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REVIEW ARTICLE

Role of tumor-associated macrophages in the progression of hepatocellular carcinoma

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Abstract Recent studies have shown that the tumor microenvironment plays an important role in cancer progression. Tumor-associated macrophages (TAMs), in particular, have been found to be associated with tumor progression. Macrophages have multiple biological roles, including antigen presentation, target cell cytotoxicity, removal of foreign bodies, tissue remodeling, regulation of inflammation, induction of immunity, thrombosis, and endocytosis. Recent immunological studies have identified two distinct states of polarized macrophage activation: the classically activated (M1) and the alternatively activated (M2) macrophage phenotypes. Bacterial moieties such as lipopolysaccharides and the Th1 cytokine interferon-γ polarize macrophages toward the M1 phenotype. The M2 polarization was discovered as a response to the Th2 cytokine interleukin-4. In general, M2 macrophages exert immunoregulatory activity, participate in polarized Th2 responses, and aid tumor progression. TAMs have recently been found to play an important role in hepatocellular carcinoma (HCC) progression. Based on the properties of TAMs, obtained from pathological examination of resected specimens, we have identified new therapeutic approaches, involving the targeting of TAMs with adjuvant therapy after hepatic resection for HCC. This review discusses the roles of TAM in HCC progression and the possibility of new therapies targeting TAMs.

Keywords Hepatocellular carcinoma · Tumor-associated macrophages · Hepatic resection · Cancer progression

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and accounts for almost 700,000 deaths annually [1]. Although surgical procedures for HCC, such as hepatic resection and liver transplantation, have progressed and the outcomes have improved, HCC is still characterized by frequent recurrence [2–4], even after liver transplantation [5]. The recent introduction of the molecular targeting agent, sorafenib, was reported to improve survival rates in patients with nonresectable or advanced HCC [6]. Sorafenib is a multikinase inhibitor, found by that study to prolong survival significantly, although still by less than 3 months [6]. Clearly, new therapeutic strategies are needed to improve the survival of patients with HCC.

For more than 100 years, pathologists have reported that solid tumors consist of malignant tumor cells and stromal cells [7]. Recent studies have revealed that the stromal cells in solid tumors are a dynamic, flexible asset to tumor progression [7]. In particular, tumor-associated macrophages (TAM) are an important component of the tumor microenvironment and can promote tumor progression. Macrophages are a major component of the leukocytes that infiltrate most tumors [8], and numerous studies have shown that a high frequency of infiltrating TAMs is associated with poor prognosis [9–12]. Macrophages have multiple biological roles, which include antigen

presentation, target cell cytotoxicity, removal of foreign bodies, tissue remodeling, regulation of inflammation, induction of immunity, thrombosis, and endocytosis. Recent immunological studies [13, 14] have identified two distinct states of polarized macrophage activation, namely, the classically activated (M1) and the alternatively activated (M2) macrophage phenotypes (Fig. 1). Bacterial moieties, such as lipopolysaccharides and the Th1 cytokine interferon (IFN)-γ, polarize macrophages toward the M1 phenotype, whereas the M2 polarization was discovered as a response to the Th2 cytokine IL-4. In general, M2 macrophages exert immunoregulatory activity, participate in polarized Th2 responses, help with parasite clearance, suppress inflammation, and promote tissue remodeling, particularly tumor progression. Although the mechanisms by which TAMs promote tumor progression are poorly understood, recent research has shed new light on their roles.

An analysis of TAMs, which interact with cancer cells, must be based on surgical specimens; hence, pathological examination of the resected specimen is particularly important for the analysis of TAMs. Therefore, surgeons will provide pivotal information to open this field and may help to develop new adjuvant therapies that target TAMs. There are many unanswered questions about TAMs: how are TAMs recruited into the tumor? How are the antitumor immune effects of TAMs impaired? What roles do TAMs play in the metastasis, angiogenesis, and endothelial–mesenchymal transition (EMT) of HCC? What is the prognostic value of TAMs in patients with surgically resected HCC? Can we develop new therapeutic strategies for HCC that specifically target TAMs?

This review discusses the recent research on TAMs in cancer, particularly in HCC, and evaluates the new therapeutic strategies in this field.

