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The molecular profile of microglia under the influence of glioma

Wei Li and Manuel B. Graeber

Brain Tumor Research Laboratories, The Brain and Mind Research Institute, University of Sydney, 94 Mallett St, Camperdown, Sydney, NSW 2050, Australia (W.L., M.B.G.)

Microglia, which contribute substantially to the tumor mass of glioblastoma, have been shown to play an important role in glioma growth and invasion. While a large number of experimental studies on functional attributes of microglia in glioma provide evidence for their tumor-supporting roles, there also exist hints in support of their anti-tumor properties. Microglial activities during glioma progression seem multifaceted. They have been attributed to the receptors expressed on the microglia surface, to glioma-derived molecules that have an effect on microglia, and to the molecules released by microglia in response to their environment under glioma control, which can have autocrine effects. In this paper, the microglia and glioma literature is reviewed. We provide a synopsis of the molecular profile of microglia under the influence of glioma in order to help establish a rational basis for their potential therapeutic use. The ability of microglia precursors to cross the blood–brain barrier makes them an attractive target for the development of novel cell-based treatments of malignant glioma.

Keywords: alternatively activated macrophage, glioma-infiltrating microglia/macrophages, immunosuppression, synthetic biology, tumor-associated macrophages.

Malignant gliomas are among the most common brain tumors. They are characterized by morphological and genetic complexity, and they infiltrate diffusely into normal brain parenchyma.[1] This feature renders all current therapeutic strategies ineffective, and patients show significant mortality, with a life expectancy of only 14 months on average from the time of diagnosis in the case of glioblastoma multiforme (GBM) despite multimodal therapy.[2–5] Furthermore, conventional strategies are limited by nonspecific damage to surrounding normal brain tissue.[6] A wide range of other therapeutic strategies, including immunotherapy and gene therapy, have been tried or are under evaluation. However, until today none of these has proven effective. As a result, there is a pressing need for better therapies that enable precise targeting of glioma cells while sparing the neighboring normal tissue.

Within a glioma, microglia/macrophages make up the largest population of tumor-infiltrating cells, contributing at least one third of the total tumor mass.[7–10] These glioma-infiltrating microglia/macrophages (the macrophage phenotype may predominate) are present in both intact glioma tissue and necrotic areas,[8] and their density in gliomas is positively correlated with glioma grade and invasiveness.[11,12] There is compelling evidence that microglial cells are involved in creating a microenvironment that favors glioma growth.[11–16] Specifically, glioma invasion[12,17–19] and the establishment of an immunosuppressive milieu[20,21] are facilitated by the presence of intratumoral microglia. However, the precise molecular mechanisms underlying these phenomena have remained unclear. Elucidation of the molecular profile of microglia, including the receptors they express and the molecules they release in response to the microenvironment created by glioma, will not only help to unravel the role of microglia in glioma, but also lead to the design of more effective treatment options for these fatal tumors. In this review, we detail the known molecular mechanisms that govern microglia–glioma interactions.

Glioma and Microglia: Worse than a Symbiosis

The presence of microglia in brain tumors was first reported by Rio-Hortega and Asua in 1921,[22] and Penfield in 1925[23] provided the first detailed description of "microglia and the process of phagocytosis in gliomas." However, the true extent of microglial infiltration in both animal and human glioma was not widely appreciated until recently.[9,10,24–29] The initial actions of glioma-infiltrating microglia, as the resident

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Corresponding Author: Manuel B. Graeber, Barnet-Cropper Chair of Brain Tumor Research, The Brain and Mind Research Institute, University of Sydney (manuel@graeber.net).

macrophages of the CNS, would be expected to be migration to the tumor site for rescue and display of properties similar to peripheral macrophages, such as phagocytosis, antigen presentation, and release of cytokines/chemokines as well as cytotoxins.[30–33] However, such glioma-cytotoxic effects of microglia have been observed in vitro only.[34,35] Most in vivo studies report that microglial cells actually promote glioma cell migration and growth.[10,16] Interestingly, a recent study by Voisin et al. found that microglia were in an activated state with phagocytic activity within the first 3 h of co-culture with C6 glioma cells.[36] However, these microglia lost their phagocytic properties when in longer contact with glioma cells, ie., after 6 h of co-culture.[36] This and other experimental work[16] have provided evidence in support of the view that the defense and immune functions of microglia are suppressed and controlled by the glioma. Other studies even suggest that in case of glioma, microglia acquire a distinct phenotype that is induced by the tumor cells and is different from the inflammatory phenotype.[11,12,37] Microglia under the influence of glioma do not release pro-inflammatory cytokines that could help fight tumors; instead they exhibit upregulation of metalloprotease-II, which facilitates tumor invasion.[38] Moreover, they have been found to secrete matrix metalloproteinase (MMP)–9,[39] epidermal growth factor (EGF),[40] and vascular endothelial growth factor (VEGF),[41,42] which are all known to promote tumor proliferation. In addition, microglia in glioma release interleukin (IL)-10, which helps to create an immunosuppressive microenvironment.[7,20,21,43] In turn, receptors expressed by glioma-infiltrating microglia including EGF receptor (EGFR) and Met, enable them to receive signals from the tumor, via EGF and hepatocyte growth factor/scatter factor (HGF/SF), which further increases glioma invasion and migration. Gliomas also promote the recruitment and proliferation of microglia by releasing monocyte chemoattractant proteins 1 and 3 (MCP-1 and MCP-3), granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF).[44–46] Even the armament of microglia is tactically employed by glioma cells to facilitate their survival, growth, and spread.[47] Therefore, the close physical association of microglia and tumor cells in a glioma seems to suggest a symbiotic relationship, but it is actually highly skewed to the advantage of the glioma. As a result, microglia that infiltrate a glioma lose their defense and immune functions.

Sources of Microglia in Glioma

Developmental Aspects

The origin of microglia and whether and how they renew in healthy adult brain have been controversial for more than a century. A recent experimental study by Ginhoux et al.[48] appears to have brought previous long-running disputes to an end. Based on the expression pattern of the runt-related transcription factor 1 (Runx-1), Ginhoux and colleagues used a genetic pulse-labeling strategy to identify yolk sac macrophages between embryonic days 6.5 and 10 and found that adult microglia arise from yolk sac macrophages present between embryonic days 7.25 and 7.5 and enter the embryo right after vascularization at day 8, before primitive or definitive hematopoiesis starts in the embryo. These experiments establish that during development, microglia originate from a single population of extra-embryonic yolk sac macrophages, which are distinct with respect to the postnatal hematopoietic origin of other tissue macrophages and the fact that their numbers in the adult brain are maintained independently of circulating hematopoietic cells. Also in contrast to monocytes, microglia seem to depend during development on growth factor receptor colony-stimulating factor (CSF)–1R and its recently identified ligand, IL-34,[49] rather than CSF-1, in keeping with the presumed distinct ontogeny of microglia.[48]

Origin of Tumor-Associated Macrophages and Microglia

Despite the fact that the vast majority of microglia in the healthy adult brain have to be considered yolk sac–derived, there remains the burning question as to whether microglia in the diseased brain can develop at least in part from other sources, such as bone marrow. This is of great interest because there may be functional differences between microglia from different sources and, consequently, different treatment targets once cellular and genetic therapies for glioma have become realities. The idea of a possible "on-demand influx" of bone marrow–derived microglia precursors into the diseased brain is in keeping with the observation that microglia express several properties usually seen in stem cells and bone marrow progenitors. Both in vivo and in vitro studies on microglia that proliferate under pathological conditions demonstrate that microglia in diseased CNS harbor at least one phenotypic marker of bone marrow myeloid progenitors: CD34, the stem and progenitor cell antigen.[50,51] Moreover, a varying degree of recruitment of bone marrow–derived precursors that colonize the CNS and transform into ramified microglia under different pathological conditions has been observed, in experimental models of stroke,[52] brain ischemia,[53,54] multiple sclerosis,[55] Parkinson's disease,[56] Alzheimer's disease,[57] and others.[58–60] Notably, such microglia engraftment originating from bone marrow precursors in the adult diseased brain not necessarily associated with disruption of the blood–brain barrier (BBB) occurs only under defined host conditions. The use of irradiation chimeras for assessing microglia turnover has been questioned because irradiation itself can damage the BBB and augment or even cause cell infiltration.[61] A recent study by Alshakweer et al. has provided evidence in support of the view that bone marrow–derived microglia give rise to tumor-infiltrating microglia/macrophages in pilocytic astrocytoma in the absence of irradiation.[62] This finding is matched by experimental results demonstrating that both bone marrow–derived macrophages

and resident microglia invade glioma.[63] Thus, microglial cells populating glioma indeed originate from at least 2 sources: intrinsic parenchymal microglia and microglia freshly derived from their bone marrow precursors in the blood.[8] It should be noted that polarization properties may specifically distinguish the 2 populations of cells.[64]

Whether tumor stem cells in glioma can give rise to intratumoral microglia/macrophages potentially representing a third source is unknown at present.[65] Microglia in nondiseased CNS should be referred to as resident or ramified microglia rather than resting[66] microglial cells.

