MINI REVIEW

Immune microenvironment of gliomas

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High-grade gliomas are rapidly progressing tumors of the central nervous system (CNS) with a very poor prognosis despite extensive resection combined with radiation and/or chemotherapy. Histopathological and flow cytometry analyses of human and rodent experimental gliomas revealed heterogeneity of a tumor and its niche, composed of reactive astrocytes, endothelial cells, and numerous immune cells. Infiltrating immune cells consist of CNS resident (microglia) and peripheral macrophages, granulocytes, myeloid-derived suppressor cells (MDSCs), and T lymphocytes. Intratumoral density of glioma-associated microglia/macrophages (GAMs) and MDSCs is the highest in malignant gliomas and inversely correlates with patient survival. Although GAMs have a few innate immune functions intact, their ability to be stimulated via TLRs, secrete cytokines, and upregulate co-stimulatory molecules is not sufficient to initiate antitumor immune responses. Moreover, tumor-reprogrammed GAMs release immunosuppressive cytokines and chemokines shaping antitumor responses. Both GAMs and MDSCs have ability to attract T regulatory lymphocytes to the tumor, but MDSCs inhibit cytotoxic responses mediated by natural killer cells, and block the activation of tumor-reactive CD4⁺ T helper cells and cytotoxic CD8⁺ T cells. The presence of regulatory T cells may further contribute to the lack of effective immune activation against malignant gliomas. We review the immunological aspects of glioma microenvironment, in particular composition and various roles of the immune cells infiltrating malignant human gliomas and experimental rodent gliomas. We describe tumor-derived signals and mechanisms driving myeloid cell accumulation and reprogramming. Although, understanding the complexity of cell–cell interactions in glioma microenvironment is far from being achieved, recent studies demonstrated several glioma-derived factors that trigger migration, accumulation, and reprogramming of immune cells. Identification of these factors may facilitate development of immunotherapy for gliomas as immunomodulatory and immune evasion mechanisms employed by malignant gliomas pose an appalling challenge to brain tumor immunotherapy.

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INTRODUCTION

Gliomas are tumors of the central nervous system (CNS), originating from transformed neural stem or progenitor glial cells.¹ On the basis of histopathological characteristics World Health Organization (WHO) divided gliomas into groups: low-grade gliomas (LGG, grades I and II) are well-differentiated, slow-growing tumors, whereas high-grade gliomas (HGG, grades III and IV) are less differentiated or anaplastic, and strongly infiltrate brain parenchyma. The most common and deadliest primary brain tumor is glioblastoma (GBM, grade IV). Histological classification is currently assisted by molecular genetic studies that provide diagnostic, prognostic, and predictive values.^{2,3} The emerging molecular profile of gliomas is based on the studies of gene expression and DNA methylation, and recent discoveries of new biomarkers facilitate patient stratification, prognosis, and

prediction of treatment responses.⁴ Gliomas that harbor mutations in isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2), and display the CpG island hypermethylator phenotype constitute a subtype of gliomas with improved survival.⁴ GBM is the most aggressive and difficult to treat malignancy, due to the frequent dysfunctions of tumor suppressors or oncogenes and highly diffusive growth, which prevents a successful resection of the tumor.⁵ Primary GBMs show alterations in *EGFR*, *PDGFRA*, *PTEN*, *TP53*, *NF1*, and *CDKN2A/B*, as well as *TERT* promoter mutations, but not *IDH1/2* mutations.^{6,7} On the basis of gene expression profiles, GBMs can be further divided into four subtypes: classical, proneural, neural, and mesenchymal, that differ in tumor aggressiveness, progression, and/or prognosis. The golden standard of GBM treatment is surgery combined with chemo- and radiotherapy, however, it remains only palliative and with

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median survival time of adult patients with GBM 15 months after diagnosis.⁸

Glioma cells secrete numerous chemokines, cytokines and growth factors that promote infiltration of various cells: astrocytes, pericytes, endothelial cells, circulating progenitor cells, and a range of immune cells such as microglia, peripheral macrophages, myeloid-derived suppressor cells (MDSC), leukocytes, CD4⁺ T cells, and Treg into the tumor.^{9–12} These non-neoplastic cells create a specific niche called a tumor microenvironment, which has a crucial role in cancer growth, metastasis, and response to treatment. Locally produced cytokines and chemokines, and their crosstalk with components of the extracellular matrix, reprogram infiltrating immune cells to acquire distinct functional phenotypes, thereby directing the immune system into inflammatory or anti-inflammatory responses. Like many other non-CNS malignant cancers, GBMs developed multiple strategies to inhibit host antitumor responses (for a review see ref. 13). Several recent transcriptomic studies of GBM tissues have identified different signatures, based on immune and myeloid/macrophage genes expression, associated with GBM pathology, overall survival (OS), or response to treatment.^{14–16} These data strongly support a link between a type of the immune system response and glioma progression.

In this review, we discuss heterogeneity of the immune microenvironment of human gliomas and experimental rodent gliomas, and contribution of specific immune subpopulations to glioma pathology. We appraise recent discoveries of tumor-derived factors that stimulate migration and reprogramming of various immune cells infiltrating gliomas, and are promising novel targets for therapeutic treatments.

ORGANIZATION OF THE IMMUNE MICROENVIRONMENT OF GLIOMAS

Glioma-Associated Microglia/Macrophages in Human Gliomas

Microglia are myeloid cells residing in the CNS, which account for 10–20% of the non-neuronal cell population. Resident microglia and extra-parenchymal macrophages such as: perivascular, choroid plexus-associated, and meningeal macrophages, described collectively as 'brain macrophages', are involved in the maintenance of brain homeostasis and immunological responses.¹⁷ Detection of the CD11b antigen (integrin alpha M, Mac-1, or complement receptor CR3A) was used as a common marker for microglia in most studies on human tissues. However, bone marrow (BM) macrophages and MDSCs also express CD11b. Generally, markers used for the identification of microglia/macrophages are CD163, CD200, CD204, CD68, and Iba-1 (a lectin binding protein). Until now, no microglia-specific marker has been identified that does not also label extra-parenchymal or peripheral macrophages. The question remains unresolved whether CD11b⁺ cells detected under pathological conditions

are CNS-resident microglia or come in part from other sources, such as blood or BM (for a review see ref. 18).

