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NEW HORIZONS

# Myeloid Cells in Hepatocellular Carcinoma

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**Hepatocellular carcinoma (HCC) is highly associated with inflammation. Myeloid cells, including tumor-associated macrophages and myeloid-derived suppressor cells, are abundant in the HCC microenvironment and are often associated with poor prognosis. Myeloid cells in HCC play a vital role in supporting tumor initiation, progression, angiogenesis, metastasis, and therapeutic resistance. Here, we summarize our current knowledge about myeloid cells in HCC and focus on their immune-suppressive activities and tumor-promoting functions, as well as the relevance to potential new therapies in HCC. (HEPATOLOGY 2015;62:1304-1312)**

Inflammation contributes to all stages of hepatocellular carcinoma (HCC), including tumor initiation, progression, and dissemination, with malignant and inflammatory cells coevolving in their microenvironment.[1] Myeloid cells are abundant in the HCC tumor microenvironment and have been linked to uncontrolled malignant growth. Myeloid cells are generated from myeloid progenitors and immature myeloid cells, which terminally differentiate into mature granulocytes, monocytes/macrophages, and dendritic cells (DCs). These cells function to phagocytize dying cells, eliminate foreign substances, repair tissue, and stimulate lymphocytes to respond to pathogens. In cancer patients, tumors globally alter the differentiation and function of myeloid cells, shifting them into immunosuppressive and tumor-promoting cells, such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). TAMs and MDSCs have been well recognized for their immunosuppressive function in HCC. In addition, other functions of myeloid cells have also been documented, including the promotion of tumor incidence, invasion, metastasis, and angiogenesis. A better understanding of these cells in HCC will be critical for developing effective HCC therapy. TAMs have been noted in HCC and other liver cancers, such as cholangiocarcinoma. However, given the higher prevalence of HCC, more mechanistic data are available for myeloid cells in HCC. Therefore, for purposes of this review, we will focus on the influences of TAMs and MDSCs on HCC through their immunosuppressive and tumor-promoting functions.

## Definition of TAMs and MDSCs

### *TAMs*

Macrophages arise from bone marrow-derived circulating monocytes, which then reside in tissues. Macrophages are incredibly plastic in response to various environmental stimuli and can be in a spectrum of functional states. The two polarization states at the extreme end are the classical activation state (M1) and the alternative activation state (M2) (Fig. 1). Classically activated M1 macrophages, in response to lipopolysaccharides (LPS) and interferon-gamma (IFN-$\gamma$), produce proinflammatory cytokines, such as interleukin (IL)-12, and stimulate effector T-cell proliferation and function. When monocytes are alternatively activated, M2, by IL-4, IL-10, and IL-13 *in vitro*, they produce low IL-12, high IL-10, transforming growth factor beta (TGF-$\beta$), and chemokine (C-C motif) ligand (CCL) family members, such as CCL17, CCL18, CCL22, and CCL24. The tumor microenvironment skews macrophage differentiation and functions toward immunosuppressive and tumor-promoting cells.[3] Given that

*Abbreviations: APCs, antigen-presenting cells; CCL, chemokine (C-C motif) ligand; C/EBPβ, CCAAT/enhancer-binding protein; CSC, cancer stem cell; CSF-1, colony-stimulating factor 1; CTLs, cytotoxic T lymphocytes; CXCL, C-X-C motif chemokine; DCs, dendritic cells; DEN, diethylnitrosamine; ECs, endothelial cells; EMT, epithelial-mesenchymal transition; FDA, U.S. Food and Drug Administration; FoxQ1, forkhead box Q1; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; IDO, indoleamine-pyrrole 2,3-dioxygenase; IFN-γ, interferon-gamma; IL, interleukin; JAK, Janus kinase; LPS, lipopolysaccharides; MDSCs, myeloid-derived suppressor cells; MMPs, matrix metalloproteinases; NF-κB, nuclear factor kappa B; NK, natural killer; PD-1, programmed death 1; PDGF, platelet-derived growth factor; SDF-1α, stromal cell-derived factor 1 alpha; STAT, signal transduction and activator of transcription; TAMs, tumor-associated macrophages; TCR, T-cell receptor; TGF-β, transforming growth factor beta; Th, T helper; TIE2, tyrosine kinase with Ig and EGF homology domains 2; TIM_3, T-cell immunoglobulin domain and mucin domain 3; TNF-α, tumor necrosis factor alpha; TNM, tumor node metastasis; Treg, T regulatory; TREM-1, triggering receptor expressed on myeloid cells 1; VEGF, vascular endothelial growth factor; ZA, zoledronic acid.*