Glioma: Taking Control of the Microglia

Glioma-Derived Factors Contributing to the Establishment of an Immunosuppressive Tumor Environment

Transforming growth factor–β.— TGF-β is the best-characterized soluble immunosuppressive cytokine secreted by gliomas. TGF-β protein exists in at least 3 different isoforms in humans: TGF-β1, TGF-β2, and TGF-β3. These isoforms utilize different cell surface mechanisms to elicit distinct intracellular responses.[67] TGF-β isoforms 1–3 are differentially expressed in glioma cell cultures[68,69] and human glioma tissues,[70] with TGF-β2 as the most abundant isoform upregulated in GBM.[69,71] TGF-β2 has been implicated in glioma-associated immunosuppression,[71] whereas TGF-β1 has been shown to act as a stimulator of glioma cell motility.[72] Significantly, TGF-β inhibits development and activation of antigen-presenting cells, including microglia, represses natural killer (NK) cells, and prevents the activation and differentiation of cytotoxic T cells.[73–76] The mechanism underlying TGF-β–evoked immunosuppression may include the downregulation of major histocompatibility complex (MHC) class II antigen expression on microglial cells,[77] as well as deactivation of microglia as phagocytic cells.[78] It is thus believed that glioma-derived TGF-β induces glioma-infiltrating microglia to shift to a distinct tumor supportive phenotype.[79] Recently, Crane and colleagues[80] reported that TGF-β downregulates the activating receptor NKG2D, which is expressed by NK cells and $CD8^+$ T cells and has a role in the specific killing of transformed cells in glioma patients. In addition to its immunosuppressive function, glioma-derived TGF-β promotes tumor cell migration and invasion by inducing MMP expression, suppressing expression of the tissue inhibitor of metalloproteinase,[81–83] assisting neovascularization of tumor tissue by TGF-β–mediated expression of angiogenic factors, such as VEGF and fibroblast growth factors (FGFs),[84–86] and stimulating tumor cell proliferation by means of increasing expression of EGFR.[87] A recent study supported the invasion-promoting role of TGF-β by showing that glioma-initiating cells (glioma stem cells) pretreated with TGF-β signaling inhibitor were less aggressive and showed less lethal potency in an intracranial transplantation assay.[88] This finding was confirmed by an in vivo study using neutralizing TGF-β antibody that was shown to reduce invasion of the glioma cells into the adjacent normal brain.[89]

Prostaglandin E_2.— PGE_2 is a small lipid-soluble molecule that is produced by both immune-competent cells and tumor cells. Glioma cells have been shown to release significant amounts of PGE_2 in vitro and in vivo, compared with PGE_2 synthesis in normal brain.[90–92] Importantly, elevated levels of PGE_2 in glioma were found to downregulate the activity of lymphokine-activated killer (LAK) cells[93] and the surface expression of MHC class II, human leukocyte antigen (HLA)–DR, on antigen presenting cells such as microglia and dendritic cells.[94,95] Moreover, the increased production of PGE_2 by glioma is also associated with suppression of T-cell activation and proliferation.[96,97] Regulatory T cells are induced by PGE_2.[98] In sum, PGE_2 plays an important role in the generation of an immunosuppressive milieu in glioma. Furthermore, PGE_2 promotes glioma cell proliferation via a signaling pathway involving activation of protein kinase A.[99,100] With regard to the cellular source of PGE_2 in glioma, microglia have been found to produce PGE_2 when co-cultured with glioma cells or conditioned glioma medium, strongly suggesting that microglia contribute to local immunosuppression by glioma.[101] PGE_2 biosynthesis is regulated by inducible membrane-associated PGE_2 synthase cyclooxygenase-2 (COX-2) and microsomal PGE synthase (mPGES)–1.[102,103] Abnormal expression of COX-2 and mPGES-1 has been detected in human glioma,[100,104] and conditioned glioma medium was found to enhance the expression of COX-2 and mPGES-1 in microglial cells.[101] Thus, the mechanism underlying the elevated level of PGE_2 in glioma could be related to the increased production of COX-2 and mPGES-1 in microglia, although the exact mechanism has remained obscure.

Fas ligand.— FasL, or CD95L, is a 42-kDa transmembrane protein that belongs to the TNF family. When bound to its receptor, Fas (CD95/APO-1), FasL initiates an intracellular signaling cascade that leads to the induction of apoptosis in Fas-expressing cells. Fas–FasL ligation has been suggested to be one of the main pathways mediating programmed cell death in a variety of cell types.[43] FasL has been detected on the surface of human glioblastoma cells and was also found to be expressed by rat glioma cell lines 9L, F98, and C6.[105] Both microglia and activated T cells express Fas and thus may be susceptible to a death signal delivered by functionally active FasL expressed on astrocytoma cells.[106–108] In line with this, Jansen and colleagues[105] recently reported that FasL was responsible for the death of T lymphocytes when co-cultured with glioma cells in vitro. These authors also demonstrated that downregulation of FasL expression in glioma cells enhances tumor infiltration of T cells and inhibits tumor growth in vivo.[105] Thus, it is now recognized that FasL expressed in tumors contributes to local immunosuppression and evasion of immune surveillance by inducing apoptosis of Fas-expressing T cells. In contrast, there is so far no

evidence that glioma-derived FasL induces the apoptosis of microglial cells despite the presence of Fas on microglia.[106] In addition, FasL also functions in the regulation of glioma motility and invasion. Blocking of Fas signaling has been found to impair MMP-2 activity, resulting in a reduction of glioma invasiveness and motility.[109] Moreover, Choi and colleagues[110] found that Fas ligation induces expression of intercellular adhesion molecule 1 (ICAM-1) in human astrocytoma cells and postulated that FasL may induce angiogenesis in glioma. Glioma cancer stem cells (gCSCs) were found to be resistant to Fas-induced apoptosis, suggesting that gCSCs are an important factor of resistance to chemotherapy.[111] Therefore, targeting endogenous FasL in glial malignancies could enhance the efficacy of immune-based treatment strategies.

Role of Signal Transducer and Activator of Transcription Protein 3 in Mediating Immunosuppressive Effects in Glioma

Signal transducer and activator of transcription protein (STAT)3 is a member of a transcription factor family, which is encoded by the STAT3 gene in humans. STAT3 activation causes expression of genes that play important roles in mediating the signals of cytokines and growth factors involved in cell growth, proliferation, differentiation, and apoptosis.[112,113] STAT3 was first implicated in glioblastoma pathology through studies demonstrating that STAT3 is constitutively activated in GBM cell lines as well as human GBM tissue.[114–117] STAT3 activation has been found to reduce the expression of MHC class II molecules, CD80, and CD86 in hematopoietic cells and glioma-infiltrating microglia.[118] In addition, STAT3 activation has been implicated in inhibiting the T-cell response against glioma.[119] When STAT3 activation was inhibited, gCSC-induced IL-10 production in glioma-infiltrating microglia was reduced, and gCSC-dependent inhibition of phagocytosis by glioma-infiltrating microglia was also reversed.[79] STAT3 thus appears to be a key mediator of immunosuppression in glioma. Apart from its role in immune evasion, STAT3 also contributes to gliomagenesis and progression. STAT3 activation is required for glioma growth[120] and for proliferation and maintenance of gCSCs.[121] Inhibition of STAT3 has been shown to suppress proliferation and growth and to induce apoptosis in glioblastoma cells both in vitro[122] and in vivo.[123] Moreover, inhibition of STAT3 has also been found to enhance radiosensitivity of glioma.[124]

