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Review Article

Clinical significance of macrophage heterogeneity in human malignant tumors

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The fact that various immune cells, including macrophages, can be found in tumor tissue has long been known. With the recent introduction of the novel concept of macrophage differentiation into a classically activated phenotype (M1) and an alternatively activated phenotype (M2), the role of tumor-associated macrophages (TAMs) is gradually beginning to be elucidated. Specifically, in human malignant tumors, TAMs that have differentiated into M2 macrophages act as "protumoral macrophages" and contribute to the progression of disease. Based on recent basic and preclinical research, TAMs that have differentiated into protumoral or M2 macrophages are believed to be intimately involved in the angiogenesis, immunosuppression, and activation of tumor cells. In this paper, we specifically discuss both the role of TAMs in human malignant tumors and the cell–cell interactions between TAMs and tumor cells.

It has long been known that many leukocytes including macrophages are present in tumor tissues and that these cells, together with fibroblasts and vascular endothelial cells, form the tumor microenvironment (Fig. 1).[1–4] Previously, activated macrophages were believed to exhibit antitumor activity by directly attacking tumor cells in the tumor microenvironment.[5] However, many recent studies have indicated the protumoral functions of tumor-associated macrophages (TAMs), and thus, TAMs are believed to directly or indirectly promote tumor progression.[6–8] Great advances have been made in TAM research over the past dozen years or so, with one of the most significant breakthroughs being the development of immunohistochemical methods for identifying TAMs in tumor tissue. Numerous studies using human samples have been carried out using CD68 as a macrophage marker, whereas CD163 and CD204 have been used as markers of M2 macrophages in recent studies.[9,10] Although variability is observed according to tumor tissue type and location, over 80% of immunohistochemical studies using various human tumor tissues have shown that higher numbers of TAMs are associated with worse clinical prognosis.[9] Supporting these clinical observations, *in vitro* experiments using human tumor cells and experiments using animal models indicate that TAMs promote tumor cell growth by suppressing antitumor immunity and inducing angiogenesis.[11,12]

As the relationship between TAMs and malignant tumors becomes clearer, TAMs have begun to be seen as the target of new cancer treatments. Clarification of how TAMs are involved in tumor progression and metastasis is anticipated to lead to the development of novel treatments and drugs.

Intratumoral infiltration of TAMs

Intratumoral infiltration of monocytes/macrophages is induced by various chemokines including chemokine (C-C motif) ligand (CCL)2, CCL5, CCL7, and chemokine (C-X3-C motif) ligand (CX3CL)1, as well as cytokines such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor, and vascular endothelial growth factor (VEGF), which are produced by tumor cells.[13–15] Subsequent differentiation into TAMs is induced by various factors produced by tumor cells. While the tumor size is small, macrophages from the surrounding tissue accumulate in and around the tumor by tumor cell-derived chemotactic molecules described above, and TAMs derived from the surrounding tissue macrophages account for the majority of TAMs.[4,16] As the tumor subsequently increases in size and an intratumoral vascular network forms, monocyte-derived TAMs become the dominant source of TAMs.[4,16]

Although many macrophage chemotactic factors are secreted by tumor cells, CCL2 and M-CSF are considered to be impor-

