, HLA-F, HLA-G), which are often upregulated in cancer, can bind to NK inhibitory receptors to inhibit NK cells. HLA-G was shown to be a HIF-1 transcriptional target in cancer (74). In HCC, HLA-G was expressed in over half of human HCC tissues (75, 76). It is therefore rational to speculate that NK cell activity is partially limited in the tumor microenvironment of HCC. NK cells also crosstalk with myeloid cells during different stages of hepatocarcinogenesis and polarize M2 to M1 macrophages to suppress NASH development to fibrosis (77). Interestingly, MDSCs are able to suppress NK cells in HCC (78).

Table 2. Roles of HIFs in HCC cells

HIF transcriptional target in cancer cells	Overexpression in HCC	Direct effect on innate immune cells in the tumor microenvironment	Mechanisms of crosstalk with other immune cell types	Role in cancer	References
CCL20	Yes	Induces exhaustion marker IDO in macrophages in tumors	Enriches Tregs, suppresses proliferation and IFN-γ production of effector T cells	Cancer promoting	79
SEMA3A	Yes	Attracts NRP1$^+$ TAMs to tumors	Suppresses proliferation and recruitment of T cells	Cancer promoting	82–84
VEGF	Yes	Recruits VEGFR1$^+$ monocytes and macrophages to tumors	Not available	Cancer promoting	85–88
CCL26	Yes	Recruits CX3CR1$^+$ MDSCs to tumors	Not available	Angiogenesis promoting	89
LOXL2	Yes	Recruits MDSCs to metastatic niche	Not available	Metastasis promoting	70, 92
CD47	Yes	Prevents phagocytosis by macrophages	Not available	Cancer promoting	95, 96, 99
CD24	Yes	Prevents phagocytosis by macrophages	Not available	Cancer promoting	94, 97, 98, 100
CD39L1	Yes	Enriches MDSCs by preventing their differentiation to macrophages and dendritic cells	Suppresses T cells	Cancer promoting, immune checkpoint blockade resistance	118

These data together suggest that HIF signaling in different immunosuppressive innate immune cells generally contributes to poor outcome in HCC (Table 1). Most studies mentioned above focus on the roles of HIF in cytokine induction in different innate immune cells. HIF-1 is a central regulator of metabolism in all cell types by diverting metabolites into glycolysis. It is likely that all cell types in the hypoxic tumor niche are forced to take the glycolytic route. The current literature has not addressed how HIF-1–mediated metabolic switching affects the biology of different tumor-infiltrated immune cells in HCC, nor, importantly, whether simultaneous activation of HIF-1 in both immune and cancer cells leads to nutrient competition between different immune cells and cancer cells in the hypoxic HCC microenvironment. Currently, innate immune cells are characterized by limited markers using flow cytometry. Single-cell RNA sequencing will bring new knowledge about the heterogeneity of each innate immune cell type. Integration of this information with hypoxia gene signature will bring new insight into the roles of hypoxia and HIFs in the biology of different immune cells.

Roles of HIFs in HCC cells

HIFs in cancer cells regulate a repertoire of genes responsible for maintaining an immunosuppressive microenvironment in the tumor. These HIF-regulated genes encode for chemoattractants, members in the purinergic signaling pathway, and don't-eat-me signaling molecules that allow cancer cells to evade immune surveillance (Figure 3 and Table 2). Notably, hypoxia also induces cancer cell death, which attracts macrophages.

HIFs induce chemoattractants and lysyl oxidase (LOX) enzymes. HIFs in cancer cells transcriptionally activate CCL20, SEMA3A, and VEGF to enrich TAMs and CCL26 to recruit MDSCs. Transcriptional regulation of HIFs on these genes has been demonstrated in multiple solid cancer types, including HCC. All of these genes are overexpressed in human HCC.