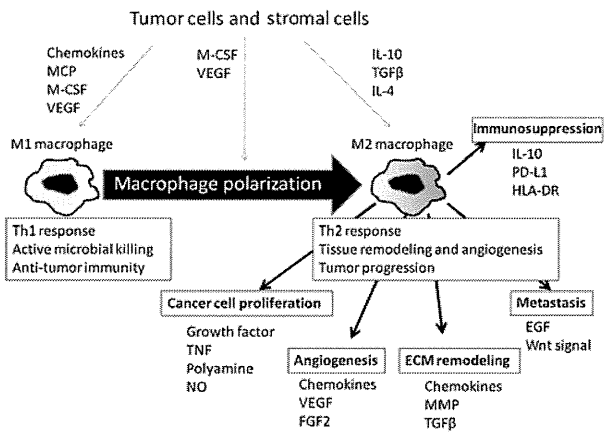

Fig. 1 Proposed functions of tumor-associated macrophages in tumor progression. Two distinct states of polarized activation for macrophages have been recognized: the classically activated (*M1*) macrophage phenotype and the alternatively activated (*M2*) macrophage phenotype. M2 macrophages are involved in cancer proliferation, angiogenesis, extracellular matrix remodeling, metastasis, and immunosuppression, producing a microenvironment that benefits cancer progression. *IL* interleukin, *MCP* macrophage chemoattractant protein, *M-CSF* macrophage colony-stimulating factor, *VEGF* vascular endothelial growth factor, *TGFβ* tumor growth factor-β, *NO* nitric oxide, *MMP* matrix metalloproteinase, *PD-L1* programmed cell death-ligand 1, *ECM* extracellular matrix, *TNF* tumor necrosis factor, *EGF* epidermal growth factor, *FGF* fibroblast growth factor

TAM recruitment

Macrophages are released from the bone marrow as immature monocytes. After circulating in the bloodstream, they migrate into tissues and undergo final differentiation into resident macrophages, including Kupffer cells in the liver. TAMs are derived from monocytes, which are recruited largely by CCL2 [chemokine (C–C motif) ligand 2; formerly known as monocyte chemotactic protein (MCP)] and chemokines [15]. CCL2 is mainly expressed by tumor cells, but also by endothelial cells, fibroblasts, and macrophages in human tumors [16]. Some studies suggest that it is highly expressed in a wide range of tumors, including glioma [17], meningioma [18], ovarian carcinoma [19], and squamous cell carcinoma of the uterine cervix [20].

Other tumor-derived signals have been reported to recruit TAMs into tumors, including macrophage-colony stimulating factor (M-CSF or CSF-1), macrophage inflammatory protein 1α (MIP-1α), vascular endothelial growth factor (VEGF), CCL3, CCL4, CCL5, CCL8, and angiopoietin-2 [21]. Zhu et al. [22] demonstrated that high expression of M-CSF in peritumoral liver tissue is correlated with macrophage density and poor survival following curative resection for HCC. Similarly, Jia et al. [23] reported that the MCSF-1 receptor expression in peritumoral tissue was associated with intrahepatic metastasis and poor prognosis following hepatectomy. Shen et al. [24] detected high levels of IL-10 and transforming growth factor (TGF)-β, and low levels of IFN-γ in the HCC microenvironment, representing an immunosuppressive state. Takai et al. [25, 26] showed that the expression of glypican-3 on the cell membrane of HCC cells was involved in TAM recruitment. Although the mechanisms involved in TAM recruitment are not yet fully understood, the microenvironment and cytokine network in HCC seem to promote TAM recruitment and M2 polarization.

TAMs and immunity

Macrophages are essential for host defense. In humans, macrophages play a crucial role in the innate and adaptive

immune responses to pathogens and are critical mediators of inflammatory processes.

Macrophages can assume a range of different phenotypes based on the environmental stimuli. The extremes of this range obtained in vitro are represented by the M1 phenotype, which is associated with active microbial killing, and the M2 phenotype, which is associated with tissue remodeling and angiogenesis. When monocytes in the tumors are exposed to tumor-derived antiinflammatory molecules, such as IL-4, IL-10, TGF-β1, and prostaglandin E_2, they develop into polarized or M2 macrophages [13].