Clinical studies showed extensive infiltration of gliomas with microglia and peripheral macrophages, collectively termed glioma-associated microglia/macrophages (GAMs). Immunohistochemical studies using anti-Mac387, KP1 antibodies, and the lectin RCA-1 demonstrated the higher abundance of GAMs in HGGs than in LGGs.¹⁹ Immunohistochemical studies of forty gliomas (grades II–IV) with an antibody against CD45RO and lectin RCA-1 showed the high number of ramified GAMs in gemistocytic astrocytomas (grade II), both the ramified and ameboid GAMs in the protoplasmic and fibrillary astrocytomas (grade II), and the greatest number of ameboid GAMs with very rare ramified GAMs in anaplastic astrocytomas (grade III) and GBM (grade IV).²⁰ Immunostaining for three microglial markers: CR3/43, Ki-M1P, and Iba1 detected the high number of GAMs in gemistocytic astrocytomas (grade II) that behave more aggressively than other grade II astrocytomas²¹ and are considered by some authors as grade III astrocytomas. Re-evaluation of GAM infiltration by staining of glioma sections with anti-HLA-DP,DQ,DR antibody (CR3/43) revealed morphological heterogeneity of immunopositive cells (more ramified GAMs were detected in grade I and II astrocytomas vs more ameboid GAMs in GBM), and the increased number of GAMs in GBM. Double staining with anti-HLA-CR3/43 and anti-CD11b antibodies demonstrated co-localization of both markers in a vast majority of positive cells.²² Interestingly, GAMs accumulation was not observed in pediatric high-grade tumors such as primitive neuroectodermal tumors (PNETs).

Flow cytometry was used to identify microglial cells within the CNS. A flow cytometry phenotype for ramified (non-activated) microglia isolated from adult CNS was defined as CD45^{low} CD11b/c⁺ in Lewis and Brown Norway rats, and clearly distinguished these cells from all blood-derived leukocytes, the latter being CD45^{high}. Isolated microglia were mostly MHC class II positive.^{23,24} The extent of microglia (CD11b^{high}, CD45^{low}), macrophage (CD11b^{high}, CD45^{high}), and lymphocyte (CD11b^{negative}, CD45^{high}) infiltration into tumors, tumor periphery, and contralateral tumor-free hemispheres was measured.²⁵ Flow cytometry studies demonstrated accumulation of both microglia and macrophages in human gliomas. The number of infiltrating GAMs ranged from 1.8 to 3.7 × 10⁷ cells per gram of tumor mass in GBM, whereas in normal brain it ranges from 4.6 to 3 × 10⁵ cells per gram of tissue.^{26,27} Intratumoural microglia/macrophage density increases during glioma progression and correlates with the grade of malignancy.²⁸ Interestingly, glioma cells expressing a marker CD133⁺, considered to be glioma-initiating cells, are more effective in recruiting GAMs to the tumor, as evidenced by enhanced Iba1 and CD68 staining around them. Glioma-initiating cells that grow *in vitro* as 3-dimensional, floating spheres, express high levels of chemoattractants (2- to 3-fold higher level of *CCL2*, *CCL5*,

and *CCL7* mRNA, 7-fold higher level of *VEGF-A* mRNA, and nearly 50-fold higher level of *NTS* mRNA) as compared to the adherent glioma cells.²⁸ Collectively those results point to a link between GAM accumulation and tumor grade. A lack of systematic studies on the abundance of amoeboid GAMs in a tumor core/periphery prevents making definite statement about its link to patient survival.

Functional Polarization of Glioma-Associated Microglia/Macrophages in Human Gliomas

Macrophages are 'plastic' and their phenotype can adapt to specific environmental cues. These cells may change their effector mechanisms along a spectrum between a 'M1' pro-inflammatory phenotype, characterized by inflammatory, antitumor responses, and a 'M2' cytoprotective, immunosuppressive phenotype, displayed by macrophages involved in tissue repair and inflammation resolution.²⁹ Cultured M2 macrophages display discrete functional states (M2a, b, c) depending on the cocktail of cytokines/factors used for stimulation, enhanced phagocytic, and cytoprotective properties, and are immunosuppressive.³⁰ M2 macrophages produce high levels of IL-10, transforming growth factor (TGF)- β and low levels of IL-12. Several markers have been proposed to distinguish between M1- and M2-like states, eg, the expression of *Arg1*, *Mrc1*, *Chi3l3*, *Socs2*, *CD163*, *Fizz-1*, and *Ccl2* mRNAs marks M2 activation, whereas M1 macrophages express *Nos2*, *IL12b*, and *Ciita*.²⁹ Despite simplification of such distinctions representing the extremes of a spectrum of discrete states, it is useful to define such states using a set of functional features and gene expression profiles. Recent data on the functions of non-CNS tumor-associated macrophages (TAMs) suggest that their activities should be better described as pro- or antitumor, rather than 'M1' or 'M2' phenotypes.^{31,32} A comprehensive analysis of gene expression profiles in TAMs isolated from various tumor models led to a surprising conclusion that TAMs from cancers associated with high amounts of endogenous commensal- and pathogen-derived molecules (such as colon cancer) will tend to the M1 phenotype, whereas tumors developing in sites with relative low inflammation such breast, head and neck tumors, developmental pediatric tumors, will have higher numbers of M2 macrophages.³²

Microglia exhibit a remarkable degree of plasticity. Upon different stimulation mimicking pathological conditions, microglia can also polarize into functional states classified as the M1 pro-inflammatory activation or the M2 cytoprotective, immunosuppressive activation.³³ M1 activation was induced by GM-CSF treatment followed by IFN γ /LPS in microglia isolated from adult brains and cultured *ex vivo*, whereas the M2 activation was induced by M-CSF treatment followed by IL-4/IL-13. High levels of Arginase-1 (*Arg1*), *CCL17*, *CCL22*, mannose and scavenger receptors, and production of anti-inflammatory cytokines such as: IL-4, IL-10, and TGF- β and low amount of IL-12 and NO were found in M2 activated microglia and macrophages. Myelin

phagocytosis was augmented in microglia in comparison to macrophages and was increased in M2 cells.³⁴ During an acute phase of brain injury microglia adopt mostly M1 phenotype and release chemical mediators such as nitric oxide (NO), reactive oxygen species (ROS), excitatory amino acids, and pro-inflammatory cytokines: interleukin-1 β (IL-1 β), IL-12, tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) (reviewed in refs 35,36).