CCL20 is a chemokine that is transcriptionally activated by HIF-1 in human HCC cells (79). CCL20 induces the expression of the immunosuppressive enzyme IDO in macrophages through the STAT pathway in the HCC microenvironment (79). IDO metabolizes tryptophan (Trp) to kynurenine (Kyn) (80). Trp supports T cell proliferation, while Kyn binds to AHR transcription factor of CD4$^+$ T cells to suppress their differentiation to Th17 cells and favor the production of Tregs (81). CCL20-treated IDO$^+$ macrophages enrich Tregs and suppress proliferation and IFN-γ production of effector T cells in HCC (79). Interestingly, HCC patients with high CCL20 expression have shorter survival. CCL20 and IDO expressions are also correlated in portal vein tumor thrombus (79).

SEMA3A is a secretory factor that attracts neuropilin-1–expressing (NRP1-expressing) macrophages. In a lung cancer model, hypoxia induced SEMA3A secretion to attract NRP1$^+$ TAMs to tumors (82). SEMA3A activates PlexinA1/PlexinA4 sig-

Table 3. HIF inhibitors used in HCC

HIF inhibitor	Mechanism of action	Effect in HCC	References
Digoxin	Suppresses translation of HIF-1α	Suppresses HCC growth and metastasis in mice	70, 89, 130
EZN-2968	Locked nucleic acid targeting HIF-1α	Partial response and stable disease in a small subset of HCC patients	135, 136
HIF-1α antisense oligonucleotide	Antisense nucleic acid targeting HIF-1α	Improves the efficiency of TAE in HCC-bearing rats	21
EF24	Inhibits HIF-1α protein	Synergizes with sorafenib to suppress HCC growth in mice	137, 138
PT-2385	Suppresses heterodimerization of the HIF-2	Synergizes with sorafenib to inhibit HCC growth in mice	139

naling to stimulate VEGFR1 on TAMs, inducing motility toward hypoxic cancer regions (82). More interestingly, Casazza et al. further showed that after NRP1$^+$ TAMs enter hypoxic zones, HIF-2α activates IKK complex (via the NF-κB pathway) to drive feed-forward repression of NRP1 expression on TAMs, preventing these TAMs from exiting the hypoxic zone (82). Multiple studies have reported that SEMA3A is overexpressed in human HCC (83, 84).

VEGF is a well-known transcriptional target of HIF that promotes tumor angiogenesis. VEGF also activates VEGFR1 (also known as FLT-1) on monocytes and macrophages to recruit them (85, 86). Hypoxia induces VEGF expression in human HCC cell lines by activating *VEGF* gene transcription and increasing *VEGF* mRNA stability. VEGF-expressing HCC cells are in close proximity to the hypoxic tumor region. Overexpression of VEGF is observed in HCC and is correlated with tumor vascularization and progression (87, 88).

CCL26 is chemotactic for eosinophils and monocytes (89, 90). Our group has reported that CCL26 is a transcriptional target of HIFs in HCC cells (89). Hypoxia-induced CCL26 in HCC cells attracts CX3CR1$^+$ MDSCs to the tumor site (89). We showed that tumor-associated MDSCs promote HCC growth by enhancing angiogenesis. Blocking communication between HCC cells and MDSCs using HIF inhibitor or CX3CR1 neutralizing antibody prevents MDSC homing and suppresses HCC development in mice (89).

Apart from regulating chemokines, HIFs were shown to recruit MDSCs to the metastatic site through transcriptional activation of the lysyl oxidase (LOX) family in cancers (91, 92). LOX family members, including LOX and LOXL1–LOXL4, are secretory amine oxidases that posttranslationally modify and cross-link collagen. In HCC, our group found that hypoxia-induced LOXL2 is responsible for cross-linking collagen in the primary tumors (70). Overexpression of LOXL2 protein is found in tissues and sera of HCC patients (70). At the primary site, cross-linking of collagen promotes the local invasion of HCC cells by enhancing tumor stiffness, which activates the Rho kinase–mediated cytoskeletal remodeling of HCC cells (70). At the metastatic site, cross-linking of collagen facilitates the attachment of myeloid cells (70, 92). These myeloid cells are known to be rich in angiogenic factors that prime the metastatic tissues for cancer cell lodging and colonization (91). It will be interesting to comprehensively examine the immunosuppressive roles of this subset of myeloid cells in the metastatic niche. An elegant study showed that HBV infection triggers the expansion of PMN-MDSCs, which protect the liver from T cell–induced inflammation and liver damage (93). HCC might represent a unique cancer type with an exceptionally high number of MDSCs due to HBV infection. Previously, our group showed that MDSCs preferentially localize at hypoxic regions of human HCC (89). Hypoxia/HIFs might act as a second trigger to further recruit MDSCs through transcriptional induction of various chemoattractants in HCC cells. It will be interesting to evaluate the differences between MDSCs in hepatitis-associated HCC and steatosis-associated HCC.

