

The roles of microglia/macrophages in tumor progression of brain cancer and metastatic disease

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1. ABSTRACT

Malignant brain tumors and brain metastases are highly aggressive diseases that are often resistant to treatment. Consequently, the current prognosis of patients with brain tumors and metastases is dismal. Activated microglia and macrophages are often observed in close proximity to or within the malignant tumor masses, suggesting that microglia/macrophages play an important role in brain tumor progression. Microglia, being resident macrophages of the central nervous system, form a major component of the brain immune system. They exhibit anti-tumor functions by phagocytosis and the release of cytotoxic factors. However, these microglia/macrophages can be polarized into

becoming tumor-supportive and immunosuppressive cells by certain tumor-derived soluble factors, thereby promoting tumor maintenance and progression. The activated microglia/macrophages also participate in the process of tumor angiogenesis, metastasis, dormancy, and relapse. In this review, we discuss the recent literature on the dual roles of microglia/macrophages in brain tumor progression. We have also reviewed the effect of several well-known microglia/macrophages-derived molecules and signals on brain tumor progression and further discussed the potential therapeutic strategies for targeting the pro-tumor and metastatic functions of microglia/macrophages.

2. INTRODUCTION

Malignant brain tumors and brain metastasis are often considered fatal in adults because of extremely poor prognosis and frequent tumor recurrence. Glioblastoma is the most common primary brain tumor in adults. In addition to glioblastoma, brain metastasis from other primary tumors including those from the lung, skin, and breast cancers also represent significant number of the central nervous system (CNS) tumors (1). It has been reported that 15–30 % of the patients with metastatic breast and lung cancers develop brain metastases (2). Even with treatments involving surgical intervention, irradiation, and chemotherapy, only a fraction of these patients with malignant brain tumor/metastasis survives longer than 2 years after diagnosis (1, 2). Recently, several drugs have shown to target neoplastic cells, which directly modulate the progression of brain tumor (3–5). Brain tumors develop a complex tumor microenvironment, which contributes to the development of drug resistance. In addition to tumor cells, non-neoplastic cells such as astrocytes, microglial cells, macrophages, endothelial cells, and lymphocytes are present in brain tumor microenvironment. Communications between the cancer cells and the non-neoplastic cells play critical roles in tumorigenesis and tumor invasion. Among the non-neoplastic cells, microglial cells and macrophages account for 30–50% of the total brain tumor mass (6, 7), suggesting that microglia/macrophages play a pivotal role in the tumorigenesis and metastasis. Most *in vitro* and *in vivo* studies have demonstrated that activated microglia/macrophages indeed accelerate growth and invasion of brain tumors (6, 8, 9). Although the depletion of microglia/macrophages does not significantly reduce the already existing tumors at the primary site in animal models (10, 11), a lack of microglia/macrophages has been observed to significantly affect metastatic spread of tumors (12–14), suggesting that these two types of cells play an essential role in the brain tumor invasion and metastasis. Therefore, understanding the mechanism of microglia/macrophage activation in the tumor microenvironment is essential for the development of novel anti-brain tumor therapies. In this review, we discuss the role and functions of microglia/macrophages in the maintenance and progression of malignant brain tumor.

3. ORIGINS OF MICROGLIA AND MACROPHAGES IN BRAIN TUMOR

Microglia, the resident macrophage of the CNS, is involved in immune surveillance and host defense against infectious agents and neoplastic tumors in the CNS. Under physiological conditions, microglia are in a resting state characterized by ramified morphology (15). After they have been exposed to infectious and traumatic stimuli, microglia rapidly change their morphology to a “amoeboid”

activated phenotype, alter gene expression and produce reactive oxygen species, and nitric oxide, pro-inflammatory cytokines and chemokines, which contribute to the clearance of pathogenic infections. However, prolonged and chronic microglial activation may result in pathological forms of inflammation that contribute to neurodegenerative and neoplastic diseases (16). Although activated microglia is believed to secrete cytotoxic factors that suppress or destroy pathogens and cancer cells, they are also capable of producing growth factors that promote cell survival, growth and enhance neuron function (17, 18).

Microglia was first characterized and reported in neural tumors by Rio-Hortegain in 1921 (6). However, later immunohistological studies have consistently revealed abundant infiltration of microglia/macrophages within the glioma and brain tumor tissues (19, 20). Moreover, the degree of activation of microglia/macrophages positively correlates with the grade of brain tumor (20), suggesting that the activated microglia/macrophages are associated with neoplastic progression.

Microglial cells and macrophages in the brain are derived from two different sources: 1) The parenchymal resident microglia and 2) Monocytes/macrophages that enter the brain from bone-marrow. Previous studies supposed that both the types of cells, microglial cells and macrophages, were myeloid-derived, based on the similarity in their surface markers and physiological functions. However, more recently, studies have demonstrated that microglia and macrophages are two distinct myeloid populations with different developmental origins (21–23). Animal studies showed that microglial cells originate from erythromyeloid progenitors that begin on embryonic day 7.5. (E7.5.)–E8.0. in the blood islands of the yolk sac. Until E9.5., erythromyeloid progenitors migrate to the developing CNS and mature into microglia (21–23). These early microglial cells reside in the brain throughout life and are thought to sustain the local microglial population. In contrast, macrophages originate from the hematopoietic stem cells that start in the aorta–gonad–mesonephros region at E10.5., and then in the fetal liver at E12.5. After the postnatal stage, macrophages are produced from monocytes in the bone marrow (fig1) (21–23). Recent studies demonstrate that microglial cells and macrophage have distinct and specific surface antigens too (24, 25). Studies on CX3CR1 (+/GFP)/CCR2 (+/RFP) knock-in fluorescent protein reporter mice demonstrated that microglial cells are CX3CR1⁺/CCR2⁻, while the macrophages are CX3CR1⁻/CCR2⁺ (24, 25), strongly supporting the notion that microglial cells and macrophages are from different populations.

In brain tumor and brain metastasis, microglial cells and macrophages are recruited either within or