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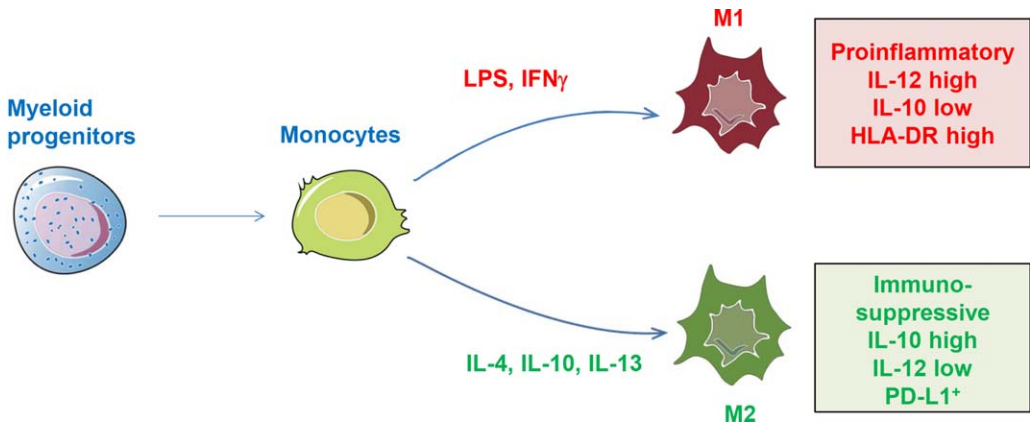


Fig. 1. Classically activated M1 and alternatively activated M2 macrophages. Macrophages originate from myeloid progenitors and circulating monocytes. Macrophages are incredibly plastic in response to various environmental stimuli and can be in a spectrum of functional states. The two polarization states at the extreme end are the classical activation state (M1) and the alternative activation state (M2). LPS and IFN-$\gamma$ generate classically activated M1 macrophages, which has proinflammatory activities, express high HLA-DR and IL-12, and stimulate effector T-cell function. IL-4, IL-10, and IL-13 generate alternatively activated M2 macrophages, which produce low IL-12, high IL-10 and PD-L1 (B7-H1), and have immunosuppressive functions.

TAM phenotypes may differ among cancer types, individual patients, and even location within the tumor, simply defining TAMs based on the classical versus alternatively activated phenotype has its limitations. TAMs comprise heterogeneous populations with diverse and mixed phenotypes.[4] In mice, TAMs are identified in tumors as F4/80$^+$ and CD11b$^+$. In humans, TAMs are identified as CD68$^+$ by immunohistochemistry and CD14$^+$ by flow cytometry. Additional markers used to define HCC TAMs include human leukocyte antigen (HLA)-DR$^+$, CD163$^+$, CD206$^+$, and high arginase activity. Frequency of infiltrating TAMs is correlated with poor prognosis in HCC.[5] Thus, a better understanding of TAMs will be important for developing future therapy for treating patients with HCC.

### *MDSCs*

In cancer, differentiation of myeloid cells is often altered, generating a population of immature myeloid cells with potent immunosuppressive activities and impaired function as antigen-presenting cells (APCs).[6] These cells are now known as MDSCs, which are a heterogeneous population of immature myeloid cells. There are two main types of MDSCs: monocytic MDSCs and granulocytic MDSCs (also called polymorphonuclear MDSCs). In mice, MDSCs are characterized by CD11b$^+$ and Gr-1$^+$, weakly or do not express other markers of mature myeloid cells (MHCII$^{-/low}$, F4/80$^{int}$, and CD11c$^{low}$). Monocytic MDSCs are further defined as Ly6G$^-$Ly6C$^{high}$, and granulocytic MDSCs are Ly6G$^+$Ly6C$^{low}$. More important, they are functionally defined as suppressors for T-cell activation.6 In human HCC, phenotype and markers used to identify MDSCs varies and includes: Lin$^-$CD14$^+$HLA-DR$^{low/-}$ (monocytic MDSCs),[7] Lin$^-$CD33$^+$CD11b$^+$HLA-DR$^-$ MDSCs,[8] and Lin$^-$CD14$^-$CD33$^+$CD11b$^+$HLA-DR$^-$ (granulocytic MDSCs).[9] Similar to TAMs, MDSCs are also plastic in response to microenvironmental signals. MDSCs can differentiate into macrophages, granulocytes, and DCs *in vitro*.[10] In the presence of tumor-derived factors or hypoxia, MDSCs can differentiate toward immunosuppressive TAMs.[11]

In summary, MDSCs and TAMs are notably diverse and plastic cells that are able to shift their functional state in response to numerous cytokines and growth factors in the tumor microenvironment. MDSCs and TAMs show considerable overlapping phenotype and functions, including inhibition of effector T cells, production of immunosuppressive cytokines, and promotion of angiogenesis. Yet, studies in HCC also demonstrate a variety of distinct mechanisms for their immunosuppressive and tumor-promoting functions.