HIFs induce don't-eat-me signals to prevent phagocytosis. Hypoxia induces the "don't-eat-me" signals CD47 and CD24 through HIF-1–mediated transcription in cancer cells to evade phagocytosis by macrophages (94, 95). CD47 expressed on cancer cells binds to SIRPα on macrophages. The cytoplasmic domain of SIRPα contains the immunoreceptor tyrosine-based inhibition motifs (ITIMs), which relay inhibitory signals to suppress phagocytosis (96). CD24 expressed on cancer cells binds to SIGLEC-10, a receptor on macrophages that inhibits phagocytosis (97). Both CD47 and CD24 are liver cancer stem cell markers (98, 99). CD24$^+$ liver cancer stem cell population is directly induced by hypoxia (100). Liver cancer stem cells are populations of liver cancer cells with self-renewal capabilities and high resistance toward chemo- and targeted therapies (101). These findings collectively suggest a new role of hypoxia-enriched liver cancer stem cells in evading surveillance from the innate immune system.

HIFs induce purinergic signaling to evade immune surveillance. ATP is released extracellularly by injured or dying cells. Extracellular ATP is generally proinflammatory and serves as a danger signal to direct phagocytic cells to the inflamed tissues and to alarm other immune cells. A pair of ectoenzymes, CD39/CD39L1 (ENTPD1/ENTPD2) and CD73 (NT5E), are responsible for the conversion of extracellular ATP to adenosine (102). CD39/CD39L1 dephosphorylates extracellular ATP to ADP to AMP, while CD73 further dephosphorylates extracellular AMP to adenosine. Adenosine is immunosuppressive and accumulates in hypoxic tissues (103, 104). Extracellular adenosine is sensed by G protein–coupled receptors (A2AR and A2BR) on immune cells. Through A2AR, adenosine inhibits the activity of M1 macrophages and NK cells, increases PD-1 and CTLA-4 expression on T cells, and enriches Tregs (105–112). A2AR also inhibits secretion of proinflammatory cytokines such as IFN-γ and IL-2 (108, 111). Through A2BR, adenosine promotes M2 macrophages and MDSCs, and prevents antigen presentation of dendritic cells (107, 113–115). CD39, CD39L1, and CD73, well-known cancer therapeutic targets (105, 116, 117), are transcriptionally activated by HIFs in different cell types, including HCC (118–120). Hypoxia also induces A2BR in cancer cells and dendritic cells in a HIF-dependent manner (115, 121). Mechanistically, CD39, CD39L1, and CD73 drive extracellular adenosine accumulation and immunosuppression in different cancer models. Our group showed that HIF-1–induced CD39L1 in HCC cells suppresses the differentiation of M-MDSCs to dendritic cells through AMP, thereby maintaining MDSCs and limiting T cell activity in the hypoxic niche (118). We further showed that CD39/CD39L1 inhibitor synergizes with anti–PD-1 to inhibit HCC development in mice (118). Effects of ATP and adenosine are broad given the ubiquitous expression of their receptors in multiple cell types leading to paradoxical effects in the pathogenesis of diseases. In *Cd39*-KO mice, it was shown that ATP activates several key oncogenic pathways in the liver; therefore CD39 loss promotes the formation of carcinogen-induced HCC in mice (122). In *Cd39l1*-KO mice, it was shown that CD39L1 protects mice from liver fibrosis (123). Interestingly, two elegant studies demonstrated that CD39 on myeloid cells protects mice from sepsis-associated liver injury and biliary fibrosis in sclerosing cholangitis (124, 125). *Cd39*-KO and *Cd73*-KO mice were more susceptible to liver injury and acute liver failure (126). Taken together, these studies suggest that, in the cancer context, conversion of extracellular ATP to AMP or adenosine creates an immunosuppressive microenvironment. Ectoenzymes involved in this conversion and receptors responding to these immunosuppressive metabolites are all induced by hypoxia and regulated by HIFs; therefore, hypoxia and HIFs play a central role in purinergic signaling and contribute to immune evasion.

