Crosstalk between tumor-associated macrophages and neighboring cells in hepatocellular carcinoma

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Short title: Tumor-associated macrophages in HCC

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Abbreviations:
αKG, alpha-ketoglutarate; CSF-1, colony-stimulating factor-1; DAA, direct-acting antivirals; DCs, dendritic cells; EMT, epithelial-to-mesenchymal transition; GPIba, glycoprotein 1b alpha; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HIF1α, hypoxia-inducible factor 1-alpha; ICIs, immune checkpoint inhibitors; KCs, Kupffer cells; LAG3, lymphocyte activation gene 3; LPS, lipopolysaccharides; MAIT, mucosal-associated invariant T cell; MDSCs, myeloid-derived suppressive cells; MIP-1α, macrophage inflammatory protein-1 alpha; MoMf, monocyte-derived macrophage; MRS, myeloid response score; NASH, non-alcoholic steatohepatitis; ORR, objective response rate; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PI3Kγ, phosphatidylinositol 3-kinase gamma; RT, radiotherapy; SIRPa, signal regulatory protein alpha; SREBP1, sterol regulatory element binding protein-1; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM-3, T cell immunoglobulin and mucin-domain containing-3; TLR, toll-like receptor; TME, tumor microenvironment;
TREM-2, triggering receptor expressed on myeloid cells 2; TKI, tyrosine kinase inhibitor VISTA, V-domain Ig-containing suppressor of T cell activation
ABSTRACT
The tumor microenvironment generally shows substantial immunosuppressive activity in hepatocellular carcinoma (HCC), explaining the suboptimal efficacy of immune-based treatments for this difficult-to-treat cancer. Crosstalk between tumor cells and various cell types in the tumor microenvironment is strongly related to the progression of HCC and treatment resistance. Monocytes are recruited to the tumor microenvironment of HCC by various factors and become tumor-associated macrophages (TAMs) with distinct phenotypes. TAMs often contribute to weakened tumor-specific immune responses and a more aggressive phenotype of malignancy. Recent single-cell RNA sequencing data have demonstrated the central roles of specific TAMs in tumorigenesis and treatment resistance by their interactions with various cell populations in the HCC tumor microenvironment. This review focuses on the roles of TAMs as well as the crosstalk between TAMs and neighboring cell types in the HCC tumor microenvironment.

Keywords: hepatocellular carcinoma, immunotherapy, tumor-associated macrophage, tumor microenvironment
INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for nearly 800,000 deaths worldwide.¹ In 2018, the estimated incidence of HCC in South Korea was 21.2 per 100,000 person-years, representing the fifth most common cancer in men and sixth in women.² HCC is associated with inflammation and stems from chronic hepatic injury.¹ Worldwide, the incidence and mortality of non-alcoholic steatohepatitis (NASH)-related HCC are increasing, reflecting the increasing prevalence of obesity.³ Although early-stage HCC can be treated with surgical resection and locoregional treatments, such as percutaneous ablation, the disease is frequently diagnosed at an advanced stage. HCC is managed by palliative systemic therapy in such cases, which in the past 10 years has relied primarily on the tyrosine kinase inhibitor (TKI) sorafenib.⁴ Recently, several TKIs for the first- and second-line management of advanced HCC, including lenvatinib, cabozantinib, and regorafenib, are approved and now clinically used.¹

Immune-based HCC treatments have various clinical advantages, beyond their high response rates. One benefit is related to quality of life. Patient-reported outcome data have demonstrated that the quality of life is better with atezolizumab plus bevacizumab treatment than with sorafenib.⁵ Recently, nivolumab was approved as a second-line therapy and atezolizumab plus bevacizumab were shown to be superior to sorafenib as a primary treatment for advanced HCC.⁶ However, nivolumab—the first approved anti-programmed death (PD)-1 antibody—has an objective response rate (ORR) of <20% in HCC.⁷,⁸ The Checkmate-040 study of unresectable HCC revealed that programmed death ligand 1 (PD-L1) expression in >1% of tumor cells was associated with a 28% ORR for nivolumab, compared with a 16% ORR with PD-L1 expression in <1% of tumor cells. These findings need to be validated because the expression of PD-L1 in non-parenchymal cells was not considered.⁹,¹⁰

The liver has a unique immunological environment comprising various immune cell types, presenting a considerable challenge for immunotherapy in HCC. The responses to immune checkpoint inhibitors (ICIs) are critically shaped by the tumor microenvironment (TME), especially these are impeded by the activity of immunosuppressive cells, including regulatory T cells, tumor-associated macrophages (TAMs), myeloid-derived suppressive cells (MDSCs), and neutrophils, which associated with an unfavorable prognosis.¹¹ Also, TAMs have central immune-regulatory roles and may limit the efficacy of immune-based therapy via crosstalk with tumor and killer cells.¹² Immune subtyping using data of The Cancer Genome Atlas describes HCC as a C4 subtype associated with an M2 macrophage enrichment.⁷,¹³,¹⁴ A single-cell RNA sequencing analyses identified two important clusters of immune-suppressive cells in the HCC TME.¹⁵ One corresponds to TAMs and the other is lysosome-associated membrane glycoprotein 3-positive dendritic cells (DCs).¹⁵ Another single-
cell analysis of primary and early-relapse HCC samples demonstrated that macrophages are enriched in tumors compared with that in adjacent non-tumors. Analyses of macrophage pro- and anti-inflammatory scores demonstrated that infiltrated macrophages have an immunosuppressive phenotype. Collectively, recent reports with single-cell RNA sequencing data clearly demonstrate that TAMs constitute a principal immunosuppressive population in TME of HCC.