Many factors found in glioma, including IL-10, IL-6, EGF, and FGF, are activators of STAT3. IL-6 was found to induce STAT3 activation in glioma cell lines[125] and in mouse models.[126,127] Furthermore, oncostatin M, a member of the IL-6 cytokine family, increases the STAT3-depedent expression and activation of VEGF and MMP-9 in human astrocytoma cell lines.[128,129] It is thus reasonable to postulate that IL-6 derived from glioma cells functions in tumor invasion and angiogenesis, at least in part through its activation of STAT3. There is also evidence to support a role for EGFR in glioblastoma-associated STAT3 activation. EGFR is amplified in approximately 50% of GBMs, and in about 50% of these amplified cases the GBMs express EGFRvIII, a mutant EGFR that lacks a portion of the extracellular ligand-binding domain. EGFRvIII was shown to be persistently autophosphorylated at low levels,[130] resulting in inefficient EGF signal attenuation and persistent activation of downstream kinase pathways, including those involving STAT3, Ras/MAPK, and Akt.[131] Moreover, as a downstream transcriptional target of FGF receptor, STAT3 can be activated by FGF via the FGF receptor.[132] Interestingly, STAT3 target molecules, such as IL-10, can also activate STAT3 in various cells. Therefore, a feed-forward mechanism appears to account for the constitutive activation of STAT3 in glioma cells and glioma-infiltrating microglia so that these STAT3-regulated molecules and STAT3-regulating molecules continue to accumulate. However, gene mutations of STAT3 have not been detected in glioblastoma. Aberrant STAT3 activity may also be caused by dysregulation of upstream tyrosine kinases or loss of negative feedback.[133] Therefore, in spite of the fact that multiple cytokines and growth factors may contribute independently to glioblastoma pathology, substantial data gathered from cell lines, rodent models, and patient samples support a role for STAT3 as a critical "molecular hub" that links multiple pathways involved in glioblastoma pathology. [133]

Polarization of Microglia (M2)

Heterogeneity of macrophage activation provides a basis for the conceptual classification of macrophages into 2 polarized functional categories: M1 (classically activated macrophages) and M2 (alternatively activated macrophages). As illustrated in Fig. 1, these 2 types of macrophage functional states differ in terms of activating signals, cytokine/chemokine production, receptor expression, and biological effects. Originally, macrophages were found to be activated by exposure to microbial products, such as lipopolysaccharides, along with exposure to interferon gamma (IFNγ). Activated type 1 polarized macrophages are characterized by preferential production of high levels of pro-inflammatory cytokines, such as IL-12, and oxidative metabolites, such as nitric oxide (NO),[134] and by displaying elevated expression levels of MHC class II and co-stimulatory molecules CD80 and CD86.[135] Thus, these macrophages, which are also referred to as classically activated macrophages, exhibit potent microbicidal/tumoricidal activities but also cause damage to healthy tissue as a side effect.[136] The first hint to the existence of alternatively activated macrophages came in the early 1990s, when Gordon and colleagues[137] treated macrophages with IL-4 and IL-13 in a study examining the regulation of mannose receptor expression on elicited macrophages. They observed signs of macrophage activation; however, these macrophages exhibited diverse biological properties that were clearly distinct from those of their classically activated

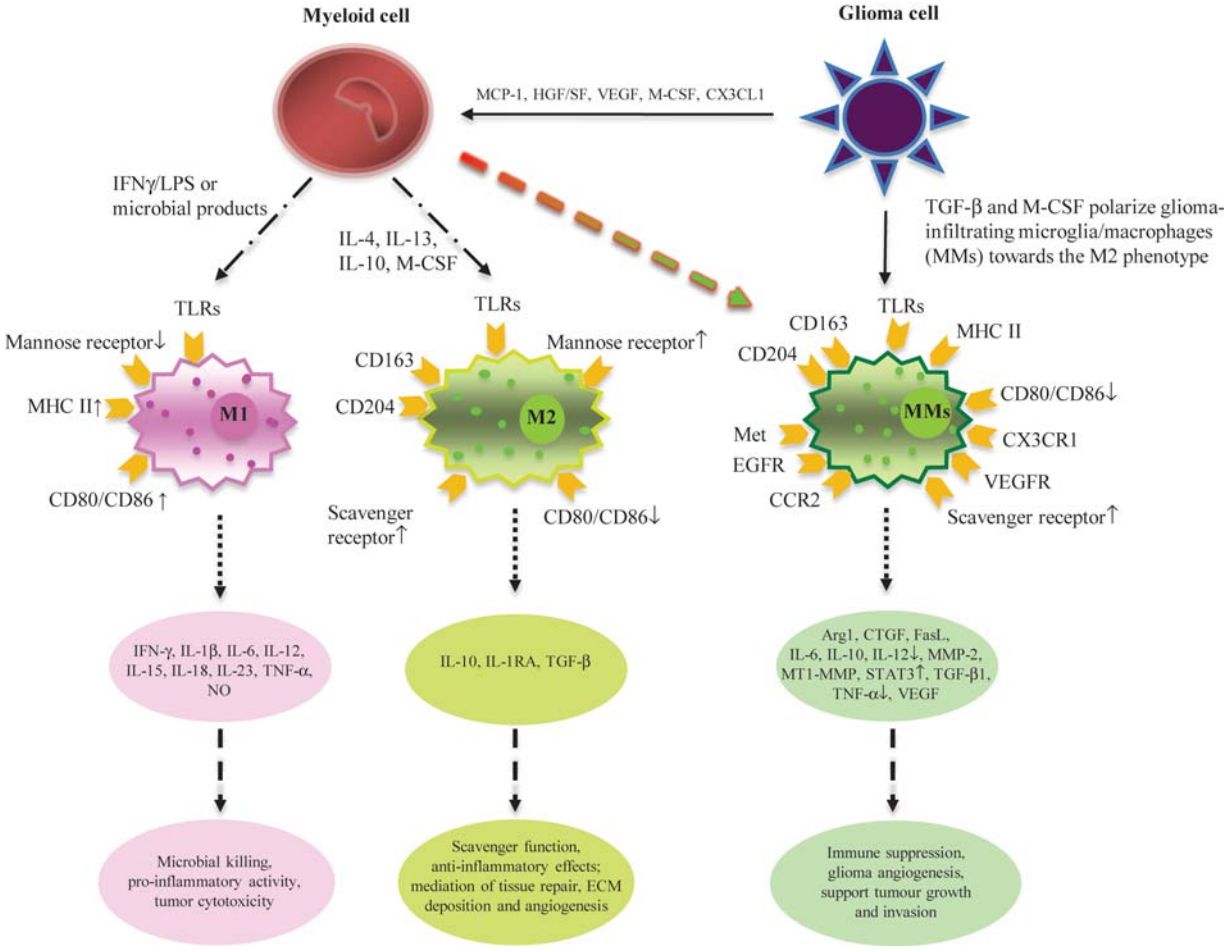

Fig. 1. Microglia in glioma are polarized. **M1** (classically activated macrophages) and **M2** (alternatively activated macrophages) differ with respect to activating signals, receptor expression, cytokine/chemokine production, and biological behavior. When mononuclear/phagocytic cells are stimulated by IFNγ, lipopolysaccharides, and other microbial products, they differentiate into the M1 phenotype. Microbial products are recognized by PRRs on the surface of M1, such as TLRs, and stimulate the production of pro-inflammatory cytokines as well as the expression of receptors that are involved in antigen presentation. When mononuclear/phagocytic cells are activated by IL-4, IL-13, IL-10, and M-CSF, they differentiate into the M2 phenotype. Tumor-derived molecules, such as TGF-β and M-CSF, can polarize glioma-infiltrating microglia/microphages (MMs) toward the M2 phenotype and accordingly stimulate the production of anti-inflammatory molecules. Some other glioma-derived molecules, such as MCP-1 and VEGF, can recruit myeloid cells into the tumor site.

counterparts. These M2 cells typically produce anti-inflammatory cytokines, such as IL-10, and show a lower level of NO production. Furthermore, although they still upregulate the expression of MHC class II molecules, they are not efficient at antigen presentation. In contrast, M2 macrophages have been found to promote tissue remodeling, angiogenesis, and repair and act to suppress tissue-destructive immune reactions.[138] The key determinant in polarizing macrophages is the microenvironment in which they dwell, and the latter selectively induces specific M1 or M2 functional programs.[136]