Hypoxia-mediated necrotic debris mediates HCC and macrophage communication. Hypoxia often results in necrosis in cancer tissues. Macrophages are dead-cell scavengers that are recruited to necrotic regions to clear cellular debris. An interesting study has demonstrated that necrotic debris from severe hypoxic HCC cells initiates crosstalk between cancer cells and TAMs in hypoxic HCC (127). The researchers observed that $CD206^+$ TAMs (M2) are preferentially located in necrotic regions in human HCC (127). The necrotic debris from hypoxic HCC cells polarizes macrophages by inducing M2 markers (CD163, ARG1, and MRC1) and repressing M1 markers (iNOS) (127). The necrotic debris from hypoxic HCC cells also induces IL-1β through TLR4/TRIF/NF-κB signaling in macrophages (127). Intriguingly, IL-1β, through the NF-κβ/COX-2 pathway, in turn induces HIF-1α transcription in HCC cells, thereby activating the epithelial-mesenchymal transition of HCC cells (127).

Compared with other cancer types, the available literature regarding the roles of HIF signaling in immune evasion in HCC is scanty. This can be attributed to the lack of reliable transgenic mouse models that can form spontaneous HCC, preventing researchers from studying the immune microenvironment of HCC. Hydrodynamic tail-vein injection enables the delivery of the genome editing systems to the mouse liver to knock out tumor suppressor genes and overexpress oncogenes to induce HCC in immune-competent mice (128, 129). This technological advancement is expected to substantially expedite research in this area.

HIF inhibitors as HCC treatment

A number of drugs have been identified to suppress different steps contributing to HIF functions. These drugs mainly inhibit the transcription, translation, dimerization, DNA binding, and transcriptional activity of HIFs. We will focus on the HIF inhibitors that have been evaluated in liver cancer (Table 3). An elegant drug library screening study in a human HCC cell line identified digoxin as an effective HIF-1 inhibitor (130). Digoxin suppresses the translation of HIF-1α and inhibits multiple HIF-1–mediated pro-tumorigenic events (130–134). We first demonstrated that digoxin is able to repress HIF-1/LOX family–mediated premetastatic niche formation, including collagen cross-linking, recruitment of $CD11b^+$ myeloid cells, and cancer cell colonization in breast cancer (70). In HCC, our group showed that digoxin effectively inhibits tumor growth and suppresses the recruitment of MDSCs to the primary tumor site (70). Digoxin is currently being evaluated in clinical trials for breast cancer (ClinicalTrials.gov NCT01763931) and pancreatic cancer as a combined treatment with FOLFIRINOX (NCT04141995). While the effects of digoxin in human HCC remain to be investigated, a phase Ib clinical trial on EZN-2968 (135), a locked nucleic acid targeting HIF-1α, showed that two of eight HCC patients had partial response and stable disease (136).