This review focuses on the origin and functional characteristics of TAMs and their crosstalk with other cell types in TME of HCC.

**Immunological heterogeneity of HCC**

HCC is typically characterized by the gradual dysfunction of innate-like and adaptive immune cells and an increase in the number of immune-regulatory cells in the TME. Recently, a trajectory from proliferative to exhausted CD8 T cells is shown in HCC by an RNA velocity analysis. When T cells are exhausted, the expression of several inhibitory receptors, including PD-1, lymphocyte activation gene 3 (LAG3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT), are upregulated and the effector function is impaired by the transcriptional changes mediated by TOX. The exhausted CD8 T cell population is heterogeneous. Two identified subgroups are PD-1**+**TCF1**+** cells capable of self-regenerating and terminally exhausted PD-1**−**TCF1**−** cells. The response to anti-PD-1 is improved when PD-1**+**TCF1**+** cells are dominant but not when the terminally exhausted PD-1**−**TCF1**−** T cells replace the precursors in TME.

Multiplex immunohistochemistry and gene expression assays of 919 regions of 158 HCC tissues revealed that the immune microenvironment in HCC is heterogeneous and can be classified into three different subtypes: immune-high, -mid, and -low. These “immune classes” are related to patient survival. The immune-high class can be further divided into two TME-based subclasses. The “active immune” subclass is supplemented with T cell effectors, such as interferon-gamma (IFN-γ) and granzyme B signatures. The “exhausted immune” subclass includes signatures of exhausted T cells, immunosuppressive TAMs, and transforming growth factor-beta (TGF-β) signaling. A subsequent broader multi-omics analysis characterized “immunocompetent,” “immunosuppressive,” and “immunodeficient” HCC subtypes. The immunocompetent subtype exhibited robust T cell infiltration, whereas immunosuppressive cells (including regulatory T cells and TAMs) and molecules such as PD-L1 were abundant in the immunosuppressive subtype. Figure 1 schematically depicts the three immune subtypes of HCC. Representative immunohistochemistry data using CD3 (T cell marker),
CD68 (macrophage marker), and PD-L1 antibodies for three immune subtypes of HCC have been acquired (unpublished data).

Based on immunohistochemical staining of various immune markers, a myeloid-specific prognostic signature referred to as the myeloid response score (MRS) for HCC was developed. MRS has remarkable discriminatory power for predicting survival in HCC. A higher MRS implies stronger pro-tumoral activity in the TME of HCC, accompanied by increased CD8+ T cell exhaustion. These findings emphasize the critical contributions of myeloid cells to the immune heterogeneity in HCC. A recent study using whole genome and RNA sequencing and cytometry by time-of-flight analysis with multiple tumor sectors demonstrated notable immune intratumoral heterogeneity in HCC. Tumors with pronounced immune intratumoral heterogeneity displayed a more immunosuppressive TME associated with worse clinical outcomes.

Clinically, the maximal tumor diameter is the only significant pre-treatment parameter for the prediction of survival outcomes after nivolumab treatment. The response to nivolumab differs according to the tumor size in patients with HCC, perhaps stemming from the intratumoral heterogeneity of larger tumors. This heterogeneity may be explained by the innate or acquired resistance of tumor cells, or by the frail immune environment and must be overcome to maximize treatment efficacy. Immune-based combination therapies with synergistic effects may be an inevitable approach. Indeed, bevacizumab plus atezolizumab, lenvatinib plus pembrolizumab, and camrelizumab plus apatinib have achieved promising ORRs in clinical trials for advanced HCC.

**Origins and functions of liver macrophages**

There is considerable heterogeneity within intrahepatic macrophage pools. Two subgroups of liver macrophages are identified depending on their origin and activity. Kupffer cells (KCs) are derived from erythromyeloid progenitor cells in the fetal yolk sac. KCs are liver-resident, non-migratory macrophages capable of self-regeneration. They are found in the hepatic sinusoids and differ from circulating monocyte-derived macrophages (MoMfs) stemming from the bone marrow. KCs and MoMfs have overlapping phenotypes and are challenging to differentiate in humans owing to the lack of lineage-specific markers. Multiple MoMfs differentiate and exhibit a phenotype that, under specific conditions, is virtually identical to that of KCs. Two markers are proposed as candidates to distinguish MoMfs from KCs: C-type lectin domain family 4 member F (Clec4F) and T cell immunoglobulin and mucin-domain containing 4 (Tim4). Clec4F and Tim4 are expressed in KCs but are not present in infiltrating MoMfs.
Accounting for approximately 20% of hepatic non-parenchymal cells, KCs are very important in host defense and coordinate the inflammatory response when they detect pathogens. They are involved in phagocytosis, antigen processing and presentation, and the production of proinflammatory mediators. Initially, they produce cytokines conducive to inflammation, including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β, IL-6, IL-12, and IL-18. CCL2 is a representative chemokine produced by activated macrophages, monocytes, and DCs when they are stimulated by proinflammatory cytokines. In the early phase of hepatic inflammation, KCs produce CCL2 to coordinate the mobilization of circulating monocytes to the liver. MoMf mobilization to the liver occurs during inflammation or following the experimental depletion of KCs in a CCL2/CCR2-dependent manner.