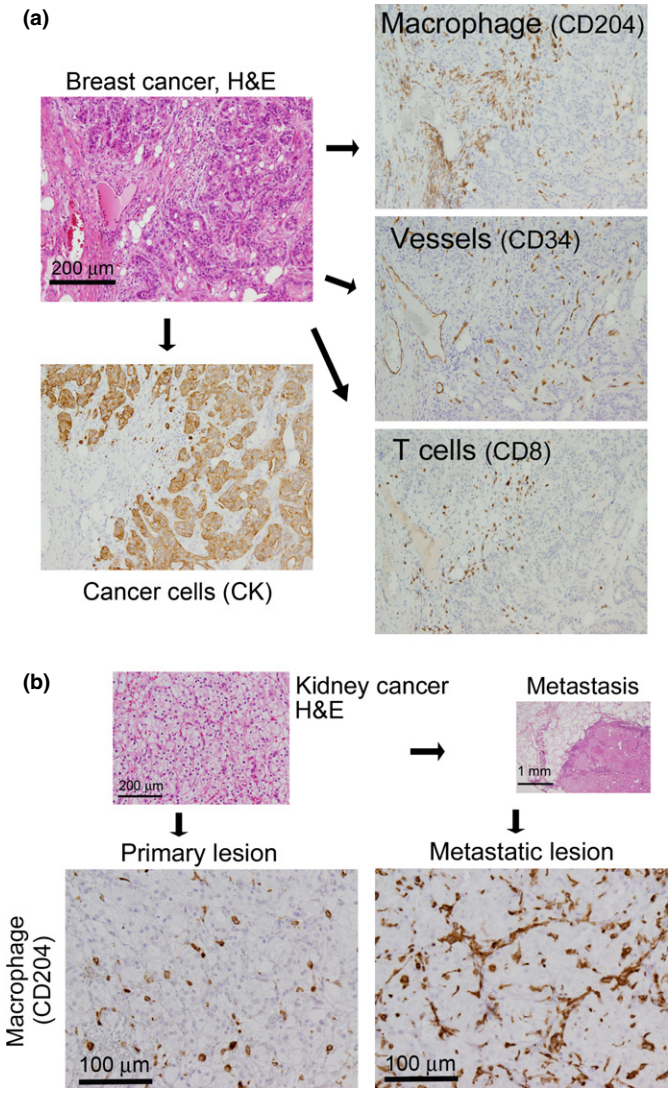

Fig. 1. Tumor microenvironment. (a) Tumor tissue contains not only tumor cells, but also large numbers of normal cells, including tumor-associated macrophages, lymphocytes, blood vessels, and fibroblasts, that affect tumor development in various ways. The photographs show an example of a clinical case of human breast cancer (invasive ductal carcinoma). The relative distributions of the above-mentioned cell types differ by organ and tissue type as well as individual case. CK, cytokeratin. (b) Metastatic tumors contain a larger number of tumor-associated macrophages. The photographs show an example of a clinical case of human kidney cancer (clear cell renal cell carcinoma). The primary tumor tissues and the metastatic (lung) tumors are shown.

tant molecules involved in macrophage infiltration. CCL2 is expressed in a wide variety of tumor cells, including gliomas, squamous cell carcinoma, ovarian cancer, prostate cancer, lung cancer, cervical cancer, and undifferentiated sarcoma, CCL2 also plays an important role in the intratumoral infiltration of monocytes.[13,17] In addition to inducing monocyte infiltration, M-CSF plays a critical role in the differentiation of monocytes into macrophages and, in particular, into M2 macrophages.[18–20]

Role of TAMs in tumor progression

Based on numerous studies using murine tumor models, activated TAMs were found to produce a variety of angiogenic, immunosuppressive, and growth-related factors.[7,8] However, few studies have been carried out using human materials, and thus the detailed mechanisms and molecular characterization of TAMs in human tumors have yet to be described. One method for studying the relationship between TAMs and tumor development is to carry out statistical analysis using clinical data related to survival rates or survival times. Studies comparing TAM infiltration into diseased tissue, using CD68 as a macrophage marker, are summarized in Table 1. The majority of studies in human malignant tumors have found that a higher level of TAM infiltration is associated with lower survival rates, and these observations indicate that TAMs may enhance tumor progression. However, other reports in certain types of cancer such as gastric, colon, and prostate cancer, have shown that a higher number of TAM infiltration results in a better outcome.