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### *Myeloid Cells and HCC Development*

The link between TAMs and HCC development has been examined in various mouse models (Table 1). In the *Md2*-knockout mouse model, mice spontaneously develop cholestatic hepatitis followed by HCC. Tumor necrosis factor alpha (TNF-$\alpha$) produced by TAMs and endothelial cells (ECs) activates nuclear factor kappa B (NF-$\kappa$B), which protects hepatocytes from apoptosis and promotes tumor growth.[12] In the chemical carcinogen diethylnitrosamine (DEN)-driven HCC model, TAM-derived TNF-$\alpha$ and IL-6 accelerate hepatocyte proliferation, leading to hepatocarcinogenesis.[13] Similar to human HCC, DEN-driven HCC in mice show gender disparity, with 100% HCC incidence in male mice and only 10%-30% HCC incidence in female littermates. This gender disparity is largely the result of higher MyD88-dependent IL-6 production by resident hepatic macrophages in male mice than in female mice. IL-6 promotes compensatory hepatocyte proliferation in response to DEN-induced tissue damage, which plays a critical role in DEN-induced HCC. Estrogen, at the concentrations present in females, inhibited IL-6 production by macrophages by decreasing activation of transcription factors NF-$\kappa$B and CCAAT/enhancer-binding protein (C/EBP)$\beta$, resulting in less incidence of HCC initiation in females.[14] Macrophages are activated, in part, by necrotic hepatocyte-derived high-mobility group box, which binds to the triggering receptor expressed on myeloid cells 1 (TREM-1). TREM-$1^{-/-}$ mice showed reduced proinflammatory cytokines IL-1$\beta$, IL-6, TNF, CCL2, and C-X-C motif chemokine (CXCL)10, as well as decreased DEN-induced liver damage, compensatory hepatocyte proliferation, and liver tumorigenesis.[15] All together, these studies support that TAM-derived cytokines play a pivotal role in HCC development and progression.

MDSCs also accumulate in mouse HCCs, but vary in different HCC models.[16] Orthotopic and subcutaneous tumors derived from mouse HCC cell lines rapidly induced MDSC expansion in liver, spleen, and blood. In contrast, in the slow-growing DEN-driven HCC or MYC-expressing spontaneous HCCs, MDSCs only increased in advanced HCC.[16] How MDSCs are involved in HCC development awaits further investigation.

## Myeloid Cells and HCC Angiogenesis

Angiogenesis plays a critical role in HCC progression. HCC tumors show extensive vasculature that provides nutrients for tumor growth. Sorafenib, a multikinase inhibitor, is the only U.S. Food and Drug Administration (FDA)-approved drug for advanced HCC. Indeed, sorafenib inhibits vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) signaling important for angiogenesis, along with the mitogen-activated protein kinase pathway for cell proliferation. Thus, drugs targeting angiogenesis show potential for treating HCC. Clinical evidence has shown a positive correlation between the frequency of TAMs and the density of microvessels, suggesting a role for TAMs in angiogenesis. TAMs secrete growth factors, including TGF-$\beta$, VEGF, fibroblast growth factor, PDGF, angiogenic factor thymidine phosphorylate, and angiogenesis-modulating enzymes cyclooxygenase-2 and matrix metalloproteinases (MMPs), including MMP-2, MMP-7, MMP-9, and MMP-12, which promote migration of ECs and angiogenesis.[17] Similar to TAMs, MDSCs can directly promote tumor angiogenesis through producing high levels of MMP-9.[18] In an HCC xenograft model, sorafenib treatment inhibits tumor growth and lung metastasis, but also increases intratumoral TAM infiltration. TAM infiltration is accompanied by increased TAM chemoattractant colony-stimulating factor 1 (CSF-1) and angiogenic factors stromal cell-derived factor 1 alpha (SDF-1$\alpha$) and VEGF. TAM depletion by zoledronic acid (ZA) or clodronate-encapsulated liposomes (clodrolip), in combination with sorafenib, further reduced tumor growth, lung metastasis, and tumor angiogenesis.19 Hence, therapies disrupting TAMs is worthy of future studies to enhance the antitumor efficacy of sorafenib.

TAMs are preferentially attracted to hypoxic areas in tumor. Hypoxia-induced factor 1 alpha in TAMs is essential for TAM infiltration and activation *in vivo*.[20] In addition, hypoxia stimulates TAM chemokine production, including CCL2, CCL5, IL-8 (CXCL8), CXCL10, CXCL12, and CXCL13, that are involved in angiogenesis and tumor progression. For example, IL-8 promotes angiogenesis and is an independent predictor of mortality in early-stage HCC patients.[21] Various TAM subsets have been described based on surface marker expression. One of the subsets is tyrosine kinase with Ig and EGF homology domains 2 (TIE2)-expressing macrophages, which exerts proangiogenic activity. TIE2 is a receptor of angiopoietins. TIE2-expressing TAMs selectively migrate toward angiopoietin 2 released by ECs, especially in hypoxic tumor areas.[22] In HCC, frequency of TIE2-expressing TAMs is positively correlated with microvessel density and may serve as diagnostic marker for HCC.[23] This suggests that TAMs are involved in angiogenesis through a network of angiogenic signaling in the HCC microenvironment.