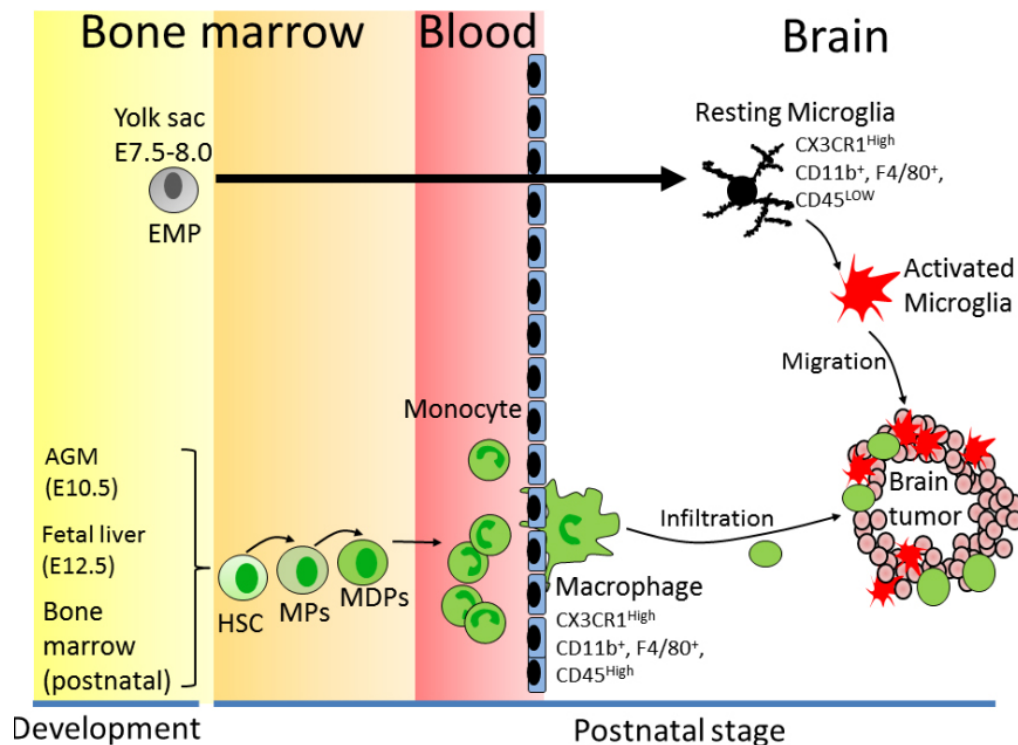


Figure 1. Different origin and lineage of microglial cells and macrophages in the brain. Microglial cells derive from erythromyeloid progenitors (EMPs) which locate in the yolk sac (embryonic day 7.5-8.0.). In contrast, brain macrophages derive from hematopoietic stem cells (HSCs), which begin at embryonic day 10.5. in the aorta-gonad-mesonephros (AGM) region and at embryonic day 12.5. in the fetal liver. After postnatal stage, HSCs generate monocytes from myeloid precursors (MPs) and macrophage and/or dendritic cell progenitors (MDPs) in the bone marrow. The mature monocytes infiltrate into different organs and differentiate to macrophage. In brain tumor microenvironment, tumor-released soluble factors recruit microglial cells and macrophages into the tumor site, which promotes tumor growth and metastasis.

in close proximity to the tumor masses. These tumor-associated microglia/macrophages can be detected by several biomarkers, including CD11b/c, CD163, CD200, CD204, CD68, F4/80, and the lectin binding protein Iba-1 (22, 23). Because there are no definitive markers to distinguish between these two cells, many investigators use the more general term “microglia/macrophages” instead of microglia or macrophage alone to describe them in the brain tumors. However, microglia and macrophages can be distinguished by the differential expression levels of certain cell-surface markers (26). Microglial cells are defined as CD45^{low} and macrophages as CD45^{high} expressing cells (16, 26, 27). Based on this observation, several studies investigated distinct populations of microglial cells and macrophages in the glioma tissue. Badie *et al.* performed flow cytometric analysis and characterized the distribution of microglial cells and macrophages in experimental gliomas and found that microglial cells (CD11b/c^{high}, CD45^{low}), mainly present at the site of tumor or tumor periphery, accounted for 13–34% of the tumor mass (27). By contrast, macrophages (CD11b/c^{high}, CD45^{high}) were less prominent within the tumors or the tumor periphery and accounted for just 4.2–12% of the tumor mass (27). These results suggest that microglial cells play a key role in mediating the tumor-related inflammatory response.

Although the reactive microglia/macrophage are frequently found both in primary brain tumor and metastatic brain tumor (28), the ratio of microglia/macrophage in these tumors is different. Previous studies showed that microglia/macrophages account for 4–70% of all cells in brain metastases, while 8–78% of cells in gliomas (29, 30). Microglia are the main responders to primary brain tumors, inhibition of microglial activation has been shown to significantly reduce glioma proliferation (6). Microglia-derived enzymes, cytokines, growth factors have been shown to directly lead to tumor proliferation and invasion, immunosuppression and angiogenesis in primary brain tumor (6). In contrast to these cancer-promoting effects, microglia has also been reported to elicit cytotoxicity toward lung cancer brain metastases in the early phase by the production of NO (31). Moreover, activated microglial cells are observed at different phase of primary and metastatic brain tumor. Microglial activation often occurs in the middle-phase of the primary tumor, whereas reaction of microglia/macrophages to metastatic brain tumor cells is usually immediate (28). The activated microglial cells have been found to be accumulated around single metastatic cancer cells that just started to extravasate into the brain from the blood stream on day 7 after intra-carotid artery injection of breast cancer cells (28),

suggesting that microglia participate metastatic cancer cell brain colonization in the very early steps.

4. CLASSICAL (M1) OR ALTERNATIVE (M2) MICROGLIA/MACROPHAGES IN BRAIN TUMOR

Microglia/macrophages can be differentiated into classical (M1) or alternative (M2) phenotype by microenvironment stimuli (32). M1 cells are activated by type I cytokines such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), lipopolysaccharide (LPS), and lipoproteins (33, 34). M1 microglia function as anti-tumor immune cells by producing pro-inflammatory cytokines and reactive oxygen species (ROS), and by expressing signal transducer and activator of transcription 1 (STAT1). The upregulation of STAT1 induces the inflammatory cytokines such as IL-1 β , TNF- α , iNOS, IFN- γ , and IL-12 that alter the function of DNA, RNA, and proteins or induce lipid peroxidation, which leads to tumor growth inhibition (16, 35, 36)._ENREF_2 The activated M1 microglia have antigen-presenting capabilities (37, 38) and are able to present antigen to Th1 cell to induce T-cell mediated cytolytic activity (38), causing tumor cell death. In benign or regressing tumors, the majority of microglia is found to be M1-like, which increases pro-inflammatory activity to promote tumor lysis and tumor killing (39–41). These M1 microglia reduce the sphereforming ability of cancer stem cells and suppress glioma growth (42), resulting in tumor inhibition. Several surface markers such as major histocompatibility complex (MHC) II, CD11c, CD74, and iNOS have been used to identify M1 microglia/macrophages (25). These M1 subtype cells also express interleukin (IL)-12^{high}, IL-23^{high}, and IL-10^{low}(35).

In contrast, M2 cells are activated by type II cytokines such as IL-4, IL-10, IL-13, and transforming growth factor- β (TGF- β) (43, 44). Chemokine stimuli including chemokine (C-C motif) ligands (e.g., CCL2, CCL17, and CCL22) and macrophage-derived chemokines can also promote M2 polarization (43). These M2 cells have a pro-tumor immune response by producing immunosuppressive factors (e.g., IL-10 and TGF- β) and exhibit a high level of intracellular STAT3 (16). STAT3 activation has also been associated with promoting immunosuppression (45). Activated STAT3 decreases the expression of surface molecules in microglia that are necessary for antigen presentation, such as MHC-II, CD80, and CD86, and also increases the expression of various M2-specific immunomodulatory mediators including IL-10, vascular endothelial growth factor (VEGF), and various matrix metalloproteinases (MMPs) (46, 47), promoting growth and invasion of the tumor. Moreover, STAT3 targets pro-proliferation genes (48) that may contribute to microglia proliferation in brain tumor. Several surface markers such as CD163, CD204 (16, 34, 49),

and arginase-1 (33) are present in M2 cells. These M2 cells promote the function of Th2 cells and frequently express IL-10, which is a strong anti-inflammatory mediator (9, 50). M2 microglia-derived IL-10 helps to create an immunosuppressive microenvironment to promote tumor survival (6, 8, 9). A recent study showed that co-culture of M2 macrophages with glioma cells significantly increased tumor proliferation when compared with co-culture with an M1 subtype, and this effect was suppressed by blocking the expression of STAT3 (51). These results suggest that the polarization of microglia/macrophage profoundly affects tumor growth in the brain (fig2).