For a localized tumor a few millimeters in size to grow larger, intratumoral angiogenesis must occur. Genetic analysis has revealed that TAMs produce VEGF, interleukin (IL)-8 (CXCL8), basic fibroblast growth factor, thymidine phosphorylase, MMP, and other molecules that are involved in angiogenesis, indicating that TAMs promote the formation of intratumoral blood vessels. Furthermore, TAMs produce immunosuppressive factors, including prostaglandin E2 (PGE_2), indoleamine 2,3-dioxygenase, and IL-10, and thus contribute to the immunosuppressed state of cancer patients.[5–7] In fact, in studies using human tissue samples, the number of intratumoral TAM infiltration is positively correlated with formation of blood vessels and the number of regulatory T cells. Tumor-associated macrophage-derived PGE_2, indoleamine 2,3-dioxygenase, and IL-10 play important roles for induction of regulatory T cells and TAM-derived CCL17, CCL18, CCL22 are chemotactic factors for regulatory T cells.[5–7] These results indicate that TAMs create environments conducive to tumor progression through their effect on angiogenesis and immunosuppression. In addition, growth factors produced by TAMs, including basic fibroblast growth factor, hepatocyte growth factor, epidermal growth factor, platelet-derived growth factor, and transforming growth factor-β (TGF-β), are considered to directly promote tumor cell growth.[5–7]

Of further interest is the suggestion, based on the results of animal model analysis, that TAMs may play a role in forming premetastatic niches in organs to which the tumor will eventually metastasize.[21–23] Specifically, tumor necrosis factor-α, VEGF, and TGF-β (VEGF and TGF-β are also produced by cancer cells), which are secreted by TAMs in cancer tissues, are believed to be transported through the bloodstream to destination organs such as the lung, where they induce macrophages to produce S100A8 and serum amyloid A3.[23] Both S100A8 and serum amyloid A3 recruit macrophages and tumor cells to these organs and promote the formation of metastatic foci.[24,25] Thus, TAMs are believed to not only influence their local environment, but also to impact macrophages throughout the body and contribute to disease progression.

CD163 and CD204 as markers for protumoral or M2 macrophages

The heterogeneity of macrophage functions was suggested as early as the late 1990s.[26,27] Macrophage activation can be broadly divided into the following two types: classically activated macrophages (M1), which promote inflammation, and alternatively activated macrophages (M2), which inhibit

Table 1. High numbers of CD68+ tumor-associated macrophages are correlated with clinical prognosis in human malignant tumors

Tumor type	Favorable prognosis	Poor prognosis
Epithelial	Gastric cancer (adenocarcinoma)[68] Colorectal cancer (adenocarcinoma)[71] Prostate cancer (adenocarcinoma)[73]	Uterine cancer (endometrioid adenocarcinoma)[69,70] Esophageal cancer (squamous cell carcinoma)[72] Liver cancer (hepatocellular carcinoma)[74] Breast cancer (invasive ductal carcinoma)[75,76] Thyroid cancer (poorly differentiated)[77] Gastric cancer (adenocarcinoma, intestinal type)[78] Bladder cancer (urothelial carcinoma)[79]
Non-epithelial		Malignant mesothelioma (sarcomatous)[80] Malignant melanoma[81] Neuroblastoma[82] Ewing's sarcoma[83]
Hematopoietic		Hodgkin's lymphoma[84] Follicular lymphoma[85]

inflammation.[27,28] Those TAMs demonstrating enhanced expression of CD163 (hemoglobin scavenger receptor), CD204 (class A macrophage scavenger receptor), CD206 (mannose receptor, C type 1), stabilin-1, arginase-1, and accelerated production of IL-10, VEGF, PGE_2, and MMP9, generally show characteristics of M2 macrophages.[6–8] The proangiogenic and immunosuppressive activity in the tumor microenvironment mediated by TAMs can also be considered the result of M2 macrophage function.[6–8] Because CD163 and CD204 are specifically expressed on macrophages, and antibodies to these antigens that are suitable for immunohistochemical analysis are commercially available,[10,29,30] many researchers have used these molecules as markers of the M2 phenotype in both *in vitro* and *in vivo* studies. The details of the functions of these molecules remain unclear; however, a few studies have indicated that these molecules are involved either in regulating the inflammatory responses or in maintaining the protumoral functions of macrophages.[31–33] The clinicopathological studies using anti-CD163 or anti-CD204 antibodies are summarized in Table 2. In malignant lymphoma, glioma, and kidney cancer, higher CD163 expression on TAMs is associated with worse clinical prognosis, but no correlation exists between clinical prognosis and the number of CD204-expressing TAMs.[10,34–36] In esophageal cancer, a higher number of CD204-expressing TAMs is associated with poor clinical outcome, but the number of CD163-positive TAMs is not.[37] These observations suggest that CD163 and CD204 are not expressed in completely identical macrophage populations. In addition, the functional significance of CD163- or CD204-positive TAMs might be different among sites and histological types of cancer. We suggest that both CD163 and CD204 should be analyzed to evaluate the polarization of TAMs and that CD163- and/or CD204-positive TAMs are considered as "protumoral" macrophages/TAMs.