The M2 phenotype appears to be the dominant macrophage phenotype in tumors because TAMs show similar molecular and functional profiles. These profiles are characterized by low expression of differentiation-associated macrophage antigens, such as carboxypeptidase M and CD51, high constitutive expression of arginase I, IL-1 decoy, IL-1ra, IL-6, and IL-10, and low expression of tumor necrosis factor (TNF) and IL-12. M2 macrophages also express chemokines, including CCL17 and CCL22 [8, 27, 28]. The polarization of M2 macrophages has not been clearly demonstrated in HCC because, in most studies, the anti-CD 68 antibody has been used for the immunohistochemical evaluation of macrophages in HCC. Takai et al. [25] used two antibodies to differentiate between resident macrophages and activated macrophages, with a pan-macrophage marker (AM-3K) to label resident macrophages, whereas PG-M1, which reacts with the human CD68 antigen, was used to label activated macrophages. Unfortunately, to our knowledge, no further functional analysis of macrophages has been performed.

The serum levels of TGF-β1 are elevated in patients with HCC, which may explain the phenomenon that the M2 phenotype macrophages, as well as TAMs, secrete down-regulatory cytokines such as TGF-β1 [29, 30]. M2 macrophages can induce the differentiation of regulatory T cells. For example, Zhou et al. [31] reported that the intratumoral prevalence of $FOXP3^+$ regulatory T cells in HCC was associated with a high density of macrophages. Macrophages exposed to tumor culture supernatants form hepatoma-derived cell lines with an increased frequency of $FOXP3^+$ regulatory T cells in vitro, and this increase is partially blocked by anti-IL-10 antibodies. Wu et al. [32] and Kuang et al. [33] demonstrated that activated macrophages in the peritumoral stroma and Kupffer cells express programmed cell death (PD) (B7-H1)-ligand 1 (L1) and suppress tumor-specific T-cell immunity. These experimental findings may explain how TAMs suppress antitumor immunity in HCC. A recent study by Ju et al. [34] showed a positive correlation between the density of peritumoral activated hepatic stellate cells (HSC) and macrophage or regulatory T cells, and that the presence of HSCs, macrophages, and regulatory T cells was associated with aggressive clinicopathological features. Taken together, these findings suggest that TAMs, regulatory T cells, and HSCs might provide an immunosuppressive environment that ultimately aids HCC recurrence.

Recent studies have revealed the important roles of TAMs and memory Th 17 cells in tumor progression. In ovarian carcinoma, $CD8^+$ T cell-derived IL-17 mediates the recruitment of myeloid cells into tumors and enhances tumor growth [35]. Zhang et al. [36] showed that intratumoral IL-17-producing cells were correlated with a poor prognosis in HCC, whereas Kuang et al. [37, 38] reported an interaction between TAMs and memory Th 17 cells in HCC and found that TAMs promote memory Th 17 cell expansion.

Role of TAMs in the metastasis of HCC

Many reports state that TAMs promote distant metastasis of cancer cells. According to Gorelik et al. [39], macrophages increased the number of lung tumor nodules following an intravenous injection of murine tumor cells. Rolny et al. [40] reported also that inhibiting TAM infiltration into tumors using antibodies to neutralize monocyte chemoattractants impaired metastases.

TAMs and cytokines such as IL-1, TNF, and IL-6 are believed to have prometastatic properties, and recent studies provide new evidence to support this. For example, the IL-6 levels in patients with liver cancer are much higher than those in healthy adults [41]. Liu et al. [42] reported that IL-6 exerted antiapoptotic activity via the STAT3 signaling pathway in human HCC cell lines. These phenomena may be related to TAMs, which can produce IL-6, and further studies in this field are necessary.

In a mammary carcinoma model, gene expression profiling of macrophages supporting cancer invasion revealed activation of the Wnt signaling pathway, which may play a key role in the prometastatic activity of TAMs [43]. Activation of the Wnt signaling pathway in cancer cells may promote cell proliferation and cell detachment, a step that is essential for cancer cells to metastasize to the distant organ.