Accumulating evidence suggests that microglia/macrophages in the brain tumor are skewed to the M2 phenotype and that the cytokines IL-4, IL-6, IL-10, and TGF- β (44, 52, 53), secreted from the tumor,_ENREF_8 induce M2 microglia/macrophage activation. Accordingly, high numbers of CD163⁺ and CD204⁺ microglia/macrophages are detected in glioma patients, (52) and their levels positively correlate with poor clinical prognosis of human glioma (51). By contrast, a higher ratio of CD74⁺ M1 cells is positively associated with better survival of human glioblastoma patients (54). Activated M2 microglial cells have been shown to promote colonization of breast cancer cells in the brain (13). In contrast, activated M1 microglial cells induced apoptosis of metastatic lung cancer cells *in vitro* (55). Both M1 and M2 microglia are detected within brain tumor mass. Even M1 microglia is able to suppress tumor growth and cause tumor cell death, the immunological functions of M1 microglia in the brain tumor including cytotoxicity, phagocytosis, and antigen presentation are impaired (56). Thus, metastatic tumor cells can escape the immune attack of M1 microglia and then colonize in the brain. The balance of upregulating M2 pro-tumor response and attenuated M1 anti-tumor immune response determines the promotion of tumor growth and invasion.”

5. CROSS-TALK BETWEEN MICROGLIA/MACROPHAGES AND TUMOR CELLS BY MULTIPLE FACTORS THAT STIMULATE BRAIN TUMOR PROGRESSION

Reactive gliosis including microgliosis is a hallmark of neurodegeneration, neuron injury and brain tumors (6, 57). In neuron, reactive microglial is known to lead local neuronal DNA damage and neuron death through secretion of proinflammatory mediators or ROS. By contrast, chronic reactive microgliosis increases production of cytokines and chemokines that accelerate growth and invasion of tumors. Reactive microgliosis creates favorable and more permissive brain microenvironment for promoting tumor growth/invasion. On the other hand, tumor-derived factors not only promote their own growth but also induce another wave of microglial activation, resulting in an autocrine self-propelling inflammatory response in the

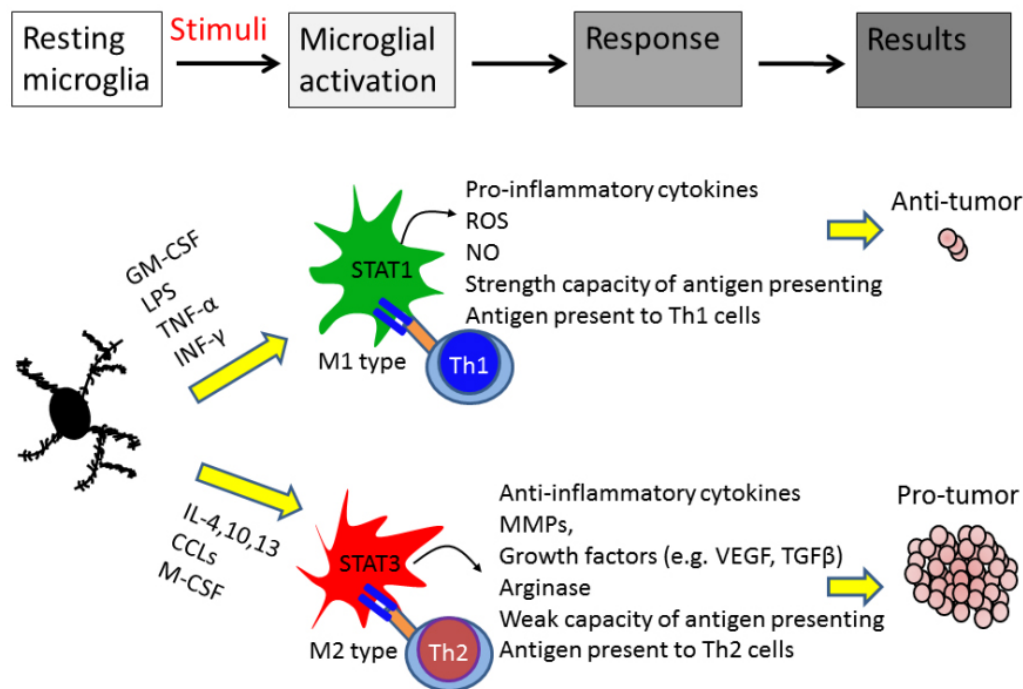


Figure 2. Differential roles of activated microglia/macrophages in the brain tumor. Microglia/macrophages have both pro- and anti-tumor potentials. In response to granulocyte-macrophage colony stimulating factor (GM-CSF), lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), and interferon- γ (INF- γ) stimuli, microglia/macrophage can be polarized to M1 phenotype. M1 cells exhibit anti-tumor immunity by producing cytotoxic factors and presenting tumor antigen to T helper type 1 (Th1) cells. STAT1 activation in M1 cells induces pro-inflammatory cytokines production and increases T-cell-mediated cytolytic activity, leading to tumor cell damage. In response to interleukin-4 (IL-4), chemokine (C-C motif) ligands (CCLs) and macrophage colony-stimulating factor (M-CSF), microglia/macrophages polarize into M2 phenotype. M2 cells express STAT3 that induces anti-inflammatory factors. M2 cells also modulate Th2 cells, which promotes tumor progression. In addition, M2 cells can promote tissue repair and angiogenesis, resulting in tumor progression.

brain. Here, we discuss several important molecules from microglia/macrophages and tumor cells that are involved in modulating reactive microgliosis in the progression of brain tumors (fig3 and tab1).

5.1. Cytokines/chemokines

Cytokines/chemokines and their receptors are thought to be important for trafficking immune cells in the peripheral nervous system (58). Although the identity of tumor-derived cytokines/chemokines that modulate the recruitment of microglia remains unclear, several common chemokines and receptors have been found to be up-regulated in brain tumors, including monocyte chemoattractant protein-1 (MCP-1), Granulocyte/macrophage-colony stimulating factor (GM-CSF), CX3CL1, and CCL (59). MCP-1/CCL2 is believed to be a major contributor in microglia/macrophage recruitment to gliomas and breast cancer brain metastases *in vivo* and *in vitro* (7, 59–64). MCP-1 is also responsible for increasing microglial proliferation in glioma (60). Brain tumor cell-derived MCP-1 binds to its specific receptor, CCR2, on the microglia, facilitating the recruitment of microglia into the tumor site (65). Expression of MCP-1 positively correlates with the higher grade of malignant glioma (66), suggesting that MCP-1 in the glioma not

only induces the recruitment of microglial cells into glioma but also increases tumor growth. MCP-1 is also implicated in breast cancer progression. A high level of MCP-1 in breast cancer cells was shown to promote migration and infiltration of macrophage into the brain through CCR2. Blocking CCL2 with neutralizing antibodies decreased macrophage infiltration and tumor growth in a mouse model of breast cancer (64, 67). GM-CSF has a similar effect as MCP-1 in enhancing microglial proliferation (68). In addition to GM-CSF, brain tumor tissues express high levels of the receptor of granulocyte-colony stimulating factor (G-CSF). Unlike GM-CSF, G-CSF does not promote microglial proliferation (68); however, it was shown to promote brain tumor proliferation by autocrine mechanisms (69). Glioma and breast tumor brain metastases exhibit a high level of mRNA and protein expressions of CX3CL1 (70, 71). By contrast, CX3CR1 expression was shown to be absent in tumor cells, but it was found to be expressed in microglia themselves that lacked expression of CX3CL1 (71). CX3CL1 functions as a chemoattractant for macrophages and microglial cells (72–74). Treating microglia with exogenous CX3CL1 increased the ability for migration and adhesion of microglia to glioma cell *in vitro*, whereas blocking of CX3CR1 by CX3CR1 antibodies reduced CX3CL1-induced adhesion ability