Polarization of glioma-infiltrating microglia toward the M2 phenotype has been well documented both in vitro and in vivo. Rodrigues et al.[139] reported that peripheral blood mononuclear cells (PBMCs) exposed to glioma, which were obtained from either normal donors or glioma patients, acquire immunosuppressive properties. This includes reduced expression of CD14 (but not of CD11b); increased expression of immunosuppressive IL-10, TGF-β, and B7-H1; decreased phagocytic capacity; and the increased ability to induce apoptosis in activated lymphocytes when monocytes were exposed to glioma cells in vitro. In addition, Wu and colleagues[79] have demonstrated that gCSC-conditioned medium polarizes glioma-infiltrating microglia toward an M2 phenotype. These M2-polarized microglia in glioma exhibited reduced phagocytic activity and secretion of immunosuppressive cytokines, such as IL-10 and TGF-β, and were less capable of stimulating T-cell proliferation. This is in line with previous findings by Hussain et al.[140] that glioma-infiltrating microglia do not secrete the pro-inflammatory cytokines IL-1β and TNF-α that play a critical role in the development of effective innate immune responses nor are capable of mediating an anti-tumor adaptive immune response.[37] It is believed that glioma-derived molecules

such as TGF-β, macrophage (M)-CSF, IL-10, and IL-4 induce the shift of glioma-infiltrating microglia toward the M2 phenotype.[141,142] This view is supported by the following in vivo studies. Using a mouse model, Gabrusiewicz et al.[63] recently demonstrated that glioma-infiltrating microglia acquire the M2 phenotype in vivo, as evidenced by upregulation of IL-10 and GM-CSF, as well as the increased expression of genes characteristic for the alternative and pro-invasive phenotype of glioma-infiltrating microglia, arginase 1 (Arg-1), membrane type 1 metalloprotease (MT1-MMP), and chemokine (C-X-C motif) ligand 14 (CXCL14). Furthermore, M-CSF expression in glioma cells was found to correlate with the expression of CD163 in glioma-infiltrating microglia in vivo, supporting the role of glioma cells in microglia polarization.[143] Specifically, expression of CD163 and CD204, both of which are considered M2 macrophage markers, by microglia/macrophages infiltrating glioma was significantly higher in grade IV glioma when compared with World Health Organization grades II and III glioma, indicating that polarization of glioma-infiltrating microglia toward the M2 phenotype correlates with a more malignant histologic grade.[143] Taken together, glioma-derived factors are able to "educate" glioma-infiltrating microglia to acquire the M2 phenotype and thus create a favorable microenvironment for glioma growth.

Molecules Promoting the Proliferation of Microglia

M-CSF, also known as CSF-1, is a growth factor that was found to be expressed in mice and human glioma tissues as well as GBM cell lines.[40,144] The M-CSF receptor (R), encoded by the c-fms proto-oncogene, is the main receptor for M-CSF[145] and is expressed on microglial cells[146] and possibly on neurons.[147] Increased M-CSFR expression in activated microglia has been reported following mouse facial nerve axotomy[146,148] and cerebral ischemia[147] and in a transgenic mouse model of Alzheimer's disease.[149] Furthermore, M-CSFR overexpression was found to result in microglial cell proliferation and increased expression of inducible NO synthase, pro-inflammatory cytokine IL-1α, macrophage inflammatory protein (MIP) 1α, IL-6, and M-CSF itself.[150] A recent study employing the rat facial nerve axotomy model reported that the expression of M-CSF in microglia was upregulated, which triggered increased expression of M-CSFR in microglia.[151] This autocrine loop between M-CSF and M-CSFR in microglia apparently underlies the proliferation of microglia in this model.[151] Despite the fact that there is so far no evidence for the expression of M-CSFR on glioma-infiltrating microglia, the production of M-CSF by glioma cells and the presence of M-CSFR on activated microglia[151] raise the possibility of a paracrine loop that could promote intratumoral proliferation of microglia in glioma. Papavasiliou et al.[152] demonstrated the existence of a paracrine loop in medulloblastoma, reporting that the tumor cells expressed M-CSF but not M-CSFR, and microglia treated with media conditioned by serum-free medulloblastoma significantly increased their proliferation in vitro. In addition, M-CSF and M-CSFR paracrine communication between GBM and microglia has been confirmed by a recent in vivo study, which further indicated that blockade of M-CSFR signaling reduces the number of glioma-infiltrating microglia and thus GBM invasion.[40] It is noteworthy that expression of the M-CSF gene may result in 2 different isoforms of the M-CSF protein, ie., the secreted form (sM-CSF) and the membrane-bound form (mM-CSF), which elicit different effects on glioma-infiltrating microglia. Glioma-derived sM-CSF promotes tumor invasion by stimulating the proliferation and recruitment of glioma-infiltrating microglia.[144] In contrast, expression of mM-CSF by tumor cells elicits anti-glioma activity in microglia, stimulating direct tumor cell killing and antigen processing.[153–155] Disruption of internal potassium ion homeostasis in mM-CSF–expressing glioma cells through channel activation of functional big potassium (BK) may represent an alternative mechanism underlying the mM-CSF–mediated anti-glioma activity of microglia.[156] Furthermore, GBM-derived sM-CSF induces polarization of glioma-infiltrating microglia toward the immunosuppressive M2 phenotype, which increases proliferation and migration of glioma cells.[143] This finding was further corroborated by a recent study on gCSCs, which are demonstrated to also produce sM-CSF that can drive polarization of microglia toward the M2 phenotype.[79]

Molecules Controlling the Recruitment/Migration of Microglia

Chemokine (C-C motif) ligand 2.— CCL2, introduced earlier in this paper as MCP-1, is a protein belonging to the CC chemokine family, encoded by the CCL2 gene. By binding to chemokine (C-C motif) receptor 2 (CCR2), MCP-1 induces the migration of monocytes, memory T cells, and dendritic cells to sites of tissue injury, infection, and inflammation. MCP-1 has been found to be produced by glioma cells,[45,157,158] and its expression is positively correlated with microglial infiltration of human gliomas.[159] This is in keeping with the results of an in vivo study by Platten et al.,[158] which provided direct experimental evidence that MCP-1 expression promotes the recruitment of microglial cells to the site of glioma. MCP-1 is therefore considered a critical chemoattractant for glioma-infiltrating microglial cells. Furthermore, MCP-1 expression is correlated with the grade of malignancy,[160] and MCP-1–expressing gliomas appear more anaplastic and vascularized than control tumors.[158] These studies suggest that the expression of MCP-1 by glioma cells not only induces the recruitment of microglial cells to the site of glioma, but also promotes tumor growth and neo-angiogenesis as a consequence of local infiltration of microglia. Interestingly, while causing migration and proliferation of microglia, MCP-1 does not appear to directly activate an inflammatory response in microglia,[161] and the authors concluded that other factors may be necessary to cause the changes that result in

the neuronal damage commonly observed in situations where MCP-1 levels are elevated. MCP-1 may stimulate CCR2-bearing microglia to produce and secrete IL-6, which in turn acts on glioma cells to promote their invasiveness.[162]

Hepatocyte growth factor/scatter factor.— HGF/SF is a protein functioning through its exclusive receptor, tyrosine kinase c-Met. In the case of GBM, HGF/SF and c-Met are expressed by both tumor cells and microglial cells.[163–165] Glioma-derived HGF/SF is able to chemotactically attract isolated microglial cells in vitro,[163] which suggests that it has a role in microglia chemotaxis in glioma in vivo. In addition, glioma-derived HGF/SF and c-Met have emerged as crucial determinants of glioma growth and angiogenesis. The expression levels of HGF/SF and c-Met in glioma cells increase with the grade of malignancy.[164,165] This autocrine HGF/SF–c-Met signaling loop in glioma has been demonstrated to be associated with tumor invasion and proliferation both in vitro[164,166–168] and in vivo.[169] Furthermore, HGF/SF has been shown to be a potent angiogenic molecule.[167,170] The angiogenic activity of HGF/SF is mediated either through direct actions on brain tumor endothelial cells (including stimulation of cell migration, proliferation, protease production, invasion, and organization into capillary-like tubes[167]) or through induction of VEGF expression and secretion in malignant glioma cells.[171] Hypoxia can induce c-Met expression in glioma cells,[172] whereas gangliosides and TGF-β have been found to stimulate the production of HGF/SF in human glioma cells.[173] Radiation enhances HGF/SF secretion, resulting in increased angiogenic potential of the tumor, which may be a factor in the radioresistance of glioma.[174] Accordingly, Buchanan and colleagues[175] recently reported that modulation of Met signaling with anti-HGF monoclonal antibody–AMG102 is able to increase the radiosensitivity of glioma cells, raising the possibility of a novel radiation sensitizing strategy. Inhibition of c-Met further enhances the chemosensitivity of glioma cell lines.[176]

Vascular endothelial growth factor.— VEGF is a signaling protein that is important for stimulating vasculogenesis and angiogenesis. Glioma cells constitutively secrete large amounts of VEGF,[42,177] and the level of VEGF production in glioma increases with the degree of tumor malignancy.[178,179] Thus, VEGF is considered to play a pivotal role in glioma growth. One possible mechanism underlying the VEGF glioma-promoting function is its chemotactic effect on microglial cells. VEGF has been found to induce the migration and proliferation of microglial cells that express VEGFR-1 in vitro.[180] Thus, it is likely that glioma-derived VEGF at least in part accounts for infiltration of microglia into glioma. Significantly, VEGF also contributes to the recruitment of bone marrow–derived cells.[181] Kerber et al.[15] reported that inducing VEGF overexpression in glioma tissues leads to a substantial infiltration of bone marrow–derived microglia/macrophages in vivo, and there was a 3.1-fold increase in infiltration compared with tumors created by implantation of wild-type glioma cells.