In a recent review, based on their location and function, Qian and Pollard[38] classified TAMs into the following six types: angiogenic; immunosuppressive; invasive; metastasis-associated; perivascular; and activated macrophages. Not all of these macrophage types of TAMs show the phenotype of M2 macrophages. Tumor-associated macrophages with M1 characteristics have also been observed in animal models of glioma and human pancreatic cancer.[39,40] Although the concept of "M1/M2 macrophages" is a convenient hypothesis simply dividing TAMs into two populations, we should note that TAMs contain various macrophage populations with a wide range of polarization statuses stimulated by complex signals in tumor microenvironment.

Significance of direct cell–cell interactions between TAMs and tumor cells

As shown in Figure 1, TAMs and tumor cells often directly contact each other, indicating that intimate cell–cell interactions exist between them. During the initial stages of tumor progression, monocyte migration factors produced by tumor cells induce infiltration of monocytes/macrophages, as described above. The macrophages that have infiltrated the tumor are

Table 2. Correlation between CD163+ or CD204+ tumor-associated macrophages and clinical prognosis in human malignant tumors

Tumor type	Favorable prognosis	Poor prognosis
Epithelial	Colorectal cancer (adenocarcinoma)[86]	Kidney cancer (clear cell type)[34] Liver cancer (hepatocellular carcinoma)[87,88] Liver cancer (cholangiocellular carcinoma)[89] Pancreatic cancer (invasive ductal carcinoma)[90,91] Lung cancer (adenocarcinoma)[92,93] Lung cancer (squamous cell carcinoma)[92,94] Oral cancer (squamous cell carcinoma)[95] Ovarian cancer (serous adenocarcinoma)[96] Esophageal cancer (squamous cell carcinoma)[37]
Non-epithelial	Osteosarcoma[97]	Leiomyosarcoma[98] Brain tumor (high-grade glioma)[10,42] Malignant melanoma[99,100]
Hematopoietic		Diffuse large B-cell lymphoma[101] Hodgkin's lymphoma[101–104] Follicular lymphoma[105] Angioimmunoblastic T-cell lymphoma[35] Adult T-cell leukemia/lymphoma[36] Multiple myeloma[106]

activated by tumor cell-derived molecules, including IL-6, M-CSF, PGE_2, and heat shock protein-27, and differentiate into protumoral/M2 macrophages.[6,20] Protumoral/M2 TAMs produce a variety of angiogenic and immunosuppressive factors, as described above, and create a microenvironment conducive to tumor progression. Signal transducer and activator of transcription 3 (Stat3) has received recent attention as an important transcription factor that mediates the interaction between TAMs and tumor cells.[12] Many angiogenic and immunosuppressive factors are transcriptionally regulated by Stat3. Therefore, activation of Stat3 not only plays an important role in the differentiation of macrophages into protumoral /M2 macrophages, it is also involved in tumor cell growth, metastasis, epithelial–mesenchymal transition, and the acquisition of resistance to anticancer drugs and radiation therapies.[12,41] Direct coculture of tumor cells and macrophages shows that Stat3 in macrophages is activated and that various factors secreted by activated macrophages, including EGF, IL-6, and IL-10, activate Stat3 in tumor cells.[18,42] Activation of the M-CSF receptor (CD115) and sphingosine-1-phosphate receptor 1 (S1PR1) on the cell surface is believed to contribute to the cell–cell interaction mediated by Stat3.[42,43] Membrane-type M-CSF on the surface of tumor cells serves as a ligand for CD115, and sphingosine-1-phosphate derived from tumor cells serves as a ligand for S1PR1. Stimulation of these receptors activates a variety of signal transduction pathways, including that of Stat3, causing TAMs to differentiate into the protumoral/M2 phenotype.[44] The activation of Stat3 through cell–cell interactions between tumor cells and macrophages contributes to the formation of the microenvironment necessary for development of primary and metastatic lesions (Fig. 2).