KCs can also be activated by interplay with platelets in NASH livers. Hyaluronic acid on the surface of KCs and the glycoprotein 1b alpha (GPIbα) platelet receptor subunit are critical for NASH and subsequent HCC. Resident KCs decrease and are replaced by a specific set of MoMfs over time in NASH. One subset of recruited MoMfs in NASH is termed hepatic lipid-associated macrophages. These cells express osteopontin. When inflammation begins in NASH livers, KCs and recruited MoMfs differentiate into proinflammatory macrophages. A recent study of human NAFLD livers demonstrated that gut-derived lipopolysaccharides (LPS) may increase liver damage by activating macrophages via the Toll-like receptor (TLR)-4 pathway. Triggering receptor expressed on myeloid cells 2 (TREM-2) is predominantly expressed in a subset of intrahepatic macrophages and inhibits TLR signaling. When chronic inflammation is not resolved, pro-fibrogenic TREM2⁺CD9⁺ scar-associated macrophages differentiate from MoMfs and expand in the fibrotic liver.

**Origins and functions of TAMs in HCC**

A recent cross-tissue, single-cell analysis of human macrophages demonstrated that every type of cancer involves the infiltration of conserved TAM populations. The IL4I1⁺PD-L1⁺IDO1⁺ and TREM2⁺ TAM subsets accumulate in various types of human cancers, including HCC. The cells display immunosuppressive phenotypes and promote infiltration of regulatory T cells. Moreover, recent single-cell RNA sequencing data for HCC tissues demonstrated that TAM subgroups are not associated with liver-resident KCs (MARCO⁺) or MDSCs (CD33⁺), suggesting that TAMs mainly originate from circulating monocytes. A recent report based on imaging mass cytometry of 562 highly multiplexed HCC tissue samples demonstrated that the regional immunity is determined by resident KCs with protumor function and infiltrating macrophages with anti-tumor
function. This suggests that specific targeting of KCs, rather than overall myeloid cell blocking, should be a novel immunotherapy for HCC.

Monocytes can be recruited by chemokines. The CCL2–CCR2 signaling axis may be a significant target for monocyte recruitment in TME of HCC. CCR2 antagonist treatment effectively impedes HCC tumor growth in different murine models. This therapeutic approach blocks Ly-6C<sup>high</sup> inflammatory monocyte infiltration, resulting in a reduction in the number of TAMs in the TME of HCC. In addition, the phenotype of residual TAMs reportedly shifts to the M1 phenotype by CCR2 targeting. An anti-tumor effect of a CCL2 neutralizing antibody via a decreased inflammatory myeloid cell population and the enhanced function of tumor-infiltrating CD8<sup>+</sup> T cells and natural killer (NK) cells was demonstrated in a murine HCC model. Several phase I/II clinical trials using CCR2/5 inhibitor in combination with ICIs for various solid tumors are expected to provide additional insight into improving the efficacy of ICIs.

Macrophages are classified into M1 or M2 macrophages according to their phenotypes. Macrophage polarization is influenced by the tumor stage and differs among tumors or among areas within a tumor. HCC tumor progression was once considered to be associated with a skew in the macrophage phenotype from M1 to M2 phenotypes. However, recent single-cell analyses have revealed co-existing M1 and M2 signatures in TAMs, indicating that the TAM phenotype may not be simply defined using the classical M1/M2 model. The TME of HCC is infiltrated by PD-L1-expressing “activated” monocytes with pro-tumoral features. Generally, PD-L1 is expressed at a higher level in macrophages/monocytes with the activated M1 phenotype. Therefore, TAMs in the TME of HCC cannot be defined as a pure M2 phenotype. Rather, they are composed of heterogeneous populations in terms of M1/M2 polarization. The expression of PD-L1 was reported to be higher in macrophages than in cancer cells, providing a potential indicator for the response to immunotherapy in HCC, and PD-L1<sup>+</sup> TAMs from the analyzed HCC samples did not exhibit complete M2 polarization. Additionally, these TAMs exhibited high HLA-DR expression, reflecting the potential immunogenic nature of tumor cells and susceptibility to therapy designed to deplete these PD-L1-expressing TAMs.

**Crosstalk between TAMs and tumor cells in HCC**

TAMs interact with tumor cells in various ways in HCC. Figure 2 schematically describes the routes of crosstalk between TAMs and neighboring cells in TME of HCC. The following subsections describe the current evidence for the crosstalk between TAMs and tumor cells.
Direct crosstalk

Liver cancer stem cells are the cause of the aggressiveness and treatment resistance in HCC. CD24 is preferentially expressed in a subset of liver cancer stem cells. Generally, CD24-expressing tumors can be protected from phagocytosis because CD24 interacts with siglec-10, an inhibitory receptor expressed by TAMs. Therefore, it can be inferred that CD24-expressing liver cancer stem cells can also evade phagocytosis by macrophages.