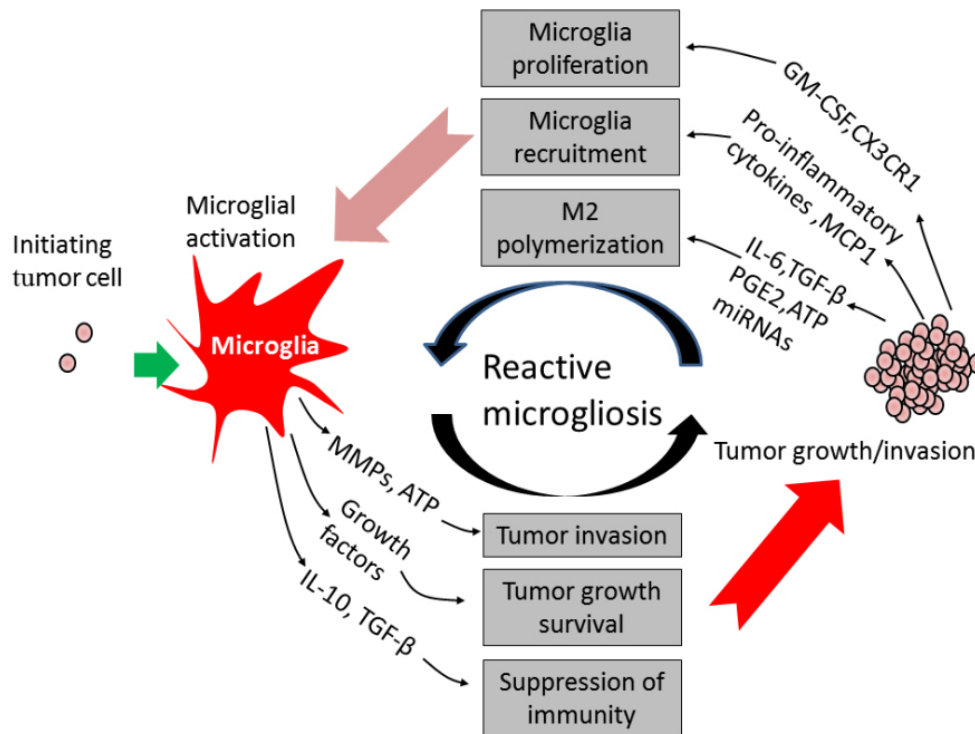


Figure 3. Reactive microgliosis promotes brain tumor progression. Microglial cells become hyper-activated through two mechanisms in brain tumor microenvironment. First, microglial cells become active, produce cytokines, growth factors and matrix-metalloproteases (MMPs) in response to initial tumor cell stimuli. Microglia-secreted factors then promote tumor growth and invasion. Second, tumor cells release growth, chemoattractant, and chemokine factors that recruit and induce another wave of microglial activation, resulting in a perpetuating cycle of microglia activation in the brain tumor. IL-6: Interleukin IL-6, IL-10: Interleukin 10, TGF-β: Transforming growth factor, PGE2: Prostaglandin E2, GM-CSF: granulocyte-macrophage colony stimulating factor, MCP-1: Monocyte chemoattractant protein-1, ATP: Adenosine triphosphate, miRNAs: microRNAs.

of microglia when compared to a control group (71). The expression of CX3CR1 is associated with tumor metastasis to the brain (75). Indeed, CX3CR1 was overexpressed in the brain metastasis of breast cancer patients (75), suggesting that CX3CR1 is associated with tumor metastasis to the brain. Increasing expression of CX3CR1 in the tumor mass is due to tumor-recruited microglia/macrophages because abundant microglia and macrophages are recruited within brain tumor mass (6, 7, 13, 76). Tumor-derived CX3CL1 attracts microglia/macrophage infiltration. These microglia/macrophages release cytokines and chemokines that promote the migratory ability of tumor cells to the brain. In view of the above facts, it is understood that these chemokines significantly increase the ability of microglia to promote the growth and invasion of brain tumor/metastasis, and therefore, these factors could serve as therapeutic targets.

The results of RNase protection assays showed that the expression of 53 genes encoding cytokines or cytokine receptors were altered in human glioma cell lines and brain tissue (77). *In situ* hybridization analyses showed that both—microglial cells and astrocytes—contribute to anti-inflammatory IL-10 gene expression in glioblastoma tissue (8). Furthermore, primary cells from human glioma

specimens showed that microglia/macrophages are the major sources of IL-10 expression in gliomas (9), suggesting that IL-10 secreted by microglia create an immunosuppressive microenvironment for growth of glioma. In addition, IL-6 secreted by glioma and microglia was shown to stimulate the production of MMP-2 that induces glioma-cell migration and invasiveness (78–81). Although tumor-derived soluble factors mainly induce polymerization of M2-like microglia/macrophages, some factors also induce a partial shift of microglia/macrophages toward the M1 phenotype in the brain tumor. Interestingly, several previous studies have shown that M1 specific markers or associated pathways are not only detected but also positively correlated with glioma growth, and that IL-1β and TNF-α directly promote glioma growth (25). In other studies, IL-1β and TNF-α were shown to stimulate brain microvessel endothelial cells, leading to increasing permeability of the blood-brain barrier (82–84) and immune cell infiltration from the peripheral system. However, several studies showed that M1 cytokines (e.g., IFN-γ, TNF-α, IL-1β, IL-2, and IL-12) and their receptors were virtually absent in glioma, brain metastatic cell lines, and in human tissues whereas M2 immunosuppressive cytokine (i.e. IL-6, TGF-β) were greatly predominant in these cells (77, 85–87). Therefore, in brain tumor, the balance of upregulating

Table 1. Tumor- and microglia-derived factors affect brain tumor progression and microglial polarization