## Myeloid Cells and HCC Metastasis

TAMs can facilitate tumor invasion and metastasis. Whereas TAMs are noted in almost all HCC patients,

**Table 1. TAMs and MDSCs in HCC: Phenotypes, Functions, and Clinical and Pathological Associations**

| Murine HCC Models | Presence/Generation | Identification/Marker | Effects Associated With the Presence of TAMs or MDSCs | Refs |
|---|---|---|---|---|
| Md2-KO mouse, inflammation-induced HCC | TAMs detected in tumors | Morphology | TAM-derived TNF-$\alpha$ activates hepatocyte NF-$\kappa$B and promotes HCC. | 12 |
| DEN-driven HCC model | TAMs detected in tumors | Isolation by centrifugation | TAM-derived TNF-$\alpha$ and IL-6 activate NF-$\kappa$B and C/EBP$\beta$ and promote HCC. | 13,14 |
| DEN-driven HCC model | TREM-1-expressing TAMs detected in liver | F4/80$^+$ CD11b$^+$ Ly6G$^-$ Ly6C$^-$ | TAM activation and TAM-derived proinflammatory cytokines IL-6, IL-1b, TNF, CCL2, and CXCL10 promote DEN-driven HCC; the myeloid cell surface receptor, TREM-1, expressed by TAMs is crucial in the development of HCC. | 15 |
| Nude mice bearing orthotopic HCC tumors | TAMs increased in tumors after sorafenib treatment | F4/80$^+$ and CD11b$^+$ by IHC and FACS | Depletion of TAMs by clodrolip or ZA in combination with sorafenib significantly inhibited tumor progression, tumor angiogenesis, and lung metastasis, compared with sorafenib alone. | 19 |
| Nude mice bearing orthotopic HCC tumors (SMMC7721, HCCLM3) | TAMs increased in tumors with high metastatic properties | F4/80$^+$ by IHC | Depletion of TAMs using clodrolip dramatically decreased FoxQ1-enhanced HCC metastasis. | 32 |
| *Abcb4* knockout mice mimicking cholangitis-associated HCC | TAMs observed at the invasive front of HCC | F4/80+ by IF | TAMs were the major source of MMP-9 at the invasive front of HCC and could be involved in the matrix remodeling and HCC invasion. | 29 |
| Orthotopic and ectopic mouse models with mouse HCC cell lines | TAMs detected in tumors | CD68$^+$ CD206$^+$ by IHC and FACS | TAMs link with HCC gender disparity. Estrogen could suppress HCC progression through inhibiting TAMs function, including reducing arginase activity, mannose receptor CD206 expression, and IL-10 production. This is dependent on the JAK1-STAT6-signaling pathway. | 63 |
| Hepa1-6 mouse HCC cell line | TAMs generated *in vitro* by culturing RAW 264.7 with IL-4 for 24 h | Macrophage lines | Conditioned media from RAW 264.7 treated with IL-4, but not LPS, plus IFN-$\gamma$ increased CSC-like properties and EMT of Hepa1-6 cells through TGF-$\beta$1. | 34 |
| Subcutaneous and orthotopic mouse models with HCC cell lines; DEN-driven HCC model; MYC-expressing spontaneous HCC model | MDSCs observed, but differs depending on the mouse models | CD11b$^+$ Gr-1$^+$ | In subcutaneous and orthotopic tumors, MDSCs increased systemically. In DEN-driven and MYC-expression tumors, MDSCs only accumulate in the livers of mice with advanced HCC. KC and GM-CSF controlled MDSC frequency. | 16 |
| **HCC Patients** | **Presence/Generation** | **Identification/Marker** | **Effects Associated With the Presence of TAMs or MDSCs** | **Refs** |
| HCC patients TMA | High density of TAMs in peritumoral liver tissue | CD68$^+$ by IHC | Peritumoral TAMs correlates with large tumor size, intrahepatic metastasis, high TNM stage, and poor survival. | 24 |
| Paraffin-embedded tissue from HCC patients | TAMs detected in tumors | CD68$^+$ by IHC | High density of TAMs was related to increased intratumoral Treg; TAMs-increased Treg was partially blocked by anti-IL-10 antibody. | 45 |
| Tumors from HCC patients | TAMs detected in tumors | CD14$^+$ by FACS and CD68$^+$ by IHC | TAM galectin-9 binds to T cell TIM-3, which induced senescence of effector T cells. | 40 |
| Tumors and peripheral blood samples from HCC patients | TAMs detected in tumors | CD14$^+$ by FACS and CD68$^+$ by IHC | TAMs-derived IDO impaired T-cell proliferation and effector cytokine production. | 47 |
| Tumors from HCC patients, peripheral blood samples from healthy donors | TAMs detected in tumors | CD14$^+$ HLA-DR$^+$ by FACS and CD68$^+$ by IHC | TAM B7-H1 binds to T cell PD-1, which suppresses effector T-cell function. | 5,49 |
| Tumors and xenograft tumors from HCC patients, HepG2 human HCC cell line | TAMs detected in tumors | CD14$^+$ by FACS | TAMs enhanced human HCC CSCs phenotype through TAMs-derived IL-6 and its downstream activation of STAT3 signaling in HCC. | 36 |
| HCC patients peripheral blood and tumor | TEMs increased in HCC patients | CD14$^+$CD16$^+$TIE2$^+$ by FACS and IF | TIE2-expressing monocytes/macrophages correlate with microvessel density and could serve as a diagnostic marker. | 23 |
| HepG2 human HCC cell line | Activated macrophage lines (RAW 264.7, THP-1, mouse peritoneal macrophages) | Macrophage lines | Conditioned media from macrophages from various sources activated by PMA or LPS, but not IFN-$\gamma$, increased migration and invasiveness of HepG2 cells by destabilizing the adherens junction *in vitro*. | 33 |
| Tumors and peripheral blood samples from HCC patients | MDSCs increased in peripheral blood and tumors of HCC patients | CD14$^+$ HLA-DR$^{-/low}$ Arginase$^{high}$ | MDSCs induced regulatory T cells and inhibited tumor-specific T-cell activation. | 7 |
| Peripheral blood samples from HCC patients | MDSCs increased in HCC patients | CD14$^+$ HLA-DR$^{-/low}$ | MDSCs inhibited autologous NK cell cytotoxicity and cytokine secretion *in vitro*. This is dependent on cell contact and NKp30 on NK cells, but not on arginase activity of MDSCs. | 56 |
| Peripheral blood samples from HCC patients | MDSC increased in HCC patients | CD14$^-$ HLA-DR$^-$ CD33$^+$ CD11b$^+$ | Augmented Tregs, MDSC, PD-1+-exhausted T cells, and immunosuppressive cytokines (IL-10, TGF-$\beta$1) in patients with HCC. Depletion of Tregs, MDSC, PD-1$^+$ T cells restored effector T-cell function *in vitro*. | 9 |