Signal regulatory protein alpha (SIRPα) is an inhibitory receptor molecule that is expressed on macrophages. SIRPα interacts with CD47 on tumor cells. It is considered a key immune-modulatory checkpoint of macrophages. SIRPα expression in macrophages is correlated with poor survival in HCC. In tumor cells, the CD47-SIRPα interaction acts as a protective signal to evade macrophage surveillance and phagocytic removal. Treatment with anti-CD47 antibodies promotes phagocytosis of tumor cells via activated macrophages. Anti-CD47 treatment combined with doxorubicin further enhances macrophage phagocytosis, suggesting that anti-CD47 antibody treatment could be complementary to transarterial chemoembolization in HCC.

Cytokines/chemokines

CD133+ liver cancer stem cells secrete IL-8. IL-8 stimulates M2 polarization of TAMs to enhance the malignant potential of the tumor. The TAMs are a principal source of paracrine IL-6. IL-6 generated by TAMs stimulates tumor cells, resulting in the intracellular activation of the STAT3 pathway to increase the proliferation of liver cancer stem cells. M2 polarization is reportedly stimulated by the activation of IL-6/STAT3 signaling in macrophages. Therefore, anti-IL-6 treatment may have both direct and indirect anti-tumor effects in HCC. TNF-α derived from TAMs promotes cancer stemness in HCC by activating the Wnt/β-catenin signaling pathway. Within a hypoxic microenvironment, the epithelial-to-mesenchymal transition (EMT) in HCC cells could be induced by IL-1β produced by TAMs via the hypoxia-inducible factor 1-alpha (HIF-1α)/IL-1β/TLR4 pathway. Furthermore, the EMT of tumor cell may also be triggered by IL-8 secreted from TAMs via the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3)/Snail pathway. EMT of tumor cells is also activated by TGF-β1 produced by TAMs.

Tumor cell autophagy can be induced by cytokines produced by TAMs and may also confer resistance to cytotoxic stimuli. Oxaliplatin cytotoxicity in SMMC-7721 and Huh-7 cell lines and HCC xenografts may be
suppressed by tumor cell autophagy activated by TAMs.\(^78\) When HCC is treated with oxaliplatin, M2-TAMs produce more IL-17, promoting autophagy in the cancer cells.\(^79\) Autophagy of macrophages themselves also has a critical impact on HCC development and progression.\(^33\) Autophagy-deficient KCs promote inflammation and HCC development by enhancing the activity of the mitochondrial reactive oxygen species/nuclear factor-kappa B (NF-κB)/IL-1α/β signaling axis.\(^80\) A recent study using mice with a myeloid lineage knockdown of ATG5 demonstrated increased hepatocarcinogenesis and an altered antitumoral immune response compared to wild-type mice.\(^81\) Collectively, these findings suggest that various cytokines produced by TAMs critically influence tumor cells and macrophages.

CCL15 is one of the most abundantly expressed chemokines in HCC cells. It recruits CCR1\(^+\) cells to the HCC TME.\(^82\) The recruited CCR1\(^+\)CD14\(^+\) monocytes express significantly higher levels of immune checkpoint molecules including PD-L1 and TIM-3.\(^82\) The activated CCL15-CCR1 axis indicates an inflammatory microenvironment infiltrated with CCR1\(^+\) monocytes and tumor cells with high metastatic potential and may become a target for HCC therapy.\(^82\)

**CSF-1/CSF1R axis**

Differentiation and functioning of macrophages can only be achieved by stimulation with colony-stimulating factor-1 (CSF-1) and its receptor, CSF-1R. Tumor cells can directly recruit TAMs via the CSF1/CSF1R axis. In HCC tumor cells, SLC7A11, whose expression is positively associated with worse tumor differentiation, upregulates the expression of CSF1 and PD-L1 via the alpha-ketoglutarate (αKG)-HIF1α cascade.\(^83\) SLC7A11 overexpression in tumor cells promotes TAM infiltration in the TME of HCC via the CSF1/CSF1R axis.\(^83\) The CSF1 pathway is activated by miR-148b depletion in HCC cells, which in turn promotes TAM recruitment into the HCC TME.\(^84\) Another microRNA, miRNA-26a, suppresses recruitment of macrophages by downregulating CSF in HCC cells.\(^85\)

The use of a competitive inhibitor of CSF-1R substantially impedes tumor growth in murine xenograft models. TAMs in CSF-1R inhibitor-treated tumors are polarized towards an M1-like phenotype, as determined by gene expression profiling.\(^86\) In mouse models of HCC, two possible consequences of blocking the CSF1/CSF1R signaling pathway were identified: TAM reprogramming from the M2 phenotype to the M1, and the suppression of PD-L1 expression, potentiating the response to PD-1/PD-L1 therapy.\(^86,87\)