The next stage in cancer metastasis is extravasation of the cancer cells. In vitro and in vivo experiments have revealed that extravasation of tumor cells into the hematogenous or lymphatic circulation requires an interaction with TAMs. Condeelis et al. [44] reported that epidermal growth factor (EGF), released by the TAMs, and CSF-1, released by tumor cells, act on the reciprocal cell types to stimulate tumor cell migration. Using in vivo multiphoton microscopy, they showed that metastasis occurs more frequently when the cancer cells are in the vicinity of TAMs and can communicate in this manner.

Role of TAMs in the angiogenesis of HCC

In human tumors, TAMs accumulate in poorly vascularized, necrotic areas. These areas become hypoxic, which in turn triggers a pro-angiogenic program in TAMs [45]. Several clinical studies have shown a significant correlation between a high density of TAMs in human tumors and microvessel density. Peng et al. [46] reported that the TAM count was significantly correlated with microvessel density in HCC. Human TAMs also express various pro-angiogenic factors in tumors, including VEGF [47] and thymidine phosphorylase [48].

Angiogenesis is also facilitated by TAM-derived proteases because extracellular proteolysis is necessary for new vessel formation. Macrophages can secrete proteinases to release a number of pro-angiogenic molecules bound to heparin sulfate in proteoglycans, and fragment fibrin and collagen, which induces angiogenesis. The most prominent proteinases that promote tumor-directed angiogenesis include matrix metalloproteinase (MMP 1, 2, 3, 9, and 12), plasmin, and urokinase-type plasminogen activator (uPA) and its receptor. TAMs were reported to be a major source of MMP-9 and synthesize uPA in various cancers [49, 50].

Role of TAMs in the EMT in HCC

The concept of the EMT, which was originally developed in embryology, has recently been extended to cancer progression and metastasis. In vitro and in vivo studies, as well as clinical samples, support the important role of EMT in cancer progression [49]. Analysis of the EMT has been aided by the development of EMT-associated markers, including epithelial-specific markers (E-cadherin and Claudin-1), mesenchymal-specific markers (vimentin, N-cadherin, and fibronectin), and transcriptional markers (SNAIL, SLUG, and Twist). Using these markers, recent studies have shown that crosstalk between cancer cells and the tumor microenvironment facilitates the EMT in cancer cells [51].

The tumor microenvironment is composed of an extracellular matrix, fibroblasts, myofibroblasts, endothelial cells, immune cells (including TAMs), and soluble factors. The tumor microenvironment is strongly related to the EMT of cancer cells. In addition to cancer cells themselves, cancer-associated fibroblasts may facilitate the EMT of cancer cells. TGF-β is an essential cytokine for the EMT [52], whereas inflammatory cytokines, such as IL-6, are important for the EMT in HCC [53]. TAMs are known to produce these cytokines, particularly the M2 macrophages that produce IL-6 and TGF-β. Recently, Werno et al. [54] demonstrated that HIF-1α-deficient macrophages exhibited the M2 phenotype and could stimulate the differentiation of stem cells toward CD31-positive cells. This finding suggests that the M2 macrophages may facilitate the EMT in cancer. Therefore, the relationship between the TAMs and the EMT in HCC warrants further research.

Prognostic value of TAMs in patients with surgically resected HCC

The prognostic value of the high density of TAMs in HCC is summarized in Table 1. All the previous related studies evaluated the prognostic value of TAMs in patients who underwent hepatic resection for HCC [22, 31, 33, 55, 56]. Most of these studies [22, 31, 36, 55] found that a high density of TAMs was associated with poor prognosis, suggesting that TAMs were related to the immunosuppressive state. However, Li et al. [56] reported that the presence of TAMs and memory T cells was associated with a good prognosis.

New therapeutic strategies for HCC that target TAMs

Three major features of TAMs are potentially amenable to therapeutic interventions: inhibition of their recruitment and/or M2 polarization, inhibition of angiogenic and tissue remodeling activities, and inhibition of their immunosuppressive effects and restoration of antitumor cytotoxicity.