Abbreviations: IHC, immunohistochemistry; FACS, fluorescence-activated cell sorting; IF, immunofluorescence; KC, keratinocytes; GM-CSF, granulocyte macrophage colony-stimulating factor; PMA, phorbol-12-myristate-13-acetate.

higher TAM densities correlate with other known HCC prognostic factors, such as tumor size, vascular invasion, number of tumor nodules, and tumor node metastasis (TNM) stage. Thus, both overall survival and disease-free survival are negatively correlated with degree of TAM infiltration.[24] High expression of peritumoral CSF-1, an important macrophage growth factor and chemoattractant, correlates with poor prognosis in HCC patients.[24] Livers bearing metastatic HCC also show higher CSF-1 gene expression, compared to livers bearing nonmetastatic HCC.[25] Tumor-derived CSF-1 stimulates TAMs to produce epidermal growth factor, which attracts tumor cells and augment metastasis. TAMs are often found at the invasive front of advanced tumors. TAMs can promote tumor invasion through their matrix remodeling capability. TAMs secrete proteases MMPs, serine proteases, and cysteine cathepsins, which cleave components of the extracellular matrix and basement membrane and disrupt cell adhesion junctions, facilitating tumor cell invasion and metastasis.[26,27] MMP-9 overexpression is associated with higher invasive potential of HCC.[28] TAMs are identified as the main MMP-9-expressing cells at the invasive tumor front in a mouse HCC model.[29] Active tumor- and stroma-derived MMP-9 can enzymatically cleave basement membrane proteins, increased at the HCC invasive front, and is significantly associated with cancer invasion to the HCC capsule and portal veins.[30] Whereas TAM recruitment to the peritumoral stroma has prognostic significance in HCC, the underlying mechanisms of the roles of TAMs and MDSCs on HCC invasiveness and metastasis require further investigation.

## Myeloid Cells and HCC Epithelial-Mesenchymal Transition and Stemness

Recent findings suggest that TAMs can enhance cancer cell migration and invasion through direct influence on cancer cell phenotypic plasticity. $CD163^+$ TAMs enhanced migration capacities of HCC tumor cells and lung metastasis; one of the underlying mechanisms could be that TAM-derived CCL22 promotes epithelial-mesenchymal transition (EMT) and increases invasive properties of tumor cells.[31] In HCC, overexpression of forkhead box Q1 (FoxQ1) induced EMT and secretion CCL2, which increased TAM infiltration. Depletion of TAMs decreased FoxQ1-enhanced HCC metastasis,[32] demonstrating the positive feedback loop between HCC cells and TAMs for tumor metastasis. Activated macrophage conditioned medium down-regulated E-cadherin and shifted liver cancer cells (HepG2 cells) from epithelial morphology toward a mesenchymal phenotype.[33] Treatment with conditioned medium from murine TAMs also induced EMT of mouse hepatoma cells (Hepa1-6), showing higher mesenchymal markers and invasion, as well as cancer stem cell (CSC)-like properties and tumorigenicity. TAM-derived TGF-$\beta$ was important for these TAM-induced EMT and CSC-like properties.[34] HCC MDSCs may utilize similar mechanisms to induce EMT.