**Crosstalk by extracellular vesicles**
Exosomes from TAMs may promote tumor growth and cancer stemness in HCC. A recent study showed that the exosome-mediated transfer of functional CD11b/CD18 protein from TAMs to tumor cells enhances the migratory potential of HCC cells.\textsuperscript{53} miR-125a/b in exosomes inhibited the activity of liver cancer stem cells by targeting CD90, although TAM-derived exosomes had decreased levels of miR-125a/b to increase tumor cell stemness.\textsuperscript{88} Conversely, emerging evidence indicates that tumor cell-derived exosomes or exosomal miRNAs contribute to HCC progression by enhancing macrophage infiltration or activation. Endoplasmic reticulum stress causes tumor cells to secrete exosomal miR-23a-3p.\textsuperscript{89} Exosomal transfer of miR-23a activates the phosphatase and tensin homolog (PTEN)-AKT pathway and increases PD-L1 expression in macrophages.\textsuperscript{89} Exosomes of tumor cells are also enriched with miR-146a-5p, which results in M2-polarization of TAMs and may cause exhaustion of T cells.\textsuperscript{90}

Crosstalk between TAMs and NK cells in HCC

In the TME of HCC, NK cells show a decreased abundance and are dysfunctional.\textsuperscript{59} A major mechanism regulating the function of NK cells is the crosstalk between various immune cell populations, such as TAMs, in the HCC TME.\textsuperscript{91} TAMs indirectly inhibit NK cells via cytokines, such as IL-10 and TGF-\(\beta\).\textsuperscript{91} Additionally, TAMs express CD48 in the TME of HCC and may directly interact with 2B4 on NK cells, resulting in NK cell dysfunction.\textsuperscript{92} Conversely, M1 macrophages may increase the number of total and activated intrahepatic NK cells in chronic liver diseases.\textsuperscript{93} This suggests that when TAMs are re-polarized to the M1 phenotype, they may stimulate NK cells to kill tumor cells.

Crosstalk between TAMs and T cells in HCC

TAMs can regulate the tumor cell killing ability of T and NK cells. This TAM-mediated suppression, rather than tumor cell-mediated suppression, may be the principal mechanism underlying tumor-specific T cell dysfunction in the TME of HCC. A decade ago, two studies independently demonstrated that macrophages are the principal non-parenchymal cells in the TME that express PD-L1 in HCC.\textsuperscript{94,95} These findings were confirmed in several subsequent studies. A recent spatial analysis revealed a positive correlation between the number of infiltrating PD-1\textsuperscript{high}TIM3\textsuperscript{CD8\textsuperscript{+}T cells and the number of PD-L1\textsuperscript{+} TAMs and the lack of correlation with the number of PD-L1\textsuperscript{+} tumor cells.\textsuperscript{96} Likewise, our group obtained similar results showing that PD-L1\textsuperscript{+} TAMs, but not PD-L1\textsuperscript{+} tumor cells, are located near infiltrating CD8\textsuperscript{+} T cell subsets.\textsuperscript{96} A recent study confirmed that PD-L1 on TAMs suppresses tumor-specific T cell responses in HCC.\textsuperscript{97} Using a myeloid-specific \(pd1\textsuperscript{-}\)-knockout mouse model, the
authors demonstrated that PD-L1 on TAMs directly suppresses intratumoral CD8+ T cells. They also found that tumor-derived Sonic hedgehog causes the upregulation of PD-L1 in TAMs.

Arginine metabolism is critically related to TAM polarization. The production of nitric oxide (NO) from arginine in M1 macrophages causes cytolysis of tumor cells, although M2 macrophages have increased enzymatic activities of arginase with both arginine level and NO production reduced. Arginine is a critical amino acid involved in anti-tumor immunity involving the activation of T cells via the upregulation of T cell receptor. Arginase produced by TAMs and MDSCs depletes extracellular arginine and suppresses T cell activation. Supplementation of arginine normalizes T cell metabolism from glycolysis to oxidative phosphorylation and promotes survival and anti-tumor activity, suggesting that arginine deficiency by M2 macrophages is a target for immunotherapy in HCC.

Phosphatidylinositol 3-kinase gamma (PI3Kγ) stimulates the activation of the “immunosuppressive program” in TAMs. Macrophage PI3Kγ suppresses T cell activation. The lack of PI3Kγ activity in TAMs reduces the production of the immunosuppressive molecules, including IL-10 and arginase, and enhances anti-tumor T cell responses. Another study demonstrated the potential value of targeting myeloid-intrinsic PI3Kγ to overcome ICI resistance. These critical roles of PI3Kγ in TAMs need to be confirmed in HCC.

In chronic hepatitis C virus (HCV) infection, rapid viral eradication by direct-acting antivirals (DAAs) changes the phenotype of infected hepatocytes and intrahepatic monocytes/macrophages and contributes to HCC. The inflammatory activity of intrahepatic macrophages, which is closely associated with mucosal-associated invariant T (MAIT) cell activity, is markedly attenuated after rapid viral clearance by DAAs. MAIT cells are activated by IL-12, IL-15, and IL-18, which are usually produced by activated macrophages/monocytes. Usually, DAA therapy causes an immediate reduction in IL-18 levels in chronic HCV infection, resulting in a rapid decrease in intrahepatic inflammation and MAIT cell cytotoxicity. The decreased cytotoxicity of MAIT cells with the reduction in proinflammatory cytokine production by macrophages after DAA treatment may be related to HCC recurrence after DAA-mediated HCV clearance.