Pharmacological drugs, such as clodronate-encapsulated liposomes or aminobisphosphonates, which knock down macrophages in vivo, have reduced angiogenesis and tumor progression in several experimental tumor models. Recently, Zhang et al. [57] showed that TAMs played an important role in tumor progression during sorafenib therapy. Clodronate-encapsulated liposomes and zoledronic acid, which deplete the macrophage population, are promising drugs that enhance the antitumor effects of sorafenib when used in combination.

Another more sophisticated strategy is to prevent the M2 polarization of TAMs or reorientate M2 TAMs to M1 TAMs. The phenotype of TAMs in most cancers is reversible. In one experimental study, the combination of CpG immunostimulatory oligonucleotide plus an anti-IL-10 receptor antibody switched infiltrating macrophages from the M2 to the M1 phenotype and triggered an innate response that debulked large tumors [58]. Moreover, TAMs lacking STAT6, the major intracellular mediator of IL-4 and IL-13, display an M1 phenotype. As a result, these mice rejected spontaneous mammary carcinoma by exhibiting adaptive immunity to cancer. Similarly, the inhibition of STAT3 activity, which is required for IL-10 activity and gene transcription, restored the expression of pro-inflammatory mediators, including IL-12 and TNF-α, and tumor inhibition.

Table 1 Tumor-associated macrophages and prognosis after hepatic resection for hepatocellular carcinoma

Study	Other factors	Location	Prognosis
Zhu et al. [22].	MCSF	Peritumoral	DFS, OS (poor)
Kwang et al. [33].	PD-L1	Peritumoral	DFS, OS (poor)
Zhou et al. [31].	T_{reg}	Intratumoral	CD68 is correlated with T_{reg}, T_{reg}: DFS, OS (poor)
Ju et al. [34].	HSC, T_{reg}	Peritumoral	DFS, OS (poor)
Ding et al. [55].		Intratumoral, marginal	DFS, OS (poor)
Li et al. [56].	T_M	Intratumoral	DFS, OS (good)

MCSF macrophage colony-stimulating factor, *T_{reg}* regulatory T cell, *HSC* hepatic stellate cell, *TM* $CD45RO^+$ memory T cell, *DFS* disease-free survival, *OS* overall survival

A recent study also showed that a DNA vaccine against the M2-associated molecule legumain, a member of the asparaginyl endopeptidase family and overexpressed by TAMs, induced a robust $CD8^+$ T-cell response against TAMs. This vaccine also suppressed angiogenesis, tumor growth and metastasis [59]. Several studies revealed the inhibition of macrophage infiltration into tumors after attenuating the effects of VEGF-A with bevacizumab or other neutralizing antibodies. In xenograft-bearing mice, the number of TAMs declined following treatment with anti-VEGF-A therapy [60]. These studies suggest that suppressing TAM accumulation in tumors may enhance the activity of anti-angiogenic therapies by preventing TAMs from secreting additional pro-angiogenic factors.

Kakinoki et al. [61] and Tsuchiyama et al. [62] recently described the biphasic antitumor effects of CCL2/MCP-1 using suicide gene therapy against HCC. They generated low and high expression models using adenoviral vectors. In the low expression model of CCL2/MCP-1, the proportion of M1 macrophages was high and marked antitumor effects were observed. In contrast, in the high expression model, VEGF-A expression and number of $CD31^+$ microvessels were increased. Clearly, further studies are needed to clarify the possible benefits of gene therapy.

Another therapeutic strategy for TAMs is to block the PD-1/PD-L1 pathway [33, 63]. Kuang et al. [33] found that tumor supernatant-treated macrophages suppressed tumor-specific T cells and an anti-PD-L1 antibody improved macrophage-mediated T-cell activation in HCC in vivo. A phase I clinical study of the anti-PD-1 antibody has been conducted in patients with cancer [64]. Taken together, anti-PD-1 antibody therapy may offer another therapeutic strategy for HCC.

Conclusion

Recent studies of TAMs have shown that TAMs play important roles in various stages in the growth and metastasis of cancer cells, including tumor angiogenesis, suppression of antitumor immunity, extravasation of cancer cells, and possibly the EMT of cancer cells. In HCC, recent studies have shown that the accumulation of TAMs is an important cause of cancer cell progression.