Tumor-derived factor	Microglia-derived factor	Receptor	Outcome	Ref.
MCP-1	-	CCR2 in microglia	Recruitment of microglia	7, 59–67
GM-CSF	-	GM-CSFR in microglia	Microglial proliferation	68
G-CSF	-	GM-CSFR in microglia	Promoting brain tumor proliferation	68, 69
CX3CL1	-	CX3CR1 in microglia	Recruitment of microglia	70–75
IL-10	IL-10 (Major)	IL-10R in both	Immunosuppression, M2 activation, Promoting cancer stem cell growth	8, 9
IL-6	IL-6	IL-6R in both	Tumor migration, M2 activation	78–81
IL-1- β , TNF- α	IL-1- β , TNF- α	IL-1R, TGFR in both	Tumor growth, increasing BBB permeability	25, 82–84
Prostaglandin	Prostaglandin	PGER in both	Immunosuppression	88, 89, 91
-	TGF- β	TGFR in tumor	Stem cell proliferation, immunosuppression	90–94
TGF- β	-	TGFR in microglia	Recruitment of microglia	90
GDNF	-	GDNFR in microglia	Recruitment of microglia	95
HGF/SF	-	c-Met in microglia	Microglia migration	96
-	HGF/SF	c-Met in tumor	Angiogenesis	96–99
MMPs	MMPs		Tumor migration, invasion, infiltration of microglia, degradation of extracellular matrix, angiogenesis	100–105
-	MT1-MMP		Pro-MMP2 cleavage	
ATP	-	P2X7R in microglia	M2 activation, enhancing motility of microglial cells	106–116
microRNAs	-		Promotion or inhibition of tumor	117–129
-	miR-124		Microglia quiescence	127
Soluble factors	-	TLRs in microglia	Degradation of extracellular matrix,	131
S100B	-	RAGE in microglia	M2 activation, angiogenesis	132, 133
Wnt signaling	Wnt signaling	WntR in Both	Brain metastasis, cytokines production	13, 134–136
-	VEGF	VEGFR ⁺ in endothelial cells	Building functional vascular tubes	140–142

M2 pro-tumor response and attenuated M1 anti-tumor immune response determine the promotion of tumor growth and invasion.

5.2. Prostaglandin and transforming growth factor- β

Prostaglandin E2 and TGF- β are important immunosuppressants in brain tumor. Previous studies have shown that prostaglandin E2 inhibits the innate and adaptive immune responses of immune cells via the activation of adenosine monophosphate (88, 89). Both, glioma and microglial cells, are known to produce prostaglandin. Microglial cells are the major source of cyclooxygenase-2, which is the key enzyme responsible for arachidonic acid conversion to active prostaglandins (90, 91). Microglial activation increases the expression of cyclooxygenase-2, which may contribute to the increase in prostaglandin production in the brain tumor. Tumor-released TGF- β has been shown to recruit microglia/macrophage to glioma (90). Furthermore, microglia-derived TGF- β stimulates the migration of glioma cell (92) and increases the proliferation of CD133⁺ glioma cells (glioma stem-like

cells) (90), whereas the blocking of TGF- β abolishes the effects of microglia on glioma invasiveness (90, 91). Moreover, several reports have shown that TGF- β 1 is required for the maintenance of self-renewal property of glioma stem-like cells (93, 94), suggesting that TGF- β secreted from microglia/macrophage plays a role in the maintenance of cancer stem-like cells associated with growth, migration, and invasion of brain tumors.

5.3. Growth factors

Glial cell-derived neurotrophic factor (GDNF) secreted by tumor cells was identified as a strong chemoattractant for microglia. The downregulation of GDNF by siRNA in mouse glioma cells was shown to diminish the attraction of microglia, whereas the overexpression of GDNF promoted microglia-attraction of glioma cells (95). In addition to GDNF, other growth factors (e.g., hepatocyte growth factor/scatter factor (HGF/SF)) from tumor cells are able to chemotactically attract microglial cells *in vitro* (96). The glioma-released HGF/SF targets the transmembrane tyrosine kinase receptor, c-Met, on microglia, thereby

increasing the migratory ability of microglia in a co-culture model with glioma cells (96). This effect can be readily abolished with HGF antibody treatment (96), suggesting that the tumor-derived growth factors play a role in microglia chemotaxis. Interestingly, activation of HGF/SF and c-Met have been shown to promote angiogenesis by stimulating endothelial cell migration and proliferation (96, 97). Microglial cells also produce HGF/SF and express c-Met (96, 98, 99), which may contribute to angiogenesis in the brain tumor. These results provide a new insight into the role of growth factors not only in promoting tumor growth but also in enhancing tumor invasion and angiogenesis by modulating the activation of microglia.

5.4. Matrix metalloproteinases

MMPs are known to degrade extracellular matrix, which promotes tumor migration and invasion. The expression analysis of MMPs in the glioma model revealed that MMP-1, 2, 3, 8, 9, 13, and 14 genes are upregulated in the tumor and microglial cells. Among them, MMP2 is also one of the major proteases found in mouse and human gliomas (100, 101). Both, microglial and tumor cells produced MMP2. Secreted MMP2 is in a pro-form that needs to be cleaved to an active form by membrane type 1-matrix metalloproteinase 1 (MT1-MMP) to facilitate glioma cell motility. In the normal brain, MT1-MMP expression in microglia is relatively low and is detectable only in the white matter (102). However, the expression of MT1-MMP is elevated in the event of occurrence of brain tumors (103). Consistent with these results, 80% of brain metastasis from lung adenocarcinoma and 50% of that from breast cancer were positive in MT1-MMP immunostaining (104). Markovic and colleagues found that MT1-MMP was especially expressed in microglia that was in close contact with glioma cells, whereas glioma cells expressed MT1-MMP in brain tumor only at a lower level (103). Knockdown of MT1-MMP in microglia by shRNA effectively reduced the growth of glioma *in vivo*, suggesting that MT1-MMP expression in microglial cells plays an essential role in glioma progression. Furthermore, upregulating MMP-2 and MMP-9 induced degeneration and retraction of astrocytic end-feet (105), which increases the blood-brain barrier permeability, resulting in the infiltration of macrophages, T cells, and cancer cells into the brain parenchyma (19, 20). Thus, microglia can serve as activators for the degradation of extracellular matrix for primary brain tumor invasion and brain metastasis. Moreover, upregulating MMPs on microglia contributes to peripheral cancer and immune cell infiltration.

5.5. Cell-cell communication through extracellular vesicles

Tumor and microglia/macrophages are able to release extracellular microvesicles known

as exosomes into the microenvironment and circulation. The released exosomes may serve as carriers for cell-cell communication, which affects brain tumor progression/metastasis and controls microglia activation in an autocrine and paracrine fashion. Exosomes are ~40 to 100 nm in size and can encapsulate various molecules, including metabolites, proteins, and nucleic acids (106, 107). For example, the exosomal adenosine triphosphate (ATP), abundantly released from tumor cells, has been shown to bind to the purinergic receptor P2X₇ (P2X₇R) on the surface of microglia/macrophages, resulting in microglia/macrophage activation and the production of macrophage inflammatory protein-1 (MIP-1 α) and MCP-1 (108). Treatments with P2X₇R antagonists or oxidized ATP reduced the expression of MIP-1 α and MCP-1 in microglia/macrophages, while it suppressed brain tumor progression (108). Exosomal ATP was also found to induce microglia ramification (109), enhance the motility of microglial cells (110, 111), and promote M2 phenotype (112), but at the same time inhibit M1 phenotype activation (113). In addition to tumor cells, microglial cells produce and release ATP (114) which may directly induce microglial activation through an autocrine mechanism. The microglial cell-derived exosomal ATP stimulates the production of MCP-1 in tumor cells (115), which in turn promotes microglia cell infiltration into the tumor mass. ATP is a well-known mitotic factor for glioma cells that promotes tumor growth. Although high levels of ATP (5 mM) induce cell death in normal cells, glioma cells present resistance to death, induced by ATP stimulation (116). These results suggest that tumor cell- and microglial cell-derived exosomal ATP induces cell death of the normal tissue surrounding the tumors, which may potentially set the stage for tumor cells for rapid growth and invasion.