**Crosstalk between TAMs and regulatory T cells in HCC**

Regulatory T cells are a major immunosuppressive cell population and have various effects on tumor progression. A recent study in which the CIBERSORT algorithm was used to estimate the relative frequencies of 22 subsets of tumor-infiltrating immune cells in 1090 HCC cases revealed four clusters of tumors. Tumors with increased regulatory T cells and decreased M1 macrophages were associated with worse prognosis.
However, M2 TAMs, not M1 macrophages, tend to promote the recruitment of regulatory T cells to the TME. The infiltration of TREM-1+ TAMs in the HCC TME is critical for resistance to anti-PD-L1 within the hypoxic TME by the recruitment of CCR6+Foxp3+ regulatory T cells via CCL20 production. The recruited or induced regulatory T cells can further enhance the immunosuppressive properties of TAMs. Regulatory T cells promote the sterol regulatory element binding protein-1 (SREBP1)-mediated metabolic processes of M2 TAMs by repressing the production of IFN-γ by CD8+ T cells. This means that regulatory T cells indirectly sustain M2 TAM survival, forming a positive feedback pool.

**Crosstalk between TAMs and T follicular helper cells/plasma cells in HCC**

HCC is a typical example of inflammation-associated cancer. TAMs also cause cancer-related inflammation, resulting in the generation of an inflammatory helper T cell subset, such as T follicular helper cells, in the TME of HCC. TLR4-induced monocyte activation is critical for the generation of IL21+ T follicular helper cells with a CXCR5 PD-1 BTLA CD69high tissue-resident phenotype in TME of HCC. These cells induce plasma cells, resulting in ideal conditions for M2 TAM induction and cancer progression.

Liver IgA+ plasma cells directly suppress cytotoxic T cell activation in vitro and in vivo, inducing their exhaustion. Moreover, recent data have demonstrated that IgA complex-stimulated monocytes/macrophages also show an inflammatory phenotype and express higher PD-L1 levels in the HCC TME. These IgA+ TAMs may be targets for improving the efficacy of immune-based therapy in HCC.

**Crosstalk between TAMs and neutrophils in HCC**

Tumor-associated neutrophils (TANs) might support the progression of tumors by hampering anti-tumor immunity. Monocyte-derived CXCL2 and CXCL8 play critical roles in recruiting neutrophils into the HCC TME. A recent study demonstrated that the glycolytic switch in monocytes mediates the upregulation of CXCL2 and CXCL8 via the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3-NF-kB pathway. Moreover, the proinflammatory cytokine IL-17 contributes to the recruitment of TANs to the TME. Neutrophils also have critical functions in the recruitment of macrophages and regulatory T cells to the HCC TME via the production of cytokines or chemokines. Besides, a positive correlation is observed between the extent of TAN infiltration and angiogenesis at the tumor-invading margin in patients with HCC. The chemokines robustly produced by TANs and peripheral blood neutrophils are CCL2 and CCL17, which
strongly recruit macrophages into the TME of HCC.\textsuperscript{120} Therefore, the infiltration of TANs is a poor prognostic factor because they further recruit TAMs and block tumor-specific immunity in HCC.

**Modulation of TAM population by current HCC therapies**

Although many innovative approaches to modulate TAMs are now being developed, no specific methods targeting TAMs in HCC are currently in clinical use. The following sections focus on the effects of current HCC therapies on TAMs in TME. Figure 3 schematically describes the various HCC treatments and their immune-modulating effects.

**Locoregional treatments/radiotherapy**

There is strong evidence that the combinatorial approach of locoregional therapies with ICIs enhances the immune-modulatory effect of locoregional therapies in HCC.\textsuperscript{122} Transcriptomic analysis and deep immunophenotyping of tissues from patients undergoing Y\textsuperscript{90}-radioembolization demonstrated strong immune activation in the TME and in the peripheral blood of patients with prolonged responses.\textsuperscript{123} Interestingly, a significantly higher proportion of peripheral CD14\textsuperscript{+}HLADR\textsuperscript{+} monocytes was observed 3 months after therapy in patients with sustained responses, suggesting that the number of antigen-presenting cells is higher in these patients.\textsuperscript{123} Interestingly, a recent study demonstrated the local immune-boosting effects (CTL infiltration with enhanced cytotoxicity) of transarterial chemoembolization.\textsuperscript{124} Future studies should reveal the precise effects of locoregional treatments, such as transarterial chemoembolization, transarterial radioembolization, radiofrequency ablation, and hepatic arterial infusion chemotherapy, on TAMs in TME of HCC.

Radiotherapy (RT) has local immune-modulatory effects in HCC. Some studies have reported that irradiation hinders tumor growth and triggers the ongoing mobilization of F4/80\textsuperscript{+}CD68\textsuperscript{+} macrophages to irradiated tumors.\textsuperscript{125} This stimulates TNF-\(\alpha\) and IL-6 production and an inflammatory response.\textsuperscript{125} In murine models, lung metastasis was reportedly prevented and survival improved when irradiation was delivered with intravenously administered recombinant macrophage inflammatory protein-1 alpha (MIP-1\(\alpha\)).\textsuperscript{126} More research is needed to determine the effect of RT on TAMs in TME.

The synergistic effects of RT and ICIs are highlighted by numerous preclinical studies. Many ongoing clinical trials are testing the efficacy of this combined approach.\textsuperscript{127} The abscopal effect, which describes the regression of tumors outside the RT field, may be boosted by the combined use of ICIs.\textsuperscript{128} A recent study using a syngeneic murine model of HCC demonstrated the potential abscopal effects with the increased infiltration of
CTLs in both irradiated and non-irradiated tumors. The RT-ICI combination is also identified as a potential HCC treatment based on an observed correlation between soluble PD-L1 levels after RT and the patient prognosis.