One well-known mechanism underlying the VEGF glioma-promoting function is the major role VEGF plays in angiogenesis in glioma. Endothelial cells in the vicinity of a glioma coexpress both VEGFR-1 and VEGFR-2, which serve a paracrine-signaling loop with glioma-derived VEGF that stimulates endothelial cell migration and proliferation and consequently new blood vessel formation.[181,182] This finding confirms the results of a previous study on primary brain tumors, which demonstrated that VEGF expression was correlated with vascularization in gliomas.[178] Glioma cancer stem cells have been proposed to be critical in sustaining tumor progression due to their capacity for self-renewal and their proliferative potential. Folkins and colleagues[183] found that tumors high in gCSCs express increased levels of VEGF and exhibit increased microvessel density and blood perfusion, as well as increased mobilization and recruitment of bone marrow–derived endothelial progenitor cells into the tumor. Blockade of the VEGF–VEGFR pathway alone or in combination with cytokines such as IL-6 has been reported to inhibit tumor growth.[184]

Abnormal energy metabolism is one feature of glioma. Glioma cells require a higher rate of glucose and glutamine uptake and metabolism than normal to maintain their survival and growth.[185–188] Significantly, human glioma cells secrete glutamate,[189,190] and high glutamate secretion conveys a growth advantage.[191] Therefore, glutamate release is thought to contribute to glioma spread as well as excitotoxicity.[191] Glucose and glutamine are also important fuels for microglia, macrophages, and other cells of the immune system.[192,193] Intriguingly, glutamate has been found to induce directed chemotaxis of microglia.[194] Both radiation and chemotherapy can induce necrosis and inflammation, which will increase tissue glutamate levels.[195,196]

Microglia Supporting Glioma Growth

Microglia Listening to Glioma

Receptors involved in microglia chemotaxis and proliferation.— The exact mechanisms underlying the recruitment of microglial cells into a glioma are still not entirely clear, but chemotactic factors released by tumor cells likely play a pivotal role. Several such factors have been identified, with MCP-1 turning out to be the most powerful. MCP-1 is constitutively produced by glioma cells as evidenced by both in vitro and in vivo studies,[45,157] whereas the specific MCP-1 receptor, CCR2, is expressed on glioma-infiltrating microglia/macrophages.[197,198] Importantly, an in vitro study by Platten et al.[158] provided direct experimental evidence that MCP-1 expression recruits microglial cells to the site of glioma. Thus, MCP-1 locally produced by glioma cells binds to CCR2 receptors expressed on the surface of microglial cells, and MCP-1/CCR2 binding facilitates recruitment of resident microglia into the site of glioma. In contrast, Okada and

colleagues[44] recently claimed that it is tumor-derived MCP-3, not MCP-1, that facilitates the infiltration of microglia into glioma tissues. However, MCP-1 binds to CCR2 exclusively, whereas MCP-3 binds to CCR1, CCR2, and CCR3.[199] The study did not report which receptor(s) respond to MCP-3 production in glioma.

Met. Met is a transmembrane tyrosine kinase receptor encoded by the proto-oncogene MET. Badie et al.[163] detected Met immuoreactivity in BV-2 murine microglia, and this is in keeping with what had been reported by Yamada et al.,[200] who described the expression of Met in microglia, predominantly in the white matter of human brain tissue. A more recent study also confirmed the expression of Met in human glioma–associated microglia.[165] As discussed earlier, the cognate ligand of c-Met, HGF/SF, is expressed by glioma cells.[163–165] Badie et al.[163] have demonstrated that glioma-derived HGF/SF is able to induce the migration of microglial cells in vitro, suggesting the existence of a paracrine loop of HGF/SF–Met signaling that plays a role in microglia chemotaxis in glioma in vivo. The existence of paracrine HGF/SF effects is supported by the results of an in vitro study that analyzed the effects of a panel of chemokines on glioma motility, demonstrating that HGF/SF was the most potent chemotactic factor for 3 glioblastoma cell lines.[201] Interestingly, microglia have also been reported to produce HGF/SF.[163,200,202,203] These findings raise the possibility of an autocrine motility loop in microglia, which HGF/SF–Met underlie.

Epidermal Growth Factor Receptor. EGFR is a 170-kDa transmembrane protein characterized by its ligand-dependent tyrosine kinase activity.[204] Activation of EGFR results in increased tumor cell proliferation and angiogenesis and decreased apoptosis and is associated with invasiveness of the tumor. Ilschner and colleagues[205] have described the transient appearance of K+ outward currents in murine microglial cells that were induced by EGF, confirming the presence of EGFR on microglia.[206] Nolte et al.[207] from the same group demonstrated the expression of EGFR on microglial cells in vitro and further showed a dose-dependent effect of EGF on microglial motility and chemotaxis. Interestingly, glioma cells, especially in GBM, also express EGFR,[208,209] usually as a result of EGFR gene amplification.[210,211] A recent study demonstrated that EGFR signaling in GBM is necessary for microglial stimulation of GBM invasion.[40] This indicates the existence of an EGF–EGFR paracrine loop linking GBM cells and microglia.

VEGF signals via 2 tyrosine kinase receptors, VEGFR1 (Fms-like tyrosine kinase 1 [FLT-1]) and VEGFR-2 (kinase insert domain receptor [KDR]/fetal liver kinase 1 [FLK-1]).[182] Both receptors are expressed on endothelial cells,[212] while only VEGFR-1 is found on cells of the monocyte/macrophage lineage.[213] Forstreuter et al.[180] reported that both rat microglial cells and mouse BV-2 microglia cell lines express VEGFR-1, but not VEGFR-2. Using in vitro assays, Forstreuter and colleagues in the same study further demonstrated that VEGF increases the chemotaxis and proliferation of microglial cells. Thus, apart from CCR2, Met, and EGFR, VEGFR-1 may be another candidate receptor involved in microglia chemotaxis.

Receptors involved in tumor immunity.— Many cytokines and cytokine receptors are expressed by microglia in the immunosuppressive microenvironment of glioma, and the binding of the respective cytokines to their receptors plays a key role in tumor immunity. Chemokine receptors represent a subclass of cytokine receptors that are expressed on the surface of microglia. They have been observed to mediate an efficient cross talk between glioma-infiltrating microglia and glioma cells.

Chemokine (C-X3-C Motif) Ligand 1. CX3CL1 is one of the most highly expressed chemokines in the CNS. It can be expressed as a membrane-bound form mediating cell–cell adhesion or as a soluble form sustaining chemotaxis.[214] Human glioma cells express both forms and, significantly, according to Sciume and colleagues,[215] the tumor cells also express the cognate receptor for CXC3CL1, CX3C chemokine receptor 1 (CX3CR1), on their surface. These authors further reported that disruption of CX3CR1/CX3CL1 interaction by means of an anti-CX3CL1 neutralizing antibody enhances glioma cell invasion, indicating that CX3CL1 inhibits glioma invasion.[215] In contrast, in a study on the expression and function of CX3CR1/CX3CL1 in human glioma, Held-Feindt et al.[216] demonstrated that CX3CR1 (also termed RBS11 or V28) was exclusively expressed in glioma-infiltrating microglia/macrophages, whereas its ligand CX3CL1 was expressed solely in glioma cells. The latter results are in agreement with previous observations on the expression of CX3CR1 by microglial cells in a murine glioma model,[217] as well as human glioma.[218] In addition, Held-Feindt and colleagues found that glioma-derived CX3CL1 not only promotes recruitment of human glioma-infiltrating microglia/macrophages, but also enhances expression of MMP2, -9, and -14 in these cells. This finding is significant because the enhanced expression of MMPs might favor adhesion and migration not only of glioma-infiltrating microglia but also of glioma cells.[11] Taken together, CXC3CL1 may act in an autocrine as well as paracrine fashion to promote the adhesion and chemotaxis of CX3CR1-expressing glioma and microglial cells during tumor growth.