The quantification of TAMs in HCC involves immunohistochemical techniques that can be performed on formalin-fixed paraffin-embedded tissues obtained from surgically resected specimens. Determining the density of TAMs in the tumor may help to predict the recurrence of HCC and to identify patients who may benefit from future immunotherapies targeting this pathway. In the near future, pathologists may play critical roles in the diagnostic assessment of TAM infiltration, thereby providing prognostic information and guidance in selecting an appropriate immunotherapeutic strategy for each patient.

A phase I/II clinical trial of an anti-PD-1 antibody for cancer patients is underway in the United States of America [64]. New concepts in adjuvant immunotherapy, such as targeting TAMs following hepatectomy or liver transplantation, may also be introduced to improve the outcome of patients with HCC in the near future.

Conflict of interest The authors have no conflict of interest.

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Kinetics of Anti-Blood Type Isoagglutinin Titers and B Lymphocytes in ABO-Incompatible Living Donor Liver Transplantation With Rituximab and Plasma Exchange

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Background. A novel immunosuppression protocol using rituximab and plasma exchange treatment was developed for ABO-incompatible living donor liver transplantation (ABO-I LDLT). The aim of this study was to investigate the kinetics of anti-blood type isoagglutinin titers and the number of blood B lymphocytes in ABO-I LDLT with the new protocol and their impact on the outcomes after ABO-I LDLT.
Methods. Fifteen patients underwent ABO-I LDLT plus splenectomy with the new protocol between November 2005 and December 2010, and their data were retrospectively analyzed.
Results. CD19-positive lymphocytes in the blood rapidly disappeared after rituximab treatment and began to recover approximately 6 months later. Anti-blood type isoagglutinin titers were lowered by pretransplant plasma exchange ($2^3 \sim 2^{12} \rightarrow 2^1 \sim 2^8$). Although the anti-donor blood type isoagglutinin titers remained consistently low after transplantation in comparison to the pretreatment levels, they persisted long after LDLT, whereas posttransplant biopsy specimens showed sustained A/B antigens on the graft livers. ABO-I hepatitis C virus-positive patients were prone to acceleration of hepatitis C viremia and cytomegalovirus antigenemia in comparison to the control patients.
Conclusions. Although the new protocol for ABO-I LDLT yielded great success with 100% graft survival, the acceptable anti-blood type isoagglutinin titers just before LDLT, and its application to hepatitis C-positive patients must be determined.

Keywords: ABO-incompatible, Allograft rejection, Isoagglutinin mediated-rejection, Living donor liver transplantation, Plasma exchange, Rituximab.

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Many patients with end-stage liver disease have to undergo living donor liver transplantation (LDLT) in Japan because there is a shortage of cadaveric organ donation. It is often difficult for recipients to find a suitable ABO-identical or -compatible living-related donor for LDLT. An ABO-incompatible (ABO-I) living-related donor is often the only way to save a patient with end-stage liver disease (*1*). ABO-I liver transplantation was considered a relative contraindication because of severe anti-blood type isoagglutinin-mediated rejection that resulted in graft necrosis (*2, 3*). However, the outcomes of ABO-I LDLT have dramatically improved since the introduction of rituximab, an anti-CD20 antibody (*4–8*). A new protocol for ABO-I LDLT using rituximab, intravenous immunoglobulin (IVIG), and plasma exchange (PE) without graft local infusion treatment has been established and successfully applied with 100% graft survival (*9*), which was superior to the recently reported results (*4*). Despite the better short outcomes of the ABO-I LDLT patients, the mid- and long-term outcomes of ABO-I LDLT patients, the kinetics of anti-blood type isoagglutinin titers, the restoration of B lymphocytes, and the impact of this protocol on defense systems to various pathogen remain to be elucidated. The aim of this study was to retrospectively review the data of ABO-I patients to determine these factors.

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RESULTS

Overall Outcomes of 15 Patients of ABO-I LDLT

All but 1 of the 15 patients were alive after ABO-I LDLT at the last follow-up (follow-up time ranging from 5 months to 5 years 6 months). One patient died of accidental drowning