RNA molecules in exosomes include mRNAs, microRNAs (miRNAs), and long non-coding RNAs. MicroRNAs function as novel classes of oncogenes or tumor suppressors and are frequently located at the chromosomal fragile sites in cancer genomes (117, 118). For example, the exosomal miR-223 plays an oncogenic role by promoting the invasive potential of breast cancer cells (119). In contrast, other exosomal miRNAs including miR15b, miR124, miR-137, miR-146b, and miR-451 inhibit brain tumor invasion and regulate the tumor cell-cycle progression (120–124). Among these, miR-124 is the brain-enriched miRNA that modulates neuronal development, tumor progression, and microglial activation (125–127). Compared with that in the normal brain tissues, the expression of miR-124 is significantly down-regulated in brain tumor tissues and cell lines (122, 123, 128). Hongping *et al.* showed that the overexpression of miR-124 reduced tumor sphere formation, inhibited stemness, and suppressed tumor cell invasion (122). Interestingly, high expression of miR-124 is detected in resting microglia of the normal CNS, whereas it appears

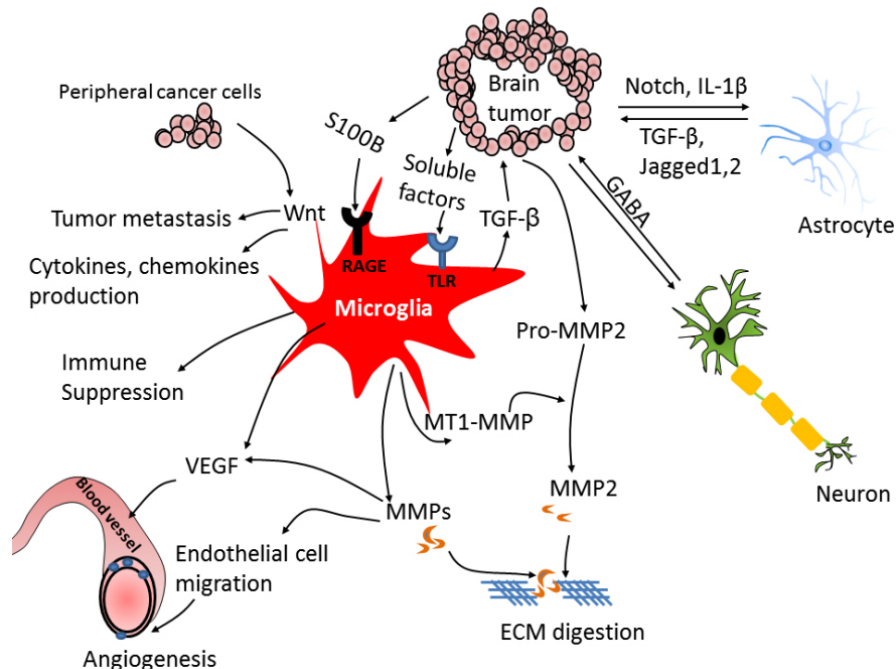


Figure 4. Cross-talk between tumor cells and resident cells in the brain. Various secreted soluble factors from tumor cell stimulate microglia and astrocyte activation. The tumor-derived soluble factors bind toll-like receptors (TLRs) on microglia that induces p-38 MAPK activation, resulting in up-regulation of matrix-metalloproteases (MMPs) and membrane type 1-matrix metalloproteinase (MT1-MMP). Microglia-released MMPs then cause extracellular matrix (ECM) digestion that promotes tumor invasion and macrophages/T cells infiltration. In addition, secreted transforming growth factor (TGF- β) from microglial cells triggers the release of pro-MMP2 from tumor cells which is cleaved into active form MMP2 by microglia-released MT1-MMP. Microglia-secreted vascular endothelial growth factor (VEGF) enhances angiogenesis. S100 calcium-binding protein B (S100B) induces receptor for advanced glycation end products (RAGE) activation on microglial cells that induces production of anti-inflammatory cytokines, leading to immune suppression. The metastatic tumor cell induces production of cytokines and chemokines in activated microglia via activation of Wnt signal. Upregulating Wnt signaling in microglia promotes tumor colonization and metastasis. Tumor cells induce astrocyte activation by production of interleukin-1 β (IL-1 β). Activated astrocytes release TGF- β , Jagged and other factors, which promotes tumor growth and mediates cancer stem cells self-renewal. Neuron-released neurotransmitter gamma-aminobutyric acid (GABA) promotes tumor progression. The interaction of tumor and resident cells induces multiple pathway activation that creates favorable microenvironment for tumor growth. BBB: blood–brain barrier, EGFR: epidermal growth factor receptor, HER: human epidermal growth factor receptor, IL: interleukin, JAG: jagged, MMP: matrix metalloproteinase, TGF: transforming growth factor, VEGF: vascular endothelial growth factor.

to be downregulated in activated microglial cells (127). Transfection of miR-124 in microglia/macrophage and animals directly inhibited the immunogen-induced microglia/macrophage activation and suppressed cytokine production (127). Furthermore, knockdown of miR-124 in microglia/macrophages resulted in microglia/macrophage activation *in vitro* and *in vivo* (127), suggesting that miR-124 could be a key regulator of microglia quiescence. In addition to tumor-derived miRNAs, astrocyte-secreted miRNAs also affected microglia activation. Loss of PTEN, an important tumor suppressor, in the tumor cells significantly increased IBA1⁺ expression on microglia, promoted tumor growth and elevated the risk of breast cancer brain metastasis through miR-19a secretion from astrocytes (129). These results suggest that miRNAs in the brain microenvironment mediate microglial activation and tumor progression.

Proteins in exosomes have also been shown to play critical roles in controlling microglia/macrophage activation. For example, heat shock

proteins (HSPs) such as HSP90, HSP70, and HSP32, induce the production of IL-6 and TNF- α , and increases the rate of phagocytosis in microglial cells (130). Taken together, exosomal miRNAs and proteins in brain tumor are important not only for promoting tumor progression/metastasis but also for modulating microglia/macrophage activation.

6. SIGNALING PATHWAYS OF MICROGLIA/MACROPHAGES FOR BRAIN TUMOR GROWTH AND INVASION

Reciprocal communications between microglia and tumor cells activate multiple key signaling pathways as summarized in fig4. Several important pathways that play pivotal roles in brain tumor growth and invasion are discussed below.