CSCs, owing to their self-renewal and tumor-initiating capacity, are involved in tumor survival, chemoresistance, recurrence, and metastasis. Our group has observed a mechanistic link between TAMs and HCC CSCs.[35] We found that TAMs enhanced the human HCC CSCs phenotype, including higher stemness-related gene expression and sphere-forming capacity, resulting in larger tumor volume *in vivo*. This CSC-promoting effect was mediated by TAMs through the IL-6- and signal transduction and activator of transcription (STAT)3-signaling pathways. This effect was disrupted by tocilizumab, a humanized anti-IL6R antibody. MDSCs have also been shown to enhance cancer cell stemness and metastasis in ovarian carcinoma by microRNA101 and corepressor gene C-terminal-binding protein 2.[36] Hence, targeting TAMs and MDSCs could interrupt CSC-mediated tumor initiation and progression.

## Myeloid Cells and HCC Immune Suppression

### *TAMs and Immune Suppression*

The degree of immune suppression in the tumor microenvironment is associated with poor prognosis in HCC patients. In HCC patients, the density of antitumor inflammatory cells, including cytotoxic $CD8^+$ T cells and neutrophils, within the tumor is correlated positively with apoptotic tumor cells and patient survival.[37] However, the majority of HCC patients lack the infiltration of these antitumor inflammatory cells owing to the immune-suppressive environment in HCC.[38] In contrast, TAMs are abundant in the HCC microenvironment, and the frequencies of TAMs in the tumor are comparable to that in the nontumor or peritumor areas.[37] TAMs actively disrupt antitumor immunity.[5,39,40] TAMs suppress T-cell effector function through multiple mechanisms. Classically activated macrophages produce IL-12, which promotes T-helper (Th)1-cell activation. However, TAMs produce limited IL-12 and instead produce IL-10, which promotes Th2 cell development and reduces cytotoxic T lymphocytes (CTLs).[41] Moreover, TAMs produce chemokines CCL17, CCL18, and CCL22, which preferentially

attract T regulatory (Treg) and Th2 cells to the tumor and, in turn, impair CTL activation.[42,43] However, this mechanism has yet to be demonstrated in HCC. Increased tumor-infiltrating Treg correlates with poor prognosis in HCC patients, and the intratumoral prevalence of Treg is associated with high density of TAMs.[44] Additionally, Treg production of IL-10, IL-4, and IL-13 can promote differentiation of monocytes into immunosuppressive TAMs.[45] Therefore, a positive feedback loop may exist between TAMs and Treg cells that further enhance their immunosuppressive effects in HCC.

In hepatitis B virus (HBV)-associated HCC, TAMs expressed a high level of galectin-9. Binding of galectin-9 to T-cell immunoglobulin domain and mucin domain 3 (TIM-3) on T cells induces senescence of T cells. Blockade of TIM-3/galectin-9 signaling restored functionality of tumor-infiltrating effector T cells.[39] Hence, TAM galectin-9 and T-cell TIM-3 could be immunotherapeutic targets in patients with HBV-associated HCC. Indoleamine-pyrrole 2,3-dioxygenase (IDO), an immunomodulatory enzyme that suppresses T-cell responses, is also highly expressed in HCC TAMs. HCC-infiltrating $CD69^+$ T cells induce IDO expression in TAMs through IFN-$\gamma$ and TNF-$\alpha$. TAMs-derived IDO, in turn, impairs T-cell proliferation and effector cytokine production, contributing to immunosuppression in HCC.[46] TAMs suppress T cells through expression of the coinhibitory molecule, B7-H1, to the ligand for programmed death 1 (PD-1) on T cells, reducing T-cell effector functions against tumor cells. B7-H1 levels in liver macrophages are higher in tumor-bearing mice than in normal mice, which, in turn, suppress T-cell activation.[47] Tumor-associated IL-10 and TNF-$\alpha$ contribute to induction of B7-H1 on HCC TAMs. Importantly, blocking B7-H1 on TAMs or PD-1 on T cells using neutralizing antibodies recovered effector T-cell function and antitumor activity.[5,48] Therefore, targeting the immunosuppressive function of TAMs provides important therapeutic implications for treatment of HCC. Indeed, anti-PD-1-targeted agents are approved by the FDA to treat patients with melanoma and are currently in phase I clinical trial in HCC patients.