Recent studies using a murine cancer model showed that Stat3 is also an important molecule in the maintenance and anticancer drug responses of cancer stem-like cells (CSCs).[45–47] The TAM-derived milk fat globule-EGF factor VIII, which is a glycoprotein belonging to an epidermal growth factor superfamily, contributes to Stat3 activation in cooperation with proinflammatory cytokines such as IL-6. And Stat3 activation is preferentially associated with tumorigenesis and drug resistance in CSCs.[46] In human colorectal cancer, overexpression of stem cell markers in tumor cells is reported to be associated with a high number of TAMs.[48] Further studies are expected to clarify the details of the relationships between TAMs and CSCs.

Tumor-associated macrophages and myeloid-derived suppressor cells

Regarding the functional analysis of TAMs, tumor xenograft mouse or rat models are more useful than human tumors. The majority of myeloid cells infiltrating tumor tissues are immature cells in some types of murine tumors.[49,50] A strong immunosuppressive response has long been known to be induced when cancer cells are transplanted into mice. In the 1980s, myeloid cells in the bone marrow of tumor-bearing mice were shown to inhibit the activation of lymphocytes.[51,52] Subsequently, the same types of cells were shown to exist in the spleen, and with the advancement of analysis resulting from the identification of myeloid markers CD11b and Gr1, these cells were also shown to exist in lymph nodes and tumor tissues.[51,52] Immature myeloid cells are derived from bone marrow myeloid cells and exhibit immunosuppressive activity; therefore, they are referred to as myeloid-derived suppressor cells (MDSCs).[51,52] Distinct from mature neutrophils and monocyte/macrophages, MDSCs have recently been divided into granulocytic MDSCs ($CD11b^+Ly6C^{int}Ly6G^{hi}$), showing characteristics similar to neutrophils, and monocytic MDSCs ($CD11b^+Ly6C^{hi}Ly6G^{neg}$), showing characteristics similar to monocytes/macrophages.[53] Despite the observed differences among tumor histopathological types, mature macrophages (TAMs, $Gr1^-$) and MDSCs ($Gr1^+$) appear to coexist in the tumor tissues of mice. As MDSCs from tumor tissues differentiate into mature macrophages in *ex vivo* assays, MDSCs are considered to be the immature phenotype of TAMs.[52,53] However, which cell type plays a greater role in angiogenesis and the activation of tumor cells remains unclear.

Systemic immunosuppression is also observed in human patients with advanced malignant tumors, suggesting the existence of cells similar in nature to the MDSCs that are found in mice. A significant increase in the number of $CD14^+HLA\text{-}DR^{low}$, $CD11b^+CD14^-CD15^+$, or $Lin^-HLA\text{-}DR^-CD33^+$ cells is observed in the peripheral blood of patients with malignant tumors.[49,53] In an *ex vivo* study using human blood or tumor samples of melanoma patients, MDSCs were shown to contribute more substantially to immunosuppression than TAMs.[54] Given that these cell types indicate immunosuppressive activity, they may correspond to the MDSCs that are found in mice. As differences in gene expression and cell markers exist between mice and humans, sufficient care must be taken when attempting to apply the results of mouse studies to humans.