Receptors involved in antigen presentation.— Antigen presentation is crucial for the generation of a specific anti-tumor response by the adaptive immune system. This process requires physical interaction between the T-cell receptor and immunogenic peptides presented via MHC class II molecules on the cytoplasmic membrane of antigen-presenting cells. A productive dialogue between microglia and T cells to result in T-cell proliferation requires a second signal, which is the simultaneous expression of co-stimulatory molecules, such as CD80 (B7-1) and CD86 (B7-2), on the surface of the antigen-presenting cells. The expression of MHC molecules has been described on microglia in both human and experimental glioma.[219–221] However, the expression of MHC class II by microglia is reduced in high-

grade glioma.[221] Therefore, it appears that microglia in glioma are deficient to helper and cytotoxic T cells in a proper antigen-presentation capacity. Using a cytotoxicity assay based on fluorescence-activated cell sorting, Flugel and colleagues[222] demonstrated that the ability of microglia was severely compromised to present C6 glioma antigen or stimulate C6-primed T cells. Furthermore, the expression of MHC class II and co-stimulatory B7 molecules in rodent glioma correlates directly with tumor immunogenicity and the extent of lymphocyte infiltration into tumors.[223] Schartner and colleagues[224] have reported downregulation of MHC class II and CD80 on the surface of microglia in glioma-bearing rats, which is consistent with a study that showed microglia isolated from human GBM exhibit downregulation of HLA-DR expression and suppression of CD80 induction by lipopolysaccharides.[225] Similarly, there was a lack of expression of the co-stimulatory molecules CD86 and CD80 in glioma-infiltrating microglia.[37] In contrast, the expression of MHC class II and B7 co-stimulatory molecules recovered after microglia were isolated from tumors and cultured for a short period of time in the absence of glioma cells.[223] These studies suggest that antigen-presenting functions of microglia are torpedoed by glioma cells.

Receptors involved in phagocytosis and cytotoxicity.— Cytotoxic and phagocytic functions of microglia are considered critical for anti-glioma responses. These defense functions require microglia to upregulate the expression of complement (CR1, CR3, CR4) and Fc-gamma receptors (CD64, CD32, CD16). Both enhance phagocytic capacity by binding to complement components and immunoglobulin fragments, respectively.[226,227] The expression of complement receptors, namely CR3 (CD11b/CD18), on the surface of microglia was found throughout glioma, and at an increased density along the tumor's periphery.[228] However, no evidence of glioma killing by CR3+ microglia was seen.[228] Furthermore, a significant positive correlation was found between the number of CR3+ cells and the proliferation rate of human glioma.[229] Taken together, these studies suggest that glioma-infiltrating microglia are in an activated state, yet exhibit tumor-supportive rather than tumor-phagocytic functions.[8]

Activation of phagocytes, including microglia, is the most common function of Fc receptors. Microglial cells are potent effector cells in antibody-dependent cell-mediated cytotoxicity in vitro,[35] which involves Fc receptor expression on the surface of microglia that stimulates microglial cells to release cytotoxic molecules that kill antibody-covered target cells.[35] Morimura and colleagues[229] have demonstrated abundant concomitant expression of Fc receptors (CD64, CD32, CD16) and CR3 on the surface of microglia in human glioma, which was found to be more intense in the area of tumor necrosis. Interestingly, Fc-gamma receptors 1 and 2 (CD64 and CD32) were detected mainly on microglia inside the tumor and to a much lower extent on microglia of peritumoral tissue, indicating that the expression of Fc-gamma receptors depends on the state of activation of microglia in glioma. These studies provide evidence that myeloid cells in human gliomas are equipped with the respective surface receptors for antibody- and complement-mediated cytotoxic actions. However, they are quite obviously not effective in real life.

Pattern Recognition Receptors. PRRs are proteins expressed by cells of the innate immune system, which allow the detection of pathogen-associated molecular patterns (PAMPs) associated with microbes or cellular stress. Toll-like receptors (TLRs) are the main family of PRRs that are necessary for the induction of an adaptive immune response to PAMPs or tumor cells through the activation and maturation of macrophages and dendritic cells.[230–232] The interaction between TLRs and their specific PAMPs induces signaling by NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), activation of the mitogen-activated protein kinase (MAPK) pathway, and consequently the secretion of pro-inflammatory cytokines, such as TNF-α, IFN, and IL-12, all of which can help to limit tumor growth.[232] Microglia have been reported to be the predominant TLR-expressing cell type in the CNS,[233] and microglia freshly isolated from human glioma tissue also express substantial levels of TLRs (TLR2, TLR3, and especially TLR4).[37] This expression can be accompanied by expression of CD14 in glioma-infiltrating microglia.[37] CD14 is otherwise expressed mainly by macrophages and acts as a co-receptor of TLR4 in the detection of bacterial lipopolysaccharides.[234] Yet, these TLR-expressing glioma-infiltrating microglia do not express cytokines that would be reflective of tumoricidal activity.[37] In contrast, microglia have been found to facilitate glioma cell invasion via TLRs.[10] Glioma-released factors stimulate the expression and activity of MT1-MMP via TLRs and their downstream molecules MyD88 and p38 MAPK in microglia, and MT1-MMP–expressing microglia in turn promote glioma expansion by degrading the extracellular matrix.[10] Independently, the high-mobility group box 1 protein (HMGB1), an alarmin protein released from dying glioma cells, has recently been identified as an endogenous ligand for TLR2.[235] Curtin et al.[235] further demonstrated that dying glioma-derived HMGB1 acts as a TLR2 agonist, induces endogenous TLR2 signaling, and initiates a CD8+ T cell-dependent anti-GBM immune response. Furthermore, a recent in vitro study reported that human microglia isolated from brain tumors exerted tumor-suppressing activities if they were pretreated with polyinosinic-polycytidylic acid.[236] These studies suggest that the tumor-supporting function of glioma-infiltrating microglia could be overridden by tumor-suppressing activities if they were activated via TLR agonists.

Scavenger Receptors. These receptors are thought to participate in the removal of many foreign substances and waste materials in the living body, and they also play a role in modulating phagocytic activity of microglia. Macrophage scavenger receptor 1 (MSR1 or CD204), a class A scavenger receptor, is a protein encoded by the MSR1 gene in human. CD204 was found to be expressed in high-grade gliomas, and tumor culture supernatants from glioma cell line T98G induced its upregulation in human macrophages in

vitro.[143] Increased MSR1 expression was also seen in ovarian cancers,[237] and targeted depletion of CD204-positive macrophages blocked ovarian tumor progression and ascites accumulation, as demonstrated in a murine model of ovarian cancer.[238] It is postulated that CD204 signaling in tumor-associated microglia cells may negatively regulate microglia activation. Therefore, CD204 may be one of the more promising targets for the immunotherapy of some cancers, including ovarian cancer and glioma.

Receptors potentially exerting an anti-tumorigenic function.— Adenosine is an endogenous ubiquitous biological mediator that regulates many physiological processes. The extracellular concentration of adenosine increases in response to cellular stress and tissue damage. Adenosine signals through 4 known adenosine receptor subtypes: A_1AR, $A_{2A}AR$, $A_{2B}AR$, and A_3AR, each featuring a different tissue distribution, ligand affinity, and signal transduction mechanism. A_1AR is found throughout the brain[239] and is expressed mainly on microglia/macrophages as well as neurons.[240] In glioma, expression of A_1AR is high in glioma-infiltrating microglia, whereas its expression in tumor cells is minimal in both human GBM and rodent glioma.[241] Synowitz et al.[241] showed that A_1AR-mediated inhibition of glioma growth depends on the presence of microglial cells because the size of the glioma was increased in microglia-depleted brain even when A_1AR was activated by the specific agonist. Furthermore, when comparing $A_1AR^{-/-}$ mice with their $A_1AR^{+/+}$ littermate controls, the tumor volume was also significantly larger, as expected, and simultaneously the number of glioma-infiltrating microglia was significantly higher in $A_1AR^{-/-}$ mice than in controls.[241] The same study further demonstrated that activation of A_1AR markedly decreases the activity of glioma-induced MMP-2 in microglia, suggesting that the inhibitory effect of adenosine on glioma invasion is at least in part due to decreased extracellular protease activity. This finding complements results of an earlier study in a model of multiple sclerosis, which revealed that A_1AR activation on microglia/macrophages interferes with the production of MMPs such as MMP-12 and of cytokines such as IL-1β.[242] In summary, A_1AR inhibition of glioma growth represents an interesting novel mechanism whereby microglia can modulate the biological behavior of a glioma.