Despite many efforts there is no consensus regarding the activation status of GAMs. The preliminary analysis of the immune functions of GAMs (CD11b/c⁺CD45⁺ cells) from post-operatives samples indicated that these cells were phagocytic, expressed substantial levels of Toll-like receptors (TLRs) but did not produce pro-inflammatory cytokines: IL-1 β , IL-6, TNF- α upon TLR stimulation.²⁶ In addition, despite the expression of the MHC class II on CD11b/c⁺CD45⁺ cells, the expression of T-cell activation factors and co-stimulatory molecules such as CD86, CD80, and CD40 was downregulated, and these cells were less capable to mediate tumor cytotoxicity compared with the microglia isolated from normal brain.²⁶

Several studies detected putative markers of M2 activation in gliomas. The expression of macrophage scavenger receptors CD163 and CD204 was increased in human gliomas, and the number of CD163 and CD204 positive cells among the GAM population was correlated with the grade of glioma and malignancy.³⁷ Combination of *in situ* hybridization with CD68 immunohistochemistry showed that microglia are a main source of *IL-10* expression in malignant gliomas.^{38,39} Moreover, *IL-10* mRNA levels were positively correlated with malignancy of the gliomas (87% of grade III and IV, and only 4% of grade II expressed *IL-10* mRNA). Elevated levels of IL-10 were found in sera of 11% of low-grade and 63.6% of high-grade human gliomas.³⁸

Applications of immunomagnetic beads or FACS immunosorting of CD11b⁺ cells allowed to isolate GAMs from gliomas, and make reasonable comparison to their counterparts in normal brain. Szulzewsky *et al*⁴⁰ determined the expression of the M1 (*Il1rn* and *Isg20*), M2a (*Clec7a*), M2b (*Tgfb11*), and M2c-specific (*Cxcr4*) genes in CD11b⁺ and CD11b⁻ cells isolated from human 11 GBMs, 5 non-tumor brains, and 2 samples of blood monocytes. High expression of tested genes was detected in GAMs isolated from GBM samples, however only *IL1RN*, *ISG20* (both M1-specific), and *TGFB1* (M2b-specific) levels were significantly higher in CD11b⁺ cells from GBMs than in CD11b⁺ cells isolated from control brains. The expression of *CLEC7A* was significantly lower in CD11b⁺ cells isolated from GBMs. The authors attributed a lack of differences to the fact that the control brain specimens were not taken from healthy patients, but patients suffering from epilepsy or and trauma injury.

In the subsequent study, they analyzed the expression profile of human GBM GAMs and CD11b⁺ cells from non-tumor samples (epilepsy and control postmortem brains), and identified 334 significantly regulated genes in human

Table 1 Phenotypic and functional characteristics of GAMs in gliomas

Source of tissue	Method used	Phenotypic markers	GAMs presence and phenotype	References
Human gliomas	Immunohistochemistry	Mac387, KP1 and galactose or <i>N</i> -acetylgalactosamine (lectin RCA-1)	More GAMs in high-grade vs low-grade gliomas	19
Human gliomas	Immunohistochemistry	CD45RO and lectin RCA-1	More ameoid GAMs in anaplastic astrocytomas (grade III) and GBM	20
Human gliomas	Immunohistochemistry	CR3/43, Ki-M1P, and IBA1	More GAMs in aggressive gemistocytic astrocytomas (grade II) than in other astrocytomas	21
Human gliomas (LGG, HGG)	Immunohistochemistry	HLA-DP,DQ,DR (CR3/43), CD11b	More ameoid GAMs in GBM	22
Human gliomas	Immunohistochemistry	IBA1, CD68	More GAMs in GBM	28
Human gliomas, normal brain	Flow cytometry	CD45 ^{low} CD11b/c ⁺ , CD11b ^{high} , CD45 ^{high}	More ameoid GAMs in GBM than in normal brain	26,27
Human gliomas, normal brain	Flow cytometry	CD11b/c ⁺ CD45 ⁺	Reduced CD86, CD80, and CD40 expression in GBM	26
Human gliomas	Flow cytometry	Scavenger receptors CD163 and CD204	Increased expression of CD163 and CD204 in GAMs	37
Human gliomas	<i>In situ</i> IL-10 hybridization and CD68 immunohistochemistry	IL-10 mRNA, CD68	Increased expression of IL-10 mRNA in CD68 ⁺ cells in HGG	38,39
Human gliomas	ELISA for IL-10	IL-10 serum level	Increased serum level of IL-10 in GBM vs LGG	39
Human gliomas, non-tumor brain	CD11b ⁺ isolated by beads; gene expression profiling; qPCR	Markers of M1 and M2 phenotypes	IL1RN, ISG20, TGFBI, GPNMB, SPP1 upregulated CD11b ⁺ cells from GBMs; CLEC7A downregulated in CD11b ⁺ cells from GBMs	40
Human gliomas, postmortem brain	CD11b ⁺ isolated by beads; global gene expression profiling; gPCR	Markers of M1 and M2 phenotypes	SPP1, ADAMDEC1, SDC3, RND3, FN1 IBSP, CTSL, and ADAMTS2, CCL2, 3, 4, 5, 8, and CXCL9 mRNAs and genes associated with 'mitotic cell cycle' and 'extracellular matrix organization' upregulated in GAMs	41
Human LGG and GBMs	CD11b ⁺ isolated by beads; gPCR	Markers of M1 and M2 phenotypes	ARG1, CXCL14, MMP14, NOS2 upregulated in HGG GAMs; IKBKB upregulated in LGG GAMs;	22
GBM, monocytes, normal brain microglia	CD11b ⁺ isolated by flow cytometry; gene expression profiling	Markers of M1 and M2 phenotypes	CD163, CD206, IL-10, TGFβ were higher in GAMs from GBM	42

Abbreviations: HGG, high-grade gliomas (WHO III–IV grade); LGG, low-grade gliomas (WHO I–II grade).