Dendritic cells in human tumor tissues

Dendritic cells (DCs) serve as other myeloid lineage cells in the tumor microenvironment, and play a critical role in integrating both innate and adaptive arms of immune responses. Myeloid DCs (mDCs) and plasmacytoid DCs constitute two major subsets of the DC population, and are distinguished from macrophages according to their unique surface marker expressions. In human DCs, mDCs are further classified as blood dendritic cell antigen (BDCA)1$(CD1c)^+CD11b^+$ and BDCA3$(CD141)^+$ C-type lectin$(CLEC)9^+$ populations, which are equivalent to $CD11b^+CD4^{+/-}$ and $CD8\alpha^+$ or $CD103^+$ tissue-resident mDCs, respectively.[55,56] The $BDCA3^+$ mDCs are specialized for cross-presentation of antigens from necrotic cells, whereas $BDCA1^+$ mDCs have pleiotropic functions to prime diverse repertories of T cell subsets, in particular, dermal and mucosa-associated T cells.[56–58] Human plasmacytoid DCs are characterized for their expression of BDCA2(CD303) and CD123 (IL3Rα), and produce large amounts of type-I interferon in response to viral or self-nucleic acids.[54] As it is difficult to identify these molecules in paraffin-embedded pathological specimens, there are few articles describing DCs in human tumor samples. However, these phenotypic differences should help clarify the distinct functions and molecular pathways of TAMs and DCs in tumor tissues.

Targeting TAMs: a novel concept of anticancer therapy

As previously explained, TAMs promote tumor progression through induction of angiogenesis and suppression of antitumor immunity. In particular, in humans, protumoral TAMs are believed to exhibit characteristics similar to M2 macrophages, and are intimately involved in the progression of malignant tumors. As such, treatment strategies aimed at local inhibition of macrophage differentiation into the M2 phenotype are anticipated to be effective. Signal transduction path-