**Multi-TKIs**

For patients with BCLC stage C or with BCLC-B who are not suitable for local or surgical treatment, systemic therapies are recommended as first-line treatments. The recent randomized phase 3 REFLECT trial demonstrated that lenvatinib is non-inferior to sorafenib in the overall survival in treatment-naïve unresectable HCC. Additionally, lenvatinib had better progression-free survival compared to sorafenib as a salvage therapy for transarterial treatment. The result may be due to the immune-modulatory effects of lenvatinib. The immune-regulatory activity of lenvatinib is an important determinant of its anti-tumor effect. This activity is mediated by a reduction in TAMs and increase in intratumoral CD8\(^+\) T cells. Lenvatinib also improves the therapeutic impact of ICIs by localized TAM reduction. It was recently demonstrated that PD-L1-expressing macrophage infiltration is a potential predictor of the response to lenvatinib in unresectable HCC. PD-L1\(^+\) TAMs may represent tumor immunogenicity and may be targeted by ICIs and by lenvatinib.

Considerable resistance to sorafenib is afforded by TAMs with the M2 phenotype via the production of hepatocyte growth factor (HGF) and activation of HGF/c-Met, mitogen-activated protein kinase/extracellular signal regulated kinase 1/2, and PI3K/AKT pathways in tumor cells. This in turn exacerbates the infiltration of M2-TAMs and generates a positive feedback loop. The number of CCL2\(^+\) or CCL17\(^+\) TANs correlates with tumor development, progression, and sorafenib resistance, as mediated by TAMs and regulatory T cells recruitment by TANs in HCC. As previously mentioned, monocyte/macrophage mobilization and TAM M2 polarization depend on the CCL2/CCR2 pathway in HCC. Interestingly, blocking the CCL2/CCR2 pathway with a specific chemical inhibitor can potentiate the effects of sorafenib by activating the anti-tumor activity of CTLs. CXCR4 and its ligand CXCL12 are critical mediators between TAMs and tumor cells in various types of cancers. Increased hypoxia after sorafenib treatment results in increased accumulation of M2 TAMs and regulatory T cells, which is partly mediated by CXCR4. In a murine HCC model, anti-PD-1 immunotherapy was effective only when administered alongside CXCR4 inhibitors when intratumoral hypoxia was caused by sorafenib.

Regorafenib and cabozantinib were recently approved as second-line treatments for unresectable HCC. A study recently demonstrated that regorafenib also enhances anti-tumor immunity by reversing M2 polarization.
of TAMs. This provides a theoretical background supporting the use of the regorafenib and ICI combination treatment for HCC. However, for cabozantinib, the combined administration of anti-PD-L1 did not exert synergistic effects in mouse models. The future discovery of novel therapeutic combinations of TKIs and ICIs is expected to improve patient outcomes by regulating TAM population in HCC TME.

Immune-based therapy

Clinical outcomes in HCC have improved significantly by ICIs. However, the monotherapy has not elicited a response in a large proportion of cases owing to the heterogeneity of the TME in HCC, as mentioned in previous sections. In an analysis of the single-cell landscape of HCC in response to immunotherapy, an increase in tumor heterogeneity was strongly linked to patient survival.

As mentioned earlier, compared to M2 macrophages, M1 macrophages display elevated expression of PD-L1/HLA-DR. CD68+CD11b+ (M1) macrophages create an antigen-presenting niche for the differentiation of stem-like, tumor-specific CD8+ T cells, which is necessary to sustain the CD8+ T cell response to human cancers. The expression of PD-L1 in macrophages may act as a marker for an anti-PD-1/PD-L1 response in HCC. The enrichment of PD-L1+ macrophages in the TME is associated with an activated immunity in TME with a considerable CD8+ T cell infiltration and immune-related gene expression, indicating that the tumor may be responsive to immune-based therapy. TAM activity in HCC is also regulated by the immune checkpoint molecule TIM-3, which is highly expressed on the surface of macrophages and activated by TGF-β in the TME. Other checkpoint ligands are also expressed on TAMs, including PD-L2 and B7-H4, galectin-9, and V-domain Ig-containing suppressor of T cell activation (VISTA). Collectively, TAMs express many checkpoint molecules in the HCC TME and may be targeted by immune-based therapies.

There are several putative ways to directly deplete TAMs in TME of HCC. In mouse models of HCC, the TAM proportion was diminished by the injection of nanoliposome-loaded C6-ceremide, stimulating CD8+ T cells to exert an anti-tumor immune response. In a murine Hepa1-6 syngeneic tumor model, the number of M2-like TAMs decreased to some extent by the administration of liposome-encapsulated clodronate, leading to a decrease in tumor size, with no impact on the number of M1-like TAMs. Zoledronate is a third-generation nitrogen-containing bisphosphonate with selective cytotoxicity towards matrix metalloproteinase-9-expressing TAMs and with ability to impair the differentiation of monocytes into TAMs. Zoledronate treatment enhanced the effects of transarterial chemoembolization by inhibiting TAM infiltration in rat
models of HCC. However, there is a concern that the general depletion of macrophages in liver causes a loss of tissue-resident macrophages mediating the immune homeostasis and bacterial clearance.