GBM GAMs (292 upregulated and 42 downregulated).⁴¹ The upregulated genes were associated with mitotic cell cycle, cell migration, cell adhesion, response to wounding, and extracellular matrix organization. Chemokines upregulated in human GAMs include *CCL2*, 3, 4, 5, 8, and *CXCL9*. Interestingly, there was no overlap of the genes upregulated in human GAMs with the genes upregulated in macrophages stimulated with IL-4. In contrast to murine GAMs that upregulated *Il-1 β* and *Tnf- α* , human GAMs did not upregulate genes associated with immune activation (ie, *IFNG*, *NOS2*, *TNF α* were barely expressed). RNA sequencing of GAMs from murine GL261 gliomas showed a fraction of upregulated similar transcripts as in human GAMs. The similar genes were mostly related to mitotic cell cycle. Among significantly upregulated genes in murine GAMs were those related to 'interferon signaling', 'complement system', and 'pattern recognition receptors'.⁴¹ The study shows that a GL261 glioma model does not recapitulate well innate and adaptive immune responses occurring in human GBMs.

We have optimized a protocol for GAM isolation based on gentle mechanical/enzymatic tumor tissue digestion, and CD11b⁺ magnetic beads to capture all microglia, monocytes, and macrophages in gliomas. Using qRT-PCR we showed higher expression of *ARG1*, *CXCL14*, *NOS2* mRNAs in GAMs from high-grade gliomas (mostly GBMs) than in low-grade gliomas.²² Interestingly, the *IKK β* mRNA and its protein IKK β were downregulated in GBM tissues, and lower *IKK β* expression was detected in CD11b⁺ sorted from HGG than LGG.²² The *IKK β* gene encodes a kinase IKK β , which phosphorylates inhibitory I κ B proteins and represents a convergence point for most signaling pathways leading to NF κ B activation. The transcription factor NF κ B is a downstream mediator of stimulation by the TLR or TNF α /IL-1 β receptors, and its activation depends on the phosphorylation of an inhibitor I κ B by IKK β resulting in release of NF κ B, translocation to the nucleus and activation of inflammatory genes. NF κ B activation, which was prominent in GAMs infiltrating pilocytic astrocytomas, was reduced in GAMs infiltrating GBMs due to reduced IKK β expression in GBMs.²² The failure of activating the IKK β -NF κ B signaling pathway could be responsible for reduced expression of immune/inflammatory genes in GAMs, and shaping the immunosuppressive microenvironment in GBMs. We performed the analysis of transcriptomes of GAMs (CD11b⁺ cells) isolated from WHO grade I pilocytic astrocytomas and GBMs. The upregulated genes in GAMs from GBM patients were associated with mitotic cell cycle, cell migration, cell adhesion, response to wounding, and extracellular matrix organization. Functional annotations showed overrepresentation of gene classes related to cell adhesion, chemotaxis, and cell proliferation. Dominating transcriptional responses in GBM CD11b⁺ cells are consistent with the protumorigenic, non-immune activation of GAMs (unpublished) which is in agreement with published studies.⁴¹ Table 1 summarizes data

on phenotypic and functional markers of microglia/macrophages in human gliomas.

In a recent study, microarray analyses and microRNA expression profiling of different myeloid populations (microglia, macrophages, and MDSCs) from GBM with matched blood monocytes, normal brain microglia, non-polarized M0, and polarized M1, M2a, M2c macrophages were performed. GBM patients have the augmented number of monocytes relative to healthy donors. Among CD11b⁺ cells, microglia and MDSCs were more abundant than macrophages. Flow cytometry showed higher levels of MHC II and CD86 (B7-2) expression on MDSCs than on microglia and macrophages.⁴² Also CD80 (B7-1) was more highly expressed on MDSCs and macrophages than on microglia. B7-1 and B7-2, ligands for CD28, are prototypic co-stimulatory molecules expressed primarily on antigen-presenting cells. Optimal activation of T cells relies upon antigen presentation to the T-cell receptor (TCR), and is enhanced by co-stimulation via CD28/B7 ligation.⁴³ Expression of CD163 and CD206 (M2 activation markers) was higher in the MDSCs and macrophages than in the microglia. The transcription factor pSTAT1 (involved in macrophage polarization) and its target a pro-inflammatory TNF- α was most commonly expressed in MDSCs. Immunosuppressive cytokines, TGF- β 1 and IL-10, were expressed by all types of cells: MDSCs, microglia, and macrophages, but the highest expression was in macrophages.⁴² The mesenchymal subtype of GBM had the lowest expression of TNF- α . The highest expression of CD163 and CD206 was detected in MDSCs within classical GBMs, it was highly variable in MDSCs and macrophages in proneural GBMs. Nanostring expression profiling of 88 genes characteristic for M0, M1, M2a, and M2c macrophages in CD14⁺ blood cells, and tumor-infiltrating CD14⁺ cells from GBM patient demonstrated separation of GBM patient CD14⁺ blood cells from other groups, and displayed similarity of the GBM-infiltrating CD14⁺ cells to non-polarized M0 macrophages. The authors concluded that GAMs exhibit distinct immunological functions than peripheral macrophages and their expression profiles are similar to non-polarized M0 macrophages.⁴²

One of the difficulties to refine definition of human GAMs phenotype lies in heterogeneity of isolated CD11b⁺ cells, which includes also CD11b⁺Gr1⁺ MDSCs bearing own transcriptomic characteristics. Altogether, gene expression profiling of human CD11b⁺ infiltrates points to a lack of the classical 'immune response' activation in GBMs, and indicates that GAM and MDSC populations assume discrete phenotypes along the M1–M2 continuum, therefore, more studies are required to ascertain their functions.