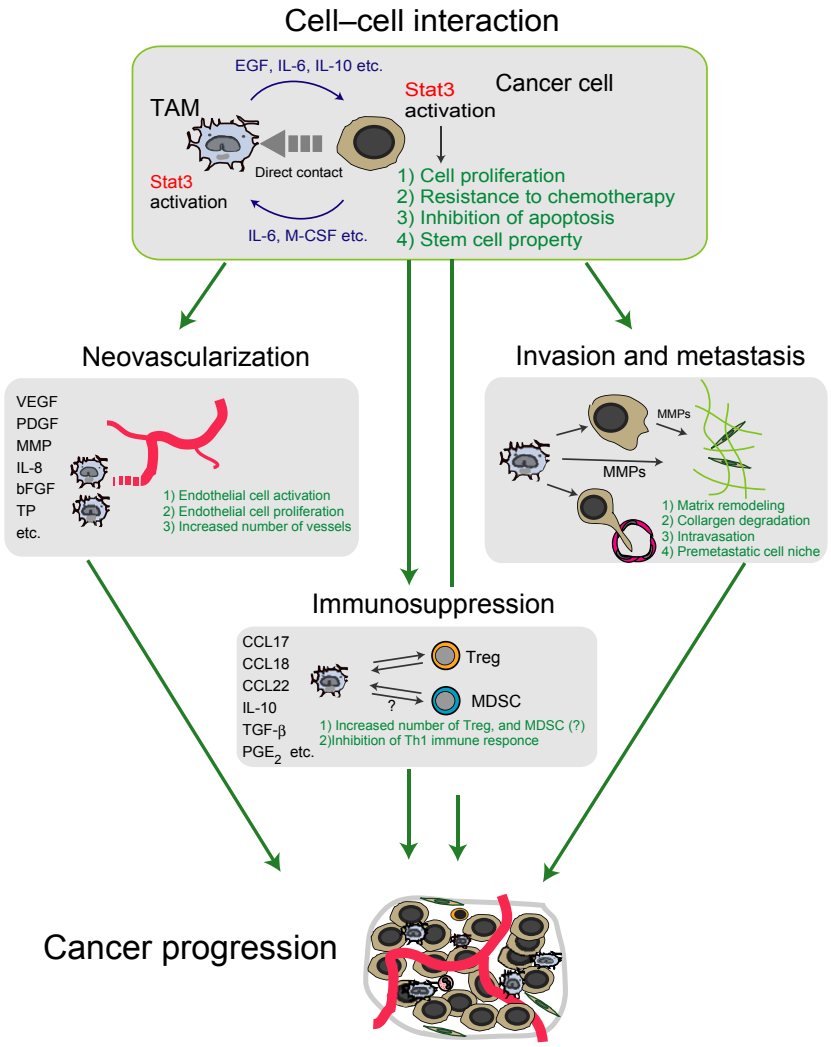

Fig. 2. Schema of the functional role of tumor-associated macrophages (TAMs). Tumor-associated macrophages are activated by macrophage colony-stimulating factor (M-CSF), interleukin (IL)-6, and other compounds secreted by tumor cells both to induce angiogenesis by producing angiogenic factors such as VEGF and platelet-derived growth factor, and to create immunosuppressive conditions by producing immunosuppressive factors such as IL-10 and prostaglandin E2 (PGE_2). At the same time, growth factors that are secreted by TAMs, such as epidermal growth factor (EGF), directly promote cancer cell growth, whereas MMP and other compounds responsible for stroma remodeling promote tumor cell infiltration and metastasis. Activation of tumor cells and TAMs induced by direct cell–cell interactions may represent an extremely important event in relation to the development of malignant tumors. bFGF, basic fibroblast growth factor; CCL, chemokine (C-C motif) ligand; MDSC, myeloid-derived suppressor cell; PDGF, platelet-derived growth factor; Stat3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β; TP, thymidine phosphorylase; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

ways, including nuclear factor (NF)-κB, Stat3, Stat6, c-Myc, and interferon regulatory factor 4, are involved in differentiation into the M2 phenotype.[44,59–61] Nuclear factor-κB and Stat3 are also strongly involved in tumor cell growth, and drugs targeting these molecules are currently being developed. Among such molecule-specific drugs, synergistic efficacy due to direct effects on tumor cells, as well as inhibition of the differentiation of TAMs into the M2 phenotype, is expected. Among drugs currently in use, some are active against TAMs. Cyclosporin A and trabectedin not only directly inhibit tumor cell growth, they also suppress activation of TAMs.[16,62] Bisphosphonates not only suppress bone resorption by osteoclasts, they also inhibit the differentiation of TAMs into the M2 phenotype.[63] The angiogenic inhibitor bevacizumab (a VEGF-inhibiting antibody) has recently been used to treat solid tumors such as colorectal adenocarcinoma, and this drug also exhibits antitumor activity by suppressing TAM migration.[64,65]

We developed a screening system of chemical compounds that suppress macrophage polarization toward the M2 phenotype. By screening a library of naturally occurring compounds, we have identified several compounds, including corosolic acid, that suppress M2 polarization of macrophages.[66] These compounds suppress Stat3 activation and NF-κB activation both in macrophages and tumor cells *in vitro*.[66] However, as the blocking effect of these compounds on Stat3 and NF-κB was not adequate in tumor cells, the direct effect on tumor cells was weaker than that of other anticancer drugs.[66] In an *in vivo* study, corosolic acid appeared not to directly suppress tumor cells, but rather to stimulate the antitumor immunity of lymphocytes by inhibiting the activation of TAMs and MDSCs.[67] Corosolic acid was therefore considered to show antitumor activity by means of indirect effects to myeloid cells.

Conclusion

With the recent introduction of the concept of macrophage differentiation into M1 and M2 macrophages, and clarification of the function of each of these cell types, the role of TAMs in malignant tumors is gradually emerging. Specifically, in human tumors, TAMs that have differentiated into the M2 phenotype act as "protumoral macrophages" and contribute to the progression of disease. Based on current basic research, TAMs that have differentiated into the M2 phenotype are believed to be intimately involved in angiogenesis, immunosuppression, and activation of tumor cells. Clarification of the mechanisms of TAM activation and the process of differentiation into the protumoral/M2 phenotype is anticipated to lead to new strategies for treating malignant tumors.

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Disclosure Statement

The authors have no conflict of interest.

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