6.1. Toll-like receptor 2 signal

The soluble factors released from a glioma can be recognized by various Toll-like receptors (TLRs),

in particular TLR 2, on microglia/macrophage. Treating the primary microglial cells with a TLR 2 agonist was shown to up-regulate the downstream molecules, MyD88 and p38 MAPK, that promoted the productions of MT1-MMP and MMP-9, leading to glioma cell invasion due to the degradation of extracellular matrix (103). In addition, blocking TLR 2 on microglial cells or deletion of microglial MT1-MMP reduced MT1-MMP expression on microglia and impaired the growth of glioma (103), suggesting that the activation of the TLR 2 signal on microglia enhances brain tumor invasion. A more recent study has identified glioma-produced versican as an endogenous TLR 2 ligand that can trigger p38 MAPK signaling activation followed by increasing MT1-MMP production in microglial cells (131). Interestingly, microglia-released TGF- β was shown to induce the production of pro-MMP2 in glioma cells, which was subsequently cleaved into active MMP2 by microglia-secreted MT1-MMP. This positive circuit between microglial cells and tumor cells promotes brain tumor growth and invasion.

6.2. S100B-RAGE-STAT3 signal

M2 microglia polarization is important for maintaining local immunosuppression. Thus, upon attracting microglia, tumors establish an immunosuppressive microenvironment to promote their growth. Several lines of evidence indicate that the activation of S100B-RAGE-STAT3 signaling stimulates polarization of M2. Tumor-secreted S100 calcium binding protein B (S100B) activates receptor for advanced glycation end products (RAGE) on microglia, which induces STAT3 activation, resulting in the suppression of microglial M1 function, in turn reflected by the inhibition of TNF- α and IL-1 β production (132). Zhang *et al.* showed that blocking RAGE expression inhibited glioma-induced STAT3 activation and suppressed the production of M2-type cytokines such as IL-10 in microglial cells (132). Importantly, activated RAGE signal in microglia/macrophage not only maintains M2-like phenotype but also affects angiogenesis. Indeed, genetic depletion of RAGE in glioma cells and mice was shown to abrogate angiogenesis by downregulating the expression of VEGF (133). Reconstitution of RAGE knockout mice with wild-type microglia or macrophages normalized glioma vascularity, suggesting that RAGE signaling in microglia/macrophages was sufficient to promote angiogenesis in glioma (133).

6.2. Wnt signal

Tobias *et al.* demonstrated that activated microglia cells significantly promote colonization of breast tumor tissues in cancer metastasis (13). Moreover, the presence of microglia also enhanced the invasion rate of human and murine breast cancer cells in the brain, when compared to a control group (13). They have also shown that the direction of microglia

movement can serve as a guiding rail for malignant cells to move toward the neighboring tissue of tumor plaques (13). Interestingly, inhibiting microglia function by a Wnt inhibitor significantly diminished total invasion of the tumor cells (13). Wnt signal is essential for communication between the microglia/macrophages and brain-metastasis tumor cells (134–136). Microglial activation-promoting brain metastasis often depends on activation of Wnt signaling (13, 136, 137), and treatment of microglial cells with a Wnt antagonist completely abolishes microglia-induced tumor invasion (13, 136). On the other hand, treating microglia/macrophages with Wnt increases the production of IL-6, IL-12, TNF- α , and MMP via the activation of AP-1/c-Jun (136, 138). Consistent with this *ex vivo* study, the Wnt signaling is elevated in breast cancer patients with brain metastasis (139). Therefore, activation of Wnt signaling in microglia promotes brain metastasis in part by upregulating microglial cytokine production.

6.3. Angiogenic factor

For the metastatic spread of cancer cells, the growth of the vascular network is important in order to supply nutrients and oxygen to tumor cells to promote their growth and invasion. The effect of microglia/macrophages on angiogenesis has been well documented. Microglia/macrophages are found to be located at vascular branching points and release VEGF that stimulates and guides the VEGFR⁺ endothelial cells to build functional vascular tubes. Deletion of macrophages by a genetic approach significantly decreases the migration ability of endothelial cells and reduces branching in the vascular plexus (140). Microglia-derived MMPs have been shown to induce production of angiogenic factors and stimulate angiogenesis in glioma (141). Moreover, microglia-derived MMPs degrade the extracellular matrix, which allows endothelial cells to invade into tumor tissues during angiogenesis in glioma (141, 142). Therefore, activated microglial cells play a pivotal role in constructing abundant vascular networks that promote tumor growth.

6.4. Signaling in other brain residential cells

In addition to microglia, tumor cells communicate with other residential brain cells that contribute to tumor growth. Beside microglia, astrocytes are the most abundant glial cells in the brain. Cancer cells stimulate the production of pro-inflammatory cytokines in astrocytes, which also promotes tumor growth and metastasis. We have recently shown that tumor-produced prostaglandin could activate astrocytes to release CCL7, which in turn promoted self-renewal of the tumor-initiating cells (143). Furthermore, we showed that breast tumor cells in the brain express high levels of IL-1 β which activates astrocytes to upregulate Jagged-1 which in turn interacts with the Notch signal in cancer stem cells

(CSCs) and promotes self-renewal of CSCs (144). Similarly, the tumor-promoting effect of Notch and the jagged-2 pathway has also been explored in other brain-metastasis models (145). The effect of the neuron-secreted neurotransmitter gamma-amino butyric acid (GABA) on brain tumor has also been explored. High levels of GABA and its receptor are detected in both, primary brain tumor and brain metastatic tissue (146). GABA is converted to a succinate form by GABA transaminase, resulting in the subsequent production of NADH to satisfy the energy and growth requirements of neuron cells (147). GABA enhances proliferation of brain-metastatic cells, whereas GABA transaminase inhibitor abolishes the proliferative effect of GABA on breast tumor (146). Together, these findings suggest that, in addition to microglia, neurons and astrocytes communicate with tumor cells to create appropriate tumor microenvironment that would promote brain tumor growth and metastasis (fig4).

7. INTERACTION BETWEEN MICROGLIA CELLS AND CANCER STEM CELLS IN THE BRAIN

It is becoming increasingly clear that CSCs can drive tumor growth, invasion, and immune evasion. Brain tumors, especially glioblastomas, are believed to arise from glioblastoma stem cells (GSCs). High-density microglia/macrophages are detected in and around the GSC niche, suggesting that the inflammatory cells and inflammatory mediators may be indispensable components for GSCs growth. GSCs-secreted chemo-attractant factors recruit microglial cells into the tumor mass, while the recruited microglial cells release soluble factors that create a favorable microenvironment to help the growth and enhance the invasiveness of GSCs (28). Liang Yi *et al.* found that the GSCs had a stronger ability to recruit microglia/macrophages than other glioma cells (148). Compared to the non-GSC glioma cells, GSCs expressed 2- to 3-fold higher level of CCL2, CCL5, and CCL7, 7-fold higher level of VEGF, and nearly 50-fold higher level of neurotensin. Among these, VEGF can induce proliferation of microglia (149) and inhibit myeloid progenitor maturation to develop tumor-associated macrophages, which promotes malignant progression (150). Neurotensin increases the migratory ability of microglia (151). Furthermore, GSCs were found to affect the polarization of microglia/macrophage. Treatment of resting microglia/macrophages with conditioned medium from GSCs promoted polarization of microglia/macrophages to an M2 phenotype and inhibited the capability of phagocytosis (42, 131). Recently, GSC-secreted periostin was found to function as a new potent chemo-attractant to recruit macrophages through the activation of integrin $\alpha\beta 3$ signal (25). Importantly, inhibiting the $\alpha\beta 3$ signal by blocking peptides impairs macrophage recruitment and suppresses GSC invasion (25). Furthermore, periostin-integrin $\alpha\beta 3$ signal has been found to maintain the microglia/macrophages

phenotype at M2 subtype, which contributes to GSC growth in brain tumors (25).