Myeloid-Derived Suppressor Cells in Gliomas

Myeloid-derived suppressor cells are a heterogeneous population of early myeloid progenitors and precursors at different stages of differentiation into granulocytes, macrophages, and dendritic cells. In mice, MDSCs are defined as CD11b⁺Gr-1/Ly-6G⁺IL4R α ⁺CD11c⁻F4/80^{+/+} cells, but based

on the relative expression levels of Ly-6G and Ly-6C two specific subsets: granulocytic (G-MDSCs) $CD11b^{+}Gr-1^{hi}Ly-6C^{low}Ly-6G^{+}CD49d^{-}$ and monocytic (Mo-MDSCs) $CD11b^{+}Gr-1^{int}Ly-6C^{hi}Ly-6G^{-}CD49d^{+}$ MDSCs were identified. A minor population of 'early stage' e-MDSC, defined as Lin^{-} (including $CD3^{-}$, $CD14^{-}$, $CD15^{-}$, $CD19^{-}$, $CD56^{-}$) HLA-DR $^{-}CD33^{+}$ cells, contains more immature progenitors.^{44,45} Gr-1 antigen is a cell-surface protein that belongs to the Ly-6 family of proteins. In rats MDSCs could be identified as $CD11b^{+}Gr-1^{+}$ cells.

Human MDSCs are identified as HLA-DR $^{-}CD11b^{+}CD14^{-}CD33^{+}$ cells that express the common myeloid marker CD33 and lack the expression of markers of mature myeloid and lymphoid cells. The ortholog to Ly6C or Ly6G has not been described in humans. In humans, precise identification of G- and Mo-MDSC subsets is more difficult, and these cells have been characterized by combination of overlapping myeloid markers. Some studies discriminated six human MDSC phenotypes: MDSC1 ($CD14^{+}IL-4R\alpha^{+}$), MDSC2 ($CD15^{+}IL-4R\alpha^{+}$), MDSC3 ($Lineage^{-}HLA-DR^{-}CD33^{+}$), MDSC4 ($CD14^{+}HLA-DR^{low/-}$), MDSC5 ($CD11b^{+}CD14^{-}CD15^{+}$), and MDSC6 ($CD15^{+}FSC^{low}SSC^{high}$) using multi-color staining.^{44,45} The prior lack of standardization led to the establishment of a human MDSC proficiency panel under the guidance of the Association of Cancer Immunotherapy (CIMI) Immunoguiding Program. The currently accepted phenotypic definitions of human MDSCs are now $CD11b^{+}CD14^{+}CD33^{+}HLA-DR^{-/low}Co-receptor^{-/low}$ for macrophage-MDSCs and $CD11b^{+}CD15^{+}CD33^{+}Lin^{-}HLA-DR^{-/low}$ for polymononuclear-MDSCs, present in the mononuclear fraction.⁴⁵ A more immature subset of human early stage e-MDSCs was characterized by the absence of staining for the lineage markers ($CD3^{-}$, $CD14^{-}$, $CD15^{-}$, $CD19^{-}$, $CD56^{-}$), HLA-DR $^{-}$ and expression of the common myeloid markers CD33 and CD11b.⁴⁵

Normal BM contains 20–30% of cells with the MDSC phenotype, a small proportion (2–4%) is present in the spleen. Immature myeloid cells generated in BM quickly differentiate into mature granulocytes, macrophages or dendritic cells, therefore, in healthy individuals, immature myeloid cells comprise ~0.5% of peripheral blood mononuclear cells. Under pathological conditions such as cancer, infectious diseases, sepsis, trauma, BM transplantation, or autoimmune disorders, the emergency myelopoiesis and/or blockade in cell differentiation leads to an expansion of this population.⁴⁵ MDSCs have ability to suppress the cytotoxic activities of natural killer (NK) cells, the adaptive immune response mediated by $CD4^{+}$ or $CD8^{+}$ T cells in antigen dependent or non-dependent manner and induce apoptosis of T-cell subsets.⁴⁵ The two MDSC subsets inhibit immune responses through different mechanisms: PMN-MDSCs suppress antigen-specific $CD8^{+}$ T cells mainly by producing reactive oxygen species, whereas M-MDSCs express nitric oxide synthase 2 (NOS2) and arginase (ARG1), that metabolize L-arginine and block translation of the T cell CD3 zeta chain, generate reactive nitrogen species,

inhibit T-cell proliferation, and promote T-cell apoptosis. Moreover, MDSCs secrete immunosuppressive cytokines and induce regulatory T-cell progression.⁴⁵

Numbers of MDSCs are frequently increased in blood, spleen, and tumor mass, and correlate with cancer stage, metastasis, and chemotherapy response.⁴⁵ Information regarding their presence and roles in gliomas is scarce.^{44,46} Gielen *et al* reported increased percentages of both M- and PMN-MDSCs in the blood of GBM patients when compared with healthy donors. The myeloid activation markers B7-1/CD80 and PD-L1 were not detected. The MDSC population in tumor cell suspensions consisted almost exclusively of $CD15^{+}$ PMN-MDSC cells. Immunohistochemistry confirmed infiltration of glioma tissues with $CD15^{+}/HLAII^{-}$ cells.⁴⁴ Raychaudhuri *et al* reported that patients with GBM have elevated levels of MDSCs compared with age-matched healthy donors and other cancer patients. The majority of the MDSCs in patients with GBM were $CD15^{+}CD14^{-}$ PMN-MDSCs (82%), followed by Lyn^{-} eMDSCs (15%) and M-MDSCs (3%).⁴⁶ Application of multicolor flow cytometry for the analysis of peripheral blood and tumor samples of 52 GBM patients revealed the significantly higher frequency of $CD14^{+}$ M-MDSCs and $CD15^{+}$ PMN-MDSCs in blood of GBM patients when compared with healthy controls. Moreover, correlation between the number of PMN-MDSCs and $CD4^{+}$ effector memory T-cells ($CD4^{+}T_{em}$) within the tumors was detected. Tumor-derived $CD4^{+}T_{em}$ expressed high levels of an inhibitory molecule PD-1 and were functionally exhausted. The expression of PD-L1 was significantly upregulated on tumor-derived MDSCs.⁴⁷ These findings show accumulation of different MDSC subsets in GBM patients and indicate that PMN-MDSCs in peripheral blood and at the tumor site may participate in GBM-induced T-cell suppression.