### *MDSCs and Immune Suppression*

MDSCs inhibit T-cell responses through diverse mechanisms. First, MDSCs deplete nutrients, L-arginine, and L-cysteine, necessary for T-cell function. MDSCs show increased activity of arginase, which depletes arginine and down-regulates CD3$\zeta$ chain on T cells, resulting in suppression of T-cell proliferation and cytokine production.[49] Cysteine is essential for T-cell activation. T cells lack the cystathionase that generates cysteine and therefore solely depend on exogenous cysteine. APCs import cystine and convert it to cysteine that is then exported outside of cells. MDSCs compete with APCs for cystine and limit the availability of cysteine in their extracellular environment by not exporting cysteine. The reduced extracellular cysteine caused by MDSCs results in impaired T-cell proliferation and activation.[50] MDSCs also generate oxidative stress through production of reactive oxygen species and reactive nitrogen species. These reactive species inhibit T-cell responses through reducing T-cell receptor (TCR)$\zeta$-chain expression, impairing IL-2 receptor signaling and disrupting TCR interaction with major histocompatibility complex.[51,52]

In human HCC, $CD14^+HLA\text{-}DR^{low/-}$ MDSCs from peripheral blood or tumors were significantly increased in HCC patients.[7] $CD14^+$ $HLA\text{-}DR^{-/low}$ MDSCs from HCC patients are unable to stimulate an allogeneic T-cell response, suppress T-cell proliferation, and have high arginase activity. Additionally, these MDSCs induce $CD4^+CD25^+Foxp3^+$ Treg expansion when cocultured with autologous T cells.[7] This suggests that MDSCs mitigate effector T-cell function by way of Tregs.[7] A recent report examined peripheral blood samples from patients with advanced HCC. They demonstrated augmented numbers of $CD14^-CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs, Tregs, and $PD\text{-}1^+$ exhausted T cells along with increased levels of immunosuppressive cytokines IL-10 and TGF-$\beta$, suggesting immune dysfunction in advanced HCC.[9] Thus, MDSCs and Tregs are important components of immunosuppressive milieu in HCC, and MDSCs can exert their immunosuppressive function through inducing Treg expansion.

Other mechanisms have been described, in which MDSCs affect T-cell function, survival, and trafficking. Similar to TAMs, MDSCs express galectin-9 that binds to TIM-3 on T cells, inducing T-cell apoptosis.[53] MDSCs also express ADAM17 that down-regulate L-selectin (CD62L) levels on T cells, limiting their homing to lymph nodes and tumors.[54] In a breast cancer mouse model, MDSCs ($CD11b^+Gr1^+$) could home to and accumulate in high numbers in the liver. Additionally, MDSCs interact with liver macrophages and cause their up-regulation of B7-H1, further strengthening the immunosuppressive phenotype.[47] MDSCs can also impair natural killer (NK) cell function. In human HCC, MDSCs ($CD14^+HLA\text{-}DR^{-/low}$) inhibit NK cell cytotoxicity and cytokine release, which is mediated by the NKp30 receptor.[55] Tumor-derived IL-1$\beta$ induces Ly6C-negative MDSCs, which also inhibit NK cell development and function.[56] In summary, MDSCs

contribute to the immunosuppressive network through multiple mechanisms and are potential immunotherapy targets for HCC.

## Myeloid Cells and HCC Therapy

Owing to the tumor-promoting and immunosuppressive roles of myeloid cells, there is great interest in targeting them to enhance the efficacy of conventional cancer therapy. A recently approved chemotherapeutic agent, trabectedin, not only targets tumor cells, but also induces rapid apoptosis in myeloid cells, whereas neutrophils and lymphocytes are not affected.[57] Noteworthy, although trabectedin has no effects on some sarcoma and ovarian cell lines *in vitro*, it still retained its efficacy on *in vivo* tumor growth from these lines, indicating that trabectedin-induced apoptosis of myeloid cells plays a key role in limiting tumor growth.[57] Trabectedin clinical trials reported reversible hepatotoxicity in human patients.[58] Trabectedin also exerts potent cytotoxicity on HepG2 liver cancer cells.[59] Therefore, trabectedin may be a promising therapy for HCC through targeting both cancer cells and myeloid cells. HCC is a male-predominant cancer with worse prognosis for men compared to women.[60] Estrogen, the primary female sex hormone, suppresses myeloid cell function in HCC.[61] Estrogen inhibited secretion of IL-6 from macrophages exposed to necrotic hepatocytes and reduced liver cancer risk in DEN-treated female mice.[14] Estrogen inhibited myeloid cell function, including reduced arginase activity, mannose receptor CD206 expression, and IL-10 production. Estrogen suppressed tumor-promoting myeloid cells through inhibiting Janus kinase (JAK)-STAT6 activation, leading to reduced tumor growth murine HCC models.[62] Hence, estrogen therapy may be useful in disrupting the development and function of myeloid cells in HCC. Myeloid cell elimination can be achieved by two well-studied agents: ZA and clodronate-containing liposome (clodrolip). ZA is an FDA-approved drug for bone metastasis, which specifically induces apoptosis of osteoclasts and macrophages. Clodrolip is a bisphosphonate clodronate-containing liposome that reduces myeloid cell number in tumors and circulating monocytes in peripheral blood. In a metastatic HCC mouse model, depletion of myeloid cells by ZA and clodrolip in combination with sorafenib