Activated microglia/macrophage also affects the growth of GSCs. Microglia/macrophage-secreted IL-10 was shown to promote the growth of GSC (131). In addition, activated microglia/macrophages produce high levels of TGF- $\beta 1$, which induces MMP-9 production and increases GSC invasiveness, whereas knockdown of the TGF receptor reduces the invasiveness of GSCs *in vivo* (90). Moreover, IL-6 has also been identified as a growth factor for glioma stem cells (152), which suggests that microglia-derived IL-6 may promote GSCs growth.

A recent study showed that naïve microglial cells curb GSC invasion. Isolated microglial cells from non-glioma patients released MCP-1 and IL-8 that reduced the sphere-forming ability of GSCs and inhibited brain tumor growth, whereas isolated microglial cells from glioma patients were unable to do so (42). Supplementing GSCs with naïve microglial-conditioned medium caused cell cycle arrest and reduced proliferation of GSCs (42). Moreover, growth- and differentiation-related genes were significantly down-regulated in GSCs when they were treated with naïve microglial-conditioned medium (42). These results suggest that the crosstalk between GSCs and microglia/macrophages promotes GSC growth and invasion.

8. INHIBITING MICROGLIA ACTIVATION AS THERAPEUTIC STRATEGY FOR BRAIN TUMOR

As described above, brain tumor growth is dependent on various signal stimulations from the microenvironment and on the various secreted factors from microglia/macrophages that promote brain tumor growth, invasion, and colonization. Thus, inhibition of the microglia/macrophages-derived signals is considered a potential anti-neoplastic-targeted therapy to block the growth of brain tumor. Here, we discuss several approaches that target and modulate the functions and activation of microglia/macrophage.

8.1. Immunotherapies

Several immunotherapies are under development for the treatment of patients with brain tumor. A recent study showed that immunotherapy using activated natural killer (NK) cells in combination with the antibody mAb9.2.2.7. diminished tumor growth by inhibiting tumor proliferation and promoting apoptosis (153). Moreover, this approach effectively increased the expression of ED1⁺ and MHC class II⁺ on microglia, which increased the function of microglia for tumor antigen presentation and cytotoxicity (153). A recombinant immunotoxin

supplement was noted to block the folate receptor β on microglia/macrophages, causing depletion of microglia/macrophages and reduce glioma growth in nude mice (154). Likewise, ablation of CD11b⁺ cells in ganciclovir-treated CD11b-*HSVTK* mice decreased the brain tumor size and improved animal survival (155). Up-regulating M1 microglia/macrophage function can be an immunotherapeutic approach to enhance anti-tumor immunity in the brain. Indeed, IL-12, LPS, and INF- γ effectively increased microglial cytotoxicity and phagocytic activity that eliminated cancer cell growth *in vivo* (156–158). Likewise, stimulation of M1 microglia/macrophage by the TLR3 agonist poly (I: C) increased tumor cell death and inhibited growth and invasion of the tumor (159). In addition, inhibiting the CSF-1 receptor has been shown to reduce M2 macrophage polarization and inhibit tumor growth (160). These findings indicate that immunotherapies or the elevation of microglial cytotoxicity function may be an amenable therapeutic strategy to treat brain tumors.

8.2. Antibiotic interference

Antibiotic drugs can also be used as anti-glioma therapies by modulating microglia/macrophage function. Minocycline hydrochloride, a small, highly lipophilic antibiotic, has been shown to suppress tumor invasion by inhibiting the expression of MT1-MMP and p38 MAP kinase in microglial cells (161, 162). Furthermore, treating mice with cyclosporine significantly down-regulated the levels of IL-10 and GM-CSF, which in turn inhibited infiltration of microglia/macrophages and decreased glioma growth (34). Moreover, amphotericin B has been shown to enhance microglia/macrophage activation, which leads to the arrest of brain tumor-initiating cell cycle and inhibition of cell differentiation (42).

8.3. Drug delivery by microglia/macrophages

Taking advantage of microglial phagocytic properties, recent studies used nanoparticles to modulate the function of microglial cells in the tumor (163, 164). Cyclodextrin-based polymer nanoparticle (CDP-NP) is taken up by microglial cells with no toxicity. The CDP-NP-labeled microglial cells are found to surround the tumor, suggesting that these labeled microglia/macrophages could potentially be used as nanoparticle drug carriers into malignant brain tumors (163).

8.4. Radiation and antiangiogenic therapy

The radiation therapy (RT) is the front-line treatment for brain tumors and metastasis; however, it eventually fails owing to the recurrence of the tumor. Whole brain irradiation is accompanied with the production of hypoxia-inducible factor-1

and stromal cell-derived factor-1 (SDF-1), which enhances recruitment of macrophage and increases angiogenesis at the tumor-invasion front (165, 166). However, using a combination of RT with a small molecule inhibitor of SDF-1, AMD3100, abrogated tumor regrowth in nude mice by preventing RT-induced macrophage recruitment (166).

Antiangiogenic therapy is able to delay brain tumor progression, but the benefit of this approach is still limited. This is because antiangiogenic therapy induces recruitment of microglia/macrophages in the tumor bulk and infiltrative regions. (167, 168). Moreover, a clinical study showed that a higher population of microglia/macrophages correlated with poor survival after anti-VEGF therapy in glioma (167). This suggests that microglia/macrophages may participate in escaping from RT and antiangiogenic therapy, which results in tumor recurrence. Thus, microglia/macrophages may be potential biomarkers for predicting resistance to RT or as a treatment target for recurrent brain tumor.

9. CONCLUSIONS AND PROSPECTIVES

Tumor-secreted soluble factors induce microglial activation and recruit microglial cells into the tumor site in the brain, while these secreted factors also modulate the immune function of microglia. Activated microglial cells release multiple pro-tumor factors, which in turn promote tumor growth and invasion. It is now evident that the paracrine loops between cancer and microglial cells profoundly affect microglial pro- and anti-tumor functions, resulting in brain tumor promotion or inhibition. However, there are still many unsolved questions. For example, the crucial factors and mechanisms that mediate the interaction of microglia/macrophages with cancer cells in the brain tumor/metastasis remain largely unknown. Likewise, the interaction of microglia/macrophages and other microenvironmental cells in the brain (e.g., astrocyte) might contribute to tumor growth, but the detailed mechanisms involved in their communications remain unclear. Although microglial cells and macrophages are recruited into the tumor mass and promote tumor growth, whether they execute distinctively different functions in brain tumor progression is still unknown. In addition, brain metastasis is a multistep process; we do not know how metastatic cells escape the immune attack of microglia/macrophages to colonize the brain microenvironment. These unanswered questions need further investigation, and understanding the role of microglia/macrophages in brain tumor could contribute to the development of new therapy for brain tumor. The functional impairment of microglia/macrophages occurs in the early stages of brain tumor/metastasis; therefore, early intervention by microglia/macrophage activation may also provide a potential therapeutic direction in brain tumor/metastasis.

10. ACKNOWLEDGMENTS

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