Growing evidence shows that the phenotype and mechanisms of action of MDSCs are tumor-dependent, therefore, it is important to determine the presence of all MDSC subsets in each cancer type and even individual patient. There is the lack of systematic studies on the abundance of specific MDSC subpopulations and their impact on numbers and subtypes of adaptive immunity cells. Furthermore, transcriptomic analyses should be carried out on MDSCs separately from microglia/macrophages to establish their functional phenotype. Molecular mechanisms responsible for MDSC migration to a tumor and inhibition of their differentiation need to be elucidated. It has to be clarified whether T-cell suppression occurs in an antigen-specific manner and whether targeting these cells in cancer patients will be of clinical significance.

LEUKOCYTE INFILTRATES IN GLIOMA MICROENVIRONMENT

Glioma Infiltrating T Helper (Th), T Cytotoxic (Tc), and Treg Cells

The glioma microenvironment is infiltrated with leukocytes, mostly $CD4^{+}$ T helper (Th), $CD8^{+}$ T cytotoxic (Tc), and $CD4^{+}CD25^{+}FoxP3^{+}$ Treg.^{9–11,26,48} $CD8^{+}$ T cells that express

the transcription factors Eomes and T-bet are destined to develop into cytotoxic effector cells that produce IFN γ , granzyme B, and perforin. CD8 $^{+}$ T cells sorted from GBM tissue were phenotypically CD8 $^{+}$ CD25 $^{-}$, which indicates a lack of activation.²⁶ It is believed that CD8 $^{+}$ T cells with a constitutive CD25 (the IL-2R α chain) expression and expressing CD45RO, CD28, and the lymph node-homing markers CD62L and CCR7 are memory T cells. CD4 $^{+}$ T cells were more numerous than CD8 $^{+}$ T cells in a GBM tissue (20 vs 11% gated lymphocytes). The CD4 $^{+}$ population increased with tumor grade, starting at 39% in WHO grade II to 73% in WHO grade III, and 98% in grade IV. Percentages of both CD4 $^{+}$ and CD8 $^{+}$ tumor-infiltrating T cells increased with tumor grade.⁴⁹ FACS analysis of freshly dissociated 93 glioma samples (II–IV grades), revealed that the CD8 $^{+}$ T-cell population consisted >93% of CD3 $^{+}$ CD8 $^{+}$ cells. Infiltration of T helper cells (Th, CD3 $^{+}$ CD8 $^{-}$) and T cytotoxic cells (Tc, CD3 $^{+}$ CD8 $^{+}$) was observed in all samples. A significant correlation between increased intratumoral numbers of CD3 $^{+}$ and CD8 $^{+}$ cells and prolonged patient survival was observed, whereas no association was found with CD4 $^{+}$ cells.⁴⁹ A large neuropathological study evaluated infiltrating CD8 $^{+}$ T cells histologically in patients with newly diagnosed GBM in relation to long-term clinical survival (>403 days). GBM patients with intermediate or extensive CD8 $^{+}$ T-cell infiltrate at the time of diagnosis were more likely to have long-term survival than patients with rare or focal CD8 $^{+}$ T-cell infiltrates.¹¹

Immunocytochemical studies demonstrate that infiltrating T cells were frequently observed in WHO IV fibrinogen-positive GBM areas, which supports a hypothesis that leaky vessels, which typically occur in GBMs, facilitate T-cell transmigration.¹⁰ On the other hand, augmented infiltration of effector CD3 $^{+}$ FOXP3 $^{-}$ T cells was associated with the expression of a cell adhesion molecule ICAM-1 on the vessels, and transmigration of T cells *in vitro* was markedly reduced in the presence of CAM-blocking antibodies.¹⁰

The lack of proper T-cell activation in tumor microenvironment is attributed to the fact that antitumor T-cell responses are suppressed by cytokines TGF- β and IL-10 secreted by glioma cells (for a review ref. 13). Moreover, glioma cells lack B7.1/2 (CD80/86) co-stimulatory molecules and overexpress B7-H1 mRNA and protein. B7-H1 expressed in glioma cells is a strong inhibitor of CD4 $^{+}$ as well as CD8 $^{+}$ T-cell activation as assessed by cytokine production (IFN- γ , interleukin-2, interleukin-10) and the expression of CD69, the T-cell activation marker.⁵⁰ GBM-infiltrating T cells had decreased INF- γ production and the increased *PD-1* mRNA level.⁴⁷ Apart from directly blocking effective activation of naive T cells, glioma cells promote the accumulation of immunosuppressive cells including Treg and regulatory dendritic cells in the tumor environment. These cells mediate inhibition of T cells, whereas MDSCs and Treg maintain inhibition of NK cells.