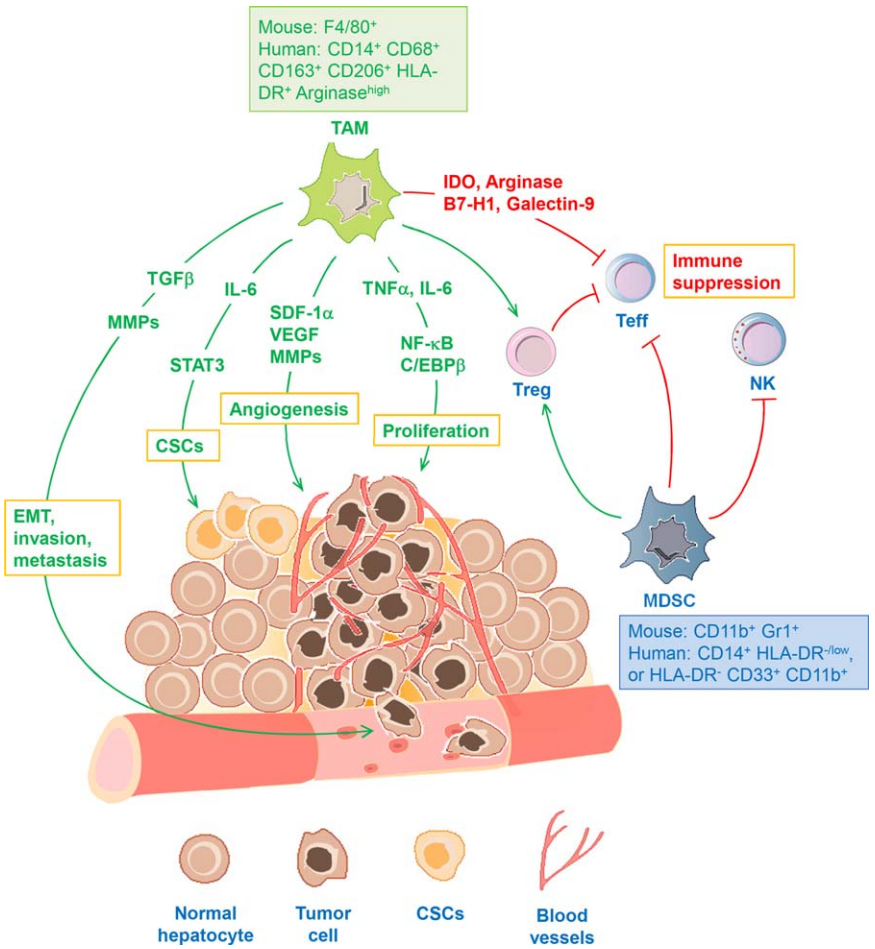


Fig. 2. Immunosuppressive and tumor-promoting functions of TAMs and MDSCs in HCC. HCC TAMs and MDSCs suppress T-cell effector functions through their expression of IDO, arginase, B7-H1 (PD-L1), and galectin-9, induction and recruitment of regulatory T cells, as well as MDSC-mediated suppression of NK cells. TAMs promote HCC development and proliferation through TNF-$\alpha$- and IL-6-activated NF-$\kappa$B and C/EBP$\beta$ pathways. TAM-derived SDF-1$\alpha$, VEGF, and MMPs induce angiogenesis in HCC. HCC TAMs enhance CSCs through IL-6-activated STAT3 signaling. HCC TAMs are found at the invasive front of tumors and associated with invasion and metastasis. TAM-derived TGF-$\beta$ induces EMT and enhances HCC metastasis. MMPs disrupt basement membrane and also facilitate tumor cell invasion. Surface markers used to identify HCC TAMs and MDSCs in mouse and human are listed in green box and blue box respectively.

significantly inhibited tumor progression, tumor angiogenesis, and lung metastasis, compared with sorafenib treatment alone.[19] Hence, targeting myeloid cells represents a point of further study as a possible adjuvant therapy to attenuate HCC progression.

## Concluding Remarks

Myeloid cells in HCC are skewed to suppress antitumor immunity and support HCC progression (Fig. 2 and Table 1). Immunosuppressive effects of myeloid cells are one of the key factors limiting the efficacy of immunotherapies that require active antitumor immune responses.[63] Therefore, disrupting these cells could counteract the immunosuppressive network and impede tumor progression. Potential methods to inhibit myeloid cells in HCC include: (1) target molecular pathways involved with suppressing effector cell function or promoting tumor growth; (2) target tumor factors that induce immunosuppressive myeloid cells from bone marrow progenitors; (3) repolarize them to become active APCs that stimulate antitumor immunity; and (4) induce apoptosis of myeloid cells or block trafficking to lymphoid organs and tumors. Targeting these common pathways utilized by immunosuppressive and tumor-promoting myeloid cells could provide novel therapeutic strategies to better treat HCC patients.

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