Molecules Derived from Glioma-Infiltrating Microglia

Molecules contributing to the immune-suppressive milieu in glioma.— *Interleukin 10.* Originally termed cytokine synthesis inhibitory factor (CSIF), IL-10 is a 17- to 21-kDa cytokine with a broad spectrum of immunosuppressive activities, including inhibition of antigen presentation, ie., antigen-specific T-cell proliferation,[243] and of inflammatory cytokine synthesis by infiltrating monocytes/macrophages.[244] Increased IL-10 mRNA expression has been reported in human glioblastomas in vivo compared with low-grade tumors, but only weak expression was seen in human glioblastoma cell lines.[245] Using in situ hybridization analyses of native tissue samples of glioblastomas, Huettner et al.[20] demonstrated that both microglia and astroglia might contribute to IL-10 gene expression in vivo. Subsequent studies using primary cultures from human glioma specimens indicated that the IL-10 gene and its protein product are expressed by glioma-infiltrating microglia, and to a much lesser extent by the glioma cells.[21] IL-10 secreted by glioma-infiltrating microglia in the immunosuppressive microenvironment of glioma not only promotes glioma cell proliferation, but also enhances the ability of glioma cells to migrate in vitro.[20] Thus, glioma-infiltrating microglia contribute to the maintenance of an immunosuppressive microenvironment in glioma by producing and secreting immunosuppressive IL-10.[7,43] The expression of IL-10 in glioma-infiltrating microglia is regulated by upstream stimulating factor 1 (USF-1)[246] and STAT3.[247] Inhibition of USF-1 derived from glioma-infiltrating microglia results in upregulation of IL-10 in the glioma-infiltrating microglia,[246] as does increased STAT3 activity in the murine microglia cell line N9.[247]

In addition to its expression by glioma cells[105] (see earlier section), FasL has been found to be expressed by glioma-infiltrating microglia[43,106] and activated T cells in GBM.[248] Using flow cytometry analysis, Badie and colleagues[43,106] showed that glioma infiltrating microglia are the major cellular source of FasL in murine glioma, and nearly every microglia cell expressed FasL on its membrane. This study thus suggests that microglia, and not the T lymphocytes or tumor cells, are responsible for the increased expression of FasL in glioma. Considering the fact that microglia can induce apoptosis of activated T cells in vitro[249] and that apoptotic T cells in GBM express Fas,[250] it is postulated that glioma-infiltrating microglia may contribute to the local immunosuppressive microenvironment in glioma by mediating T-cell apoptosis via the Fas–FasL pathway. This is further evidenced by the significant increase in the number of tumor-infiltrated lymphocytes after an injection of anti-FasL neutralizing antibody into intracranial murine G26 glioma.[43] However, in contrast to Badie's findings in murine glioma, Hussain et al.[37] determined that human glioma-infiltrating microglia did not express FasL, or expressed it at only very low levels, indicating that apoptosis mediated by Fas–microglial FasL may not be the predominant mechanism of immune evasion in human tumors. Rat glioma cell–derived FasL is also able to mediate the death of T lymphocytes when T cells are co-cultured with glioma cells.[105] Furthermore, Jansen et al.[105] demonstrated that downregulation of FasL expression in glioma cells following the application of short hairpin RNA can enhance T-cell infiltration in glioma and thus inhibit tumor growth. Moreover, T-cell apoptosis in GBM was even found to be mediated by ligation of Fas with FasL on the same T cell.[248] In spite of the uncertainty surrounding the main cellular source of FasL in glioma, Fas–FasL interaction is likely to contribute to the immunosuppressive

microenvironment of glioma. Further investigations are therefore needed to elucidate the main cellular source of FasL in glioma in vivo.

Molecules contributing to glioma angiogenesis.— Neo-angiogenesis is very conspicuous in glioblastoma, and has been widely recognized as a key process in the malignant progression of glioma.[251,252] The process is thought to be driven largely by VEGF, which is a signaling protein functioning via its 2 receptors, VEGFR-1 and VEGFR-2. These 2 receptors are co-expressed on endothelial cells. Consequently, it is a widely held view that VEGF interacts with its receptors in vascular endothelium in a paracrine fashion and that it plays a major role in the regulation of tumor neo-angiogenesis.[177,253,254] In addition to glioma cells, microglial cells also secrete VEGF.[41,42] It is therefore speculated that microglial cells play a role in tumor progression by supporting angiogenesis as well.[19,255]

Molecules contributing to glioma growth and invasion.— TGF-β1 protein is produced by microglia under certain pathological conditions, such as experimental allergic neuritis,[256] and in traumatically injured brain.[257] TGF-β signals through TGF-β type II receptor (TβRII), which recruits and activates TGF-β type I receptor (TβRI).[18] Using in situ hybridization, Kiefer and colleagues[258] revealed that both activated microglial cells and glioma cells produce TGF-β1 in the vicinity of glioma in vivo. A recent study by Wesolowska et al.[18] confirmed that cultured microglial cells secrete TGF-β1, and their exposure to glioma cells strongly enhances secretion of TGF-β1. In addition, the study also demonstrated that the invasion-promoting activity of microglia was lost in glioma cells exhibiting downregulated TβRII, indicating that the promotion of glioma invasion of microglia is mediated by TGF-β.[18] Furthermore, expression of TGF-β may upregulate the expression of its receptors, TβRI and TβRII, both of which show increased expression in gliomas compared with normal tissues, and their antagonists are able to inhibit human glioma cell line proliferation and motility.[69,259,260] Thus, additional paracrine loops seem to exist that skew the microglial cell–glioma relationship in favor of the tumor, in that glioma-derived TGF-β exerts immunosuppressive effects in and around the tumor that render the glioma-infiltrating microglia ineffective, whereas microglia-derived TGF-β promotes tumor growth and invasion by stimulating the upregulation of its cognate receptors, TβRI and TβRII, on glioma cells.

Degradation of the extracellular matrix by proteolytic enzymes, such as membrane-bound and secreted MMPs, is a key mechanism utilized by invading glioma.[39] MMP-2, also known as gelatinase A, was found to play an especially important role in this process. Expression of the MMP-2 gene in human glioma tissues is upregulated when compared with normal brain and is dramatically increased in GBM.[261–263] Immunostaining of human glioma biopsies confirmed upregulation of MMP-2 and identified tumor cells as the cellular source of MMP-2.[262] The activity of this enzyme has been directly correlated to glioma invasiveness and the survival rate of tumor-bearing mice.[11,264] Both microglial cells and glioma cells produce MMP-2, as demonstrated both in vitro[11,263] and in human glioma tissue.[265] Notably, MMP-2 released by glioma cells is in an inactive soluble form (pro-MMP-2), is found especially at the actively invasive tumor zone, and needs to be cleaved to become activated.[265] The extracellular activator of MMP-2, MT1-MMP, is required for this cleavage.[39,266] However, glioma cells themselves cannot produce MT1-MMP but have to rely on microglial cells, which are the major, though not exclusive, cellular source of MT1-MMP in glioma.[10] In normal brain, MT1-MMP expression in microglia is low and detectable only in white matter.[266] In the context of glioma, MT1-MMP expression has been found to be upregulated in human, mouse, and rat glioma-infiltrating microglia, and its immunoreactivity was particularly pronounced when the microglia were in close contact with glioma cells.[10] A recent in vitro study demonstrated that attenuating microglial MT1-MMP expression using minocycline reduces glioma growth and invasion.[267] Moreover, deletion of the TLR adapter protein, MyD88, prevented overexpression of MT1-MMP, which had been induced by glioma-conditioned medium (GCM) in microglia, suggesting that the expression of MT1-MMP in glioma is regulated by TLR signaling.[10] Furthermore, TLR-mediated MT1-MMP expression was dependent on p38 MAPK, which resides downstream of MyD88.[10] Among the major candidate mechanisms underlying glioma invasion facilitated by microglia appear to be the production of heat-shock proteins, HMGB1, and hyluronan.[268–270] These glioma-derived molecules can activate microglial TLR signaling (via MyD88) and its downstream p38 MAPK pathway and thereby trigger the upregulation of MT1-MMP in microglia. MT1-MMP expressed in microglia is then thought to convert glioma-derived pro-MMP-2 into MMP-2. MMP-2 in turn promotes glioma cell invasion and tumor expansion.[10] Thus, microglia can serve as activators for the degradation of extracellular matrix, which is key for glioma invasion.