Treg (CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$) cells are known as potent suppressors of the adaptive immune response, because of the ability of this subset to inhibit the proliferation of any cytokine-secreting effector T cells. Accumulation of Treg subsets was observed in different types of cancer and correlated with worse prognosis.¹² Treg are necessary to restrict and resolve activation of the immune system. In one of the first reports evaluating Tregs using flow cytometry, it has been reported that gliomas were infiltrated predominantly with CD8 $^{+}$ CD25 $^{-}$ T cells and CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$ regulatory T cells.²⁶ Relatively high content of Tregs (CD4 $^{+}$ FOXP3 $^{+}$ CD25 high CD127 low) accounting for 14% of total CD4 $^{+}$ T-cell population was further reported.⁵¹ Immunocytochemical studies indicated the presence of up to 10% Treg in human GBMs and contribution of mostly thymus-derived, Helios expressing Tregs.⁴⁸ In contrast to these reports, Lohr *et al*¹⁰ observed a very limited infiltration of Treg cells (detected as FOXP3 $^{+}$ CD3 $^{+}$ cells in flow cytometry) that accounted for <1% of total T cells identified in GBMs. In another study, CD8 $^{+}$ CD28 $^{-}$ FOXP3 $^{+}$ were identified in 3/5 of patients, making up $2.08 \pm 0.99\%$ of all T cells.⁵² These contrasting results likely occurred due to the use of different methods and markers for cell type detection. Gene expression profiling of human GBMs indicated overexpression of Treg markers such as: nuclear transcription factor (FOXP3), membrane-resident interleukin-2 receptor alpha (IL-2R α /CD25), cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR).¹² Despite reported differences, the FOXP3 $^{+}$ cells likely represent 10–14% of the total CD4 $^{+}$ population.¹³ The prognostic implication of Tregs accumulation in patients with GBM is not clear. Although two groups: Heimberger *et al*⁵¹ and Lohr *et al*¹⁰ found no association between Tregs and prognosis in patients with GBM, Yue *et al*⁵² reported a significant association between the density of Tregs infiltrating GBM and poor prognosis. Further analysis of Tregs, other T-cell subsets and their activation state in GBM tissue and patient blood is required to draw conclusions. Dissecting heterogeneity and specific roles of intratumoral T cells in gliomas may be of critical importance for the design of future immunotherapies.

Natural Killer Cells in Gliomas

Natural killer cells (NK; characterized as CD3 $^{-}$ CD56 $^{+}$ CD16 $^{+}$) are effective cytotoxic lymphocytes that contain both perforin-rich and granzyme-rich granules, and are capable of killing cancer cells and virally infected cells. Human NK cells can be subdivided into different populations based on the relative expression of the surface markers: CD16 and CD56. Activation of NK and effector T cells is inhibited by interactions of HLA class I antigens with certain receptors originally identified as NK cell receptors but also variably expressed on $\alpha\beta$ and $\gamma\delta$ T cells. These inhibitory NK cell receptors include isoforms of the killer cell immunoglobulin-like receptors (KIR) and immunoglobulin-like transcript/

leukocyte immunoglobulin-like receptors (ILT/LIR) that interact with HLA-A, -B, -C, or -G antigens, and a heterodimer formed by the C-type lectin CD94/NKG2A that interact with HLA-E.⁵³ Binding of inhibitory KIRs, expressed by NK cells, to the HLA class I molecules protects non-pathologic cells from being killed and maintains NK cells tolerance. After disruption of the homeostasis, HLA class I expression is reduced and this impairs the tolerance of NK cells.⁵⁴ A major barrier to effective NK cell-mediated killing of malignant gliomas is high HLA class I expression. The expression of ectopic HLA-G, a non-classical MHC molecule, was described as a different, tumor-dependent mechanism to paralyze the immune system. Primary GBMs and established glioma cell lines express ectopic HLA-G, which may protect the tumor from T and NK-mediated killing.⁵⁵ NKG2D (natural killer group 2, member D) is a C-type, lectin-like homodimeric receptor expressed by human NK, $\gamma\delta$ T, and CD8⁺ $\alpha\beta$ T cells. Ligation of specific NKG2D results in the production of pro-inflammatory cytokines such as IFN- γ and the release of cytotoxic granules that cause tumor cell lysis. NKG2D interacts with ligands that are not constitutively expressed but are inducible, such as human MHC class I chain-related A (MICA) and MICB, and UL16-binding proteins.⁵⁶ Escape from the NKG2D-mediated immune surveillance of malignant gliomas may be promoted by the inhibition of MICA and ULBP2 expression by glioma-derived TGF- β and by metalloproteinase-dependent shedding from the cell surface.⁵⁷

Moreover, percentages of NK cells among peripheral blood mononuclear cells isolated from GBM patients were reduced.⁵⁸ Poli *et al* reported that NK cells account for a minor part of infiltrating CD45⁺ cell population in GBMs. Infiltrating NK cells were non-functional, possibly due to the contact with immunosuppressive cells, such as GAMS, MDSCs, and Tregs.⁵⁹ These cells inhibit activities of NK cells by suppressing NKG2D expression and production of INF- γ .⁶⁰ Kmiecik *et al* identified NK cells ($2.11 \pm 0.54\%$) and rare B cells ($0.66 \pm 0.27\%$) in eight GBM biopsies. NK cells from GBM were characterized by the CD56^{dim}CD16⁺ phenotype and $57.45 \pm 12.05\%$ expressed NKG2D.⁴⁹ B cells (HLA-DR⁺CD19⁺) were infrequent in both GBM and normal CNS tissue.²⁶

LESSONS FROM GLIOMA EXPERIMENTAL MODELS

Glioma Experimental Models

The use of *in vivo* models of malignant glioma shed a light on composition of tumor microenvironment, its influence on disease progression and outcome, as well identified new therapeutic targets for treatment. Animal models attempt to mimic molecular profiles of gliomas as well as underlying biology. Molecular analysis of human gliomas have identified a number of mutations and alterations within the human glioma genome.⁶¹ Retroviral or transgenic glioma models allowed for incorporation of most common mutations found in GBM, creating molecular profiles as seen in the clinic.⁶²

Another models are xenografts and transplantation of established glioma cell lines. Because of the availability of the reviews describing glioma animal models,⁶³ here we only briefly outline most commonly used models underlying their usefulness for defining immune microenvironment.