**Conclusions and future directions**

Various clinical trials and real-world data for HCC indicate that immune-based therapy achieves encouraging clinical responses with manageable toxicity profiles. To maximize the therapeutic efficacy of immune-based therapies, TAMs in HCC TME are principal targets because these cells have various routes of crosstalk between tumor and other immune cells, resulting in more aggressive tumors unresponsive to the treatments. Future HCC immunotherapy strategies should identify new combination approaches using current or potential treatment options targeting TAMs in HCC TME for optimal anti-tumor efficacy.

**Acknowledgements**

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2021R1C1C1005844). This work was also partly supported by a Young Medical Scientist Research Grant through the Daewoong Foundation (DY20204P to PSS).

**Conflict of interest**

The author has no conflict of interest to declare.
References


TREM-2 defends the liver against hepatocellular carcinoma through multifactorial protective mechanisms. Gut 2021;70:1345-1361.


Sung PS, Cho SW, Lee J, Yang H, Jang JW, Bae SH, et al. Infiltration of T Cells and


82. Liu LZ, Zhang Z, Zheng BH, Shi Y, Duan M, Ma LJ, et al. CCL15 Recruits Suppressive Monocytes to Facilitate Immune Escape and Disease Progression in Hepatocellular


95. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in


Figure 1. Three immune subtypes of HCC. The three immune subtypes of HCC described in previous studies are schematically depicted. Representative immunohistochemistry using CD3 (T cell marker), CD68 (macrophage marker), and PD-L1 antibodies for three immune subtypes of HCC are also presented (unpublished data). (A) This subtype exhibits robust infiltration of T cells (CTLs and Th1 cells) and M1-dominant TAMs infiltration. High expression of PD-L1 in TAMs with or without in tumor cells reflects the immunogenic nature of the tumor. This phenotype may respond well to immune checkpoint inhibitor therapies. (B) In this subtype, immunosuppressive cells including TAMs are abundantly infiltrated. However, there are few infiltrating T cells. TAMs may show PD-L1 expression, although the level of PD-L1 is not as high as that in immunocompetent phenotype. This phenotype may not respond well to immune checkpoint inhibitor therapies and immune-based combination treatments for this subtype may be required. (C) This subtype may be referred to as “immunodeficient” subtype. Infiltration of T cells and TAMs is poor, which may stem from the poor immunogenicity of the tumor. This subtype may not respond to the immune-based therapy unless the antigen release by the locoregional or systemic therapy results in the local inflammation sufficient to cause the infiltration of immune cells.
TAMs interact with tumor cells in various ways in HCC. This figure schematically describes the crosstalk between TAMs and neighboring cells in TME of HCC. One route is crosstalk between TAMs and tumor cells. Various cytokines, chemokines, and extracellular vesicles (EVs) are produced by tumor cells. Macrophages are recruited and differentiate to TAMs. TAMs also produce various cytokines that confer malignant potential to tumor cells. Direct crosstalk between TAMs and HCC tumor cells is mediated by CD47/SIRPα or CD24/siglec-10 pathways. TAMs have critical crosstalk with CTLs, resulting in the inhibition of anti-tumor activity with the PD-1/PD-L1 or TIM-3/galectin-9 pathway involved. Moreover, PI3Kγ mediates immunosuppressive activity of TAMs by enhancing arginase-1 activity and IL-10 secretion of TAMs. L-arginine depletion by enhanced arginase-1 activity of TAMs also causes the exhaustion of antigen-specific T cells in TME. Regulatory T cells (Tregs) are also recruited to TME of HCC by CCL20. CCL20 is produced by TAMs and binds to CCR6 in Tregs. Neutrophils are recruited to TME by chemokines produced by TAMs. They also recruit TAMs by secreting CCL2 and CCL17. Collectively, TAMs usually have central immunosuppressive roles in TME of HCC, although the phenotype of these macrophages varies significantly because of the heterogeneity of HCC.
Figure 3. Modulation of TAM population in HCC TME by the current standard therapies. This figure schematically describes the various HCC treatments and their effects on TAMs. Transarterial therapy such as transarterial chemoembolization or transarterial radioembolization and radiation therapy may activate TAMs in HCC TME to exhibit M1-like phenotype and to enhance the antigen presentation of these cells. In response to TAM activation, the number and cytolytic function of infiltrated CTLs may be increased by these therapeutic modalities. One of the multi-tyrosine kinase inhibitors for HCC, lenvatinib, is known to have a robust immune-modulating effects for HCC. Lenvatinib decreases the number of infiltrated TAMs and activates CTLs in TME. Sorafenib, another multi-tyrosine kinase inhibitor that has been used for more than a decade, has been known to have little immune-boosting effects in HCC TME. Intratumoral hypoxia caused by sorafenib treatment may increase the number of TAMs in HCC TME, resulting in the decreased activity of CTLs. In immunogenic HCC, PD-L1 is preferentially expressed in TAMs, rather than in tumor cells. ICI treatment in HCC activates these PD-L1+ TAMs, resulting in the enhanced activity of CTLs.