A number of HIF inhibitors could improve the effects of existing HCC treatments (Table 3). A study showed that intraportal delivery of HIF-1α antisense oligonucleotides via adeno-associated viral vector is able to improve the efficiency of TAE in HCC-bearing rats (21). The clinical outcomes of sorafenib, lenvatinib, and cabozantinib, like many antiangiogenic inhibitors, have been disappointing, as tumors often develop resistance. One of the important mechanisms that contribute to antiangiogenic inhibitor resistance is the undesirable induction of intratumoral hypoxia and stabilization of HIFs. As demonstrated in a mouse model, sorafenib induces HIF-1α in HCC, leading to sorafenib resistance (137). A drug with antagonistic effects against HIF-1α, EF24, was able to synergize with sorafenib to suppress HCC growth in mice (137, 138). A potent HIF-2 inhibitor, PT-2385, which specifically suppresses the heterodimerization of HIF-2, synergizes with sorafenib to inhibit HCC growth (139). Meanwhile, another study showed that sorafenib promotes the recruitment of myeloid cells expressing a high level of macrophage chemoattractants in a mouse HCC model (140). Cotreatment of sorafenib and TAM-depleting agents (zoledronic acid or clodrolip) synergistically inhibits HCC angiogenesis, growth, and metastasis (140). These studies independently show that inhibition of HIFs or inhibition of TAM synergizes with sorafenib in HCC. Whether the effects of HIF inhibition are mediated through TAM inhibition in the microenvironment of HCC merits further exploration. An additional consideration is that TAM-depleting agents simultaneously remove pro-tumorigenic and anti-tumorigenic TAMs. Therapeutic agents that specifically drive the polarization of TAMs to reinvigorate their phagocytic, antigen-presenting, and other antitumoral abilities are yet to be developed.

A recent phase III clinical trial (IMbrave 50) showed that combined anti–PD-L1 (atezolizumab) and anti-VEGF (bevacizumab/Avastin) therapy as a first-line treatment remarkably extended overall and progression-free survival in advanced HCC patients (141). This is the first drug regimen to show improved survival benefits relative to sorafenib with statistical significance and will likely change the clinical treatment of HCC in the near future (141). Synergy of antiangiogenic treatment with immune checkpoint blockade was concurrently observed in a mouse HCC model (142), in which anti-VEGFR2 stimulates PD-1 and PD-L1 expression in vivo (142). Bevacizumab induces intratumoral hypoxia (143, 144). It is rational to speculate that antiangiogenic drugs like bevacizumab induce an immunosuppressive microenvironment through HIF-1 by enriching TAMs, MDSCs, and TANs and inducing PD-L1, which together limit T cell activity. Therefore, PD-1/PD-L1 blockade restores T cell activity and works synergistically with antiangiogenic drugs, explaining the strong synergy observed in atezolizumab and bevacizumab in HCC patients.

Conclusion

Hypoxia is a key microenvironmental factor in all stages of hepatocarcinogenesis. Hypoxia affects all cell types in the liver. In early stages of hepatocarcinogenesis, HIFs in Kupffer cells promote liver cirrhosis by activating HSCs to generate profibrogenic factors. In later stages of hepatocarcinogenesis, HIFs act as master regulators to dampen the innate immunity in the liver to allow cancer cells to bypass immune surveillance. There are many exciting research questions yet to be addressed. In the future, studies should focus on the functions of hypoxia/HIFs in the complement system and the antigen presentation abilities of different innate immune cells in liver cancer. Innate immunity is tightly linked with adaptive immunity, and together they affect the response to immune therapies. Due to space limitation, we have only covered the roles of hypoxia/HIFs in innate immune cells in liver cancer. HIFs also orchestrate the adaptive immune system by transcriptionally activating genes important for Treg enrichment and T cell exhaustion. In the future, researchers should take advantage of new technol-

ogy platforms such as single-cell RNA sequencing, mass cytometry (CyTOF), and fluorescent multiplex immunohistochemistry to obtain a complete understanding of the involvement of HIFs in innate and adaptive immune system interactions in different stages of hepatocarcinogenesis. As immune checkpoint blockade has emerged as a next-generation therapy for liver cancer, and blockade of the HIF signaling pathway is expected to subvert the immunosuppressive microenvironment and restore T cell infiltration, more translational efforts should be put forth to evaluate the effects of different HIF inhibitors in combination with various immune checkpoint inhibitors for liver cancer treatment.

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