Connective Tissue Growth Factor. CTGF, also known as CCN2, is a cysteine-rich, matrix-associated, heparin-binding protein encoded by an immediate early gene, which is associated with drug resistance in GBM.[271] CTGF has been found to be implicated in cancer progression[272,273] when binding the cell surface protein beta 1 integrin (ITGB1),[274] tyrosine kinase receptor type A (TrkA), and co-receptor p75NTR.[275] Edwards and colleagues[276] have shown that a CTGF-rich microenvironment increases the invasiveness of malignant gliomas. Halliday and Holland[277] have pointed out that parallels exist between brain tumors and brain injury with regard to CTGF expression and emphasize that CTGF is expressed by microglia under pathological conditions. Thus, microglia come into the spotlight again because expression levels of CTGF are prognostic of tumor progression as well as survival of patients with glioma.[278]

Interleukin-6. IL-6 is a protein of 185 amino acids encoded by the *IL6* gene in humans. It has been reported that GBMs display a significantly higher level of IL-6 expression than normal brain tissue.[279] In addition, IL-6 expression has been correlated with glioma growth invasiveness.[280,281] Glioma cells as well as microglia have been reported to secrete IL-6,[47,162,282,283] and both express IL-6 receptors.[94,284] Glioma-derived IL-6, working together with other tumor-secreted factors, such as TGFβ and PGE2, polarize glioma-infiltrating microglia toward the M2 phenotype,[47,282] and microglia-derived IL-6 has been reported to induce glioma cell migration and invasiveness.[162,283] Notably, IL-6 has been confirmed as a growth factor for glioma stem cells.[285] The mechanism by which IL-6 promotes the invasiveness of glioma cells is unclear. IL-6 has been found to increase the production of MMP-2 by glioma cells, suggesting that it may exert its tumor-promoting role via MMP-2.[281] Furthermore, it has been hypothesized that activated microglial cells synthesize IL-6 following NF-κB activation, which in turn may stimulate transcription factors STAT3 and NF-κB in glioma cells that initiate specific pathways of glioma progression such as angiogenesis, migration, and apoptosis inhibition.[94,286]

STAT3 activation of glioma-infiltrating microglia.— STAT3 activity has been shown to be higher in glioma-infiltrating microglia compared with microglia from normal animals.[247] Activation of the STAT3 pathway was found to increase expression of IL-10 and IL-6 in glioma-infiltrating microglia.[247] STAT3 blockage stimulates the immunological activation of glioma-infiltrating microglia, as evidenced by their increased expression of co-stimulatory molecules CD80 and CD86.[119] Furthermore, in vivo STAT3 inhibition in murine glioma-infiltrating microglia was shown to reduce expression of immunosuppressive cytokines, such as IL-10 and IL-6, while it stimulates production of pro-inflammatory TNF-α.[247]

Activation of STAT3 in glioma-infiltrating microglia can be induced by means of several immunosuppressive factors that are highly expressed in gliomas, such as IL-10, and VEGF.[287] Interestingly, these soluble factors can also activate STAT3 in hematopoietic cells in the tumor microenvironment.[118] In addition, Zhang et al.[288] recently reported that S100B, which is constitutively expressed by glioma cells, might be yet another factor that induces the activation of STAT3 in glioma-infiltrating microglia through interaction with RAGE, the receptor, for advanced glycation end product, in glioma-infiltrating microglia both in vitro and in vivo. Therefore, it appears that glioma-derived factors polarize glioma-infiltrating microglia toward the immunosuppressive M2 phenotype through STAT3 activation, which upregulates expression of immunosuppressive factors by glioma-infiltrating microglia while limiting their expression of co-stimulatory molecules.

Conclusions and Future Directions

GBM is the most common and aggressive primary tumor of the brain and has one of the worst 5-year survival rates among all human cancers.[289] Microglial cells and

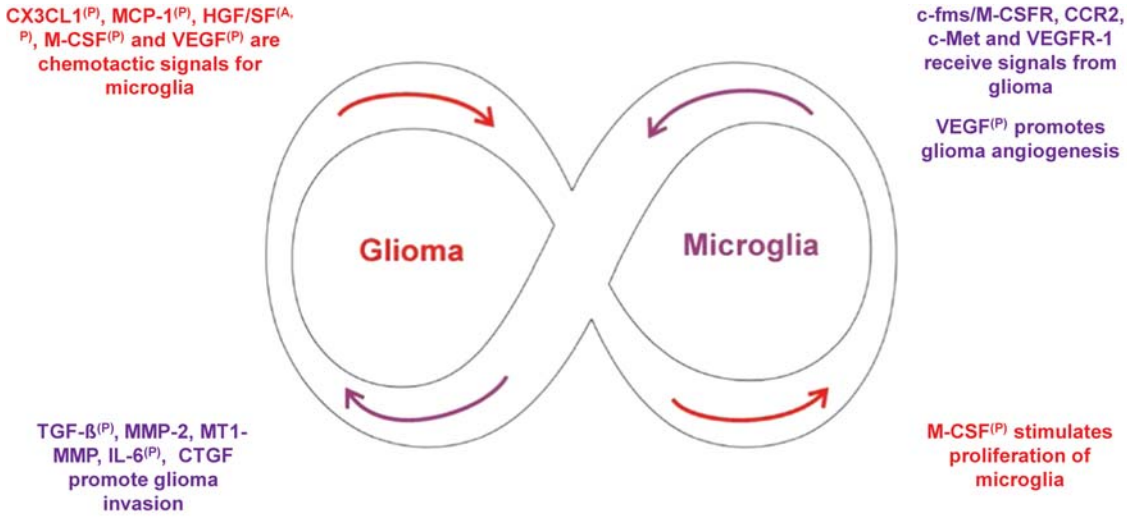

Fig. 2. Glioma-microglia synergies drive a self-amplifying process that spirals out of control. Glioma and microglial cells have a symbiotic relationship that becomes highly skewed in favor of the glioma. The immunosuppressive microenvironment created by molecules such as TGF-β, FasL, and IL-10 polarizes glioma-infiltrating microglia toward the M2 phenotype (cf. Fig. 1). Glioma (red) produces chemotactic factors, such as MCP-1, resulting in the recruitment of microglia. Glioma further promotes the proliferation of microglia. In turn, microglia (purple) promote glioma angiogenesis as well as glioma cell invasion. The cross talk between glioma and microglia is governed by multiple paracrine loops formed by glioma and microglia released molecules and their receptors, as indicated by superscript (P) in the figure. In addition, some of the molecules act in an autocrine fashion, as indicated by the superscript (A) and regulate either glioma or microglia behavior.

macrophages have been found to populate malignant glioma in large numbers, contributing one third or more to their actual tumor mass. Representing the immune effector cells of the CNS, microglia have been found to phagocytose glioma cells in vitro but their behavior is quite different in vivo, where they act more like nodding sycophants of tumor cells. As illustrated in Fig. 2, experimental studies have demonstrated that microglia express a variety of receptors on their surface, which are able to receive signals from glioma. In response, microglia under the influence of glioma release several classes of molecules that foster glioma growth and progression. There is clearly more than one self-amplifying autocrine and paracrine loop, which can spiral out of control and the tumor progresses. However, we now also know of a number of potential molecular targets in tumor-associated microglia that could be used to alter the fatal course of glioma. A significant portion of the microglia found in glioma appear to be derived from bone marrow precursors, and it may become possible to enable these bone marrow–derived microglia to track down glioma cells using the same genes that stem cells employ for sniffing out glioma in the brain.[290] Consequently, the great attraction of glioma for microglia and the principal ability of microglia precursors to cross the BBB make them attractive targets for the development of a truly novel therapeutic approach. Emerging technologies[291] may allow us to genetically enhance (reprogram) microglia[65] so that they are protected and able to turn against the glioma.

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