The method widely used in biomedical research is intracranial or subcutaneous injection of tumor cells into the mouse or rat (Figure 1). Established cell lines (eg, C6, 9 L, GL261) that originated from tumors chemically induced by methylnitrosourea or methylcholanthrene, are used to study biology of glioma or new therapeutic agents. The C6 and 9 L gliomas were created using the same method but show distinct characteristics. The first one, resembles more the molecular and histopathological profile of the human GBM.^{64,65} The diffuse nature of human GBM is well represented when C6 cells are implanted into the Wistar rats, but not to Sprague-Dawley or Long-Evans rats.⁶³

Orthotopic xenografts retain human GBM features and are considered to be a useful model for therapeutic studies.⁶⁶ Gene expression analysis of mouse xenografts growing from transplanted human U87-MG, LN18, or U251 glioma cells revealed similar pattern to that of human GBM samples, suggesting that the behavior of human U87-MG or U251 glioma cells is representative of that seen in the clinical settings.⁶⁷ Nevertheless, this was only shown for one subset of the heterogeneous human GBM. Xenograft models also lack the proper immune environment due to the use of immunocompromised mice. The BALB/c-nude strain has an innate immune system but no acquired immunity, whereas both the innate and acquired immune systems are impaired in the NOD-scid IL2Rgamma^{null} (NSG) mice. This raises an issue for the tumor immunology research in which such models should not be employed.

Figure 1 Most common glioma experimental models. Xenografts of human U87-MG and LN18 cell lines. U87-MG carries mutation in PTEN,²²⁶ wild-type p53 (p53 WT),²²⁷ wild-type IDH1,²²⁸ and overexpression of MHC I and MHC II.²²⁹ Human LN18 glioma cell line carries mutation in p53,²³⁰ has wild-type PTEN,²³¹ overexpression of TNFR1,²³² and downregulation of Kras. Mice allograft commonly used employ implantation of GL261 that carries mutation in p53 with overexpression of MHC I and c-myc.²³³ C6 rat glioma show downregulation of MHC I and MHC II,²³⁴ and carries wild-type p53.²³⁵

The advances in molecular biology and gene editing made it possible to generate models that spontaneously form tumors in immunocompetent mice. Disruption of the p53, Rb, Pten, and Nf1 pathways, and others lead to formation of tumors resembling human tumors.⁶⁸ Mice harboring heterozygous loss of function mutations for the *Nf1* and *p53* tumor suppressor genes develop glioma spontaneously, however, with different tumor incidence depending on mouse strain genetic background. A second generation of NF1-based glioma models relies on the use of conditional knockout (Cre/LoxP) system that can specify the cell type in which tumor suppressor gene inactivation is induced. These mice developed glioma with manifestation of pathological features of grades II, III, and IV tumors. An S100b-v-erbB transgenic mice and rats develop low-grade and malignant anaplastic oligodendroglioma with over 80% of tumors localized near or within the cerebellum. The use of retrovirus-mediated expression of oncogene allowed for more precise initiation of tumor formation, thus allowing to study a cell origin of GBM. This was shown by triggering GBM formation in genetically engineered mice that expressed a retroviral receptor under nestin promoter (neuronal stem cells—NSCs and early progenitors) or GFAP (primarily in glia, but also NSCs). Introduction of activated K-ras and Akt to neuronal progenitors led to formation of GBM. This was not observed when introduced to more differentiated astrocytes/glia.⁶⁹ Using a similar system two mouse models of PDGF-driven glioma were generated. First model employs a Moloney murine leukemia virus containing the human *PDGF B*-chain gene for infecting proliferating cells in the neonatal brain, which leads to c.a. 40% of mice developing tumors within 14–29 weeks with mixed histology (majority exhibit characteristics of GBM or PNET). The second strategy uses the RCAS/tv-a system that employs genetically engineered mice and a viral vector RCAS that is introduced only to the cells expressing tv-a receptor.⁷⁰ This system was used to model a human adult glioma driven by the *PDGF* oncogene in mice expressing tv-a. Newborn *tv-a* pups with *PDGFB* overexpression in different locations such as cortex, brain stem, and cerebellum have been generated to identify the role of anatomical location in tumorigenesis or were combined with a loss of various tumor suppressors such as *Ink4a-arf*, *p53*, and *Pten* (for a review see ref. 71). It is believed that these transgenic murine tumors recapitulate the three main GBM subtypes: proneural-PDGF, classical-EGFR and mesenchymal-NF1.⁷² The information about genetic background of frequently used animal glioma models is presented in the Figure 1.

IMMUNE ENVIRONMENT OF EXPERIMENTAL GLIOMAS

Microglia/Macrophages in Rodent Glioma Models

Animal models have been used to study the microenvironment of glioma and its influence on the surrounding cells.⁷³ Using flow cytometry to determine the proportion of microglia (CD11b/c^{high}, CD45^{low}), macrophages (CD11b/

c^{high}, CD45^{high}), and lymphocytes (CD11b/c⁺, CD45^{high}) in single cell suspensions of rat C6, 9 L, and RG-2 gliomas, Badie and Scharfner²⁵ demonstrated that microglia infiltration is dependent on the glioma cell line and does not correlate with the tumor size. Microglia accounted for the highest proportion of the mass (13–34%) within the tumor periphery. The highest infiltration was seen in C6 gliomas. Macrophages accounted for 5–12% of cells and showed no significant difference between C6, 9 L, and RG-2 gliomas.²⁵ Gliomas generated by an intracranial transplantation of GL261 cells to the CSF-1 R-GFP(+) macrophage Fas-induced apoptosis (MAFIA) transgenic mice showed accumulation of both cell populations, with macrophages accumulating preferentially within the tumor center, rather than periphery.⁷⁴

The RCAS-PDGFB tumor model, in which primary RCAS-PDGFB tumors were re-transplanted into *Cx3cr1^{GFP/wt}* *Ccr2^{RFP/wt}* mice, was used to evaluate GAM infiltration. The system allows to isolate RFP⁺/GFP⁺ microglia and RFP⁺/GFP^{low} macrophages/monocytes from tumors. Flow cytometry demonstrated 14% microglia and 8.5% macrophages/monocytes in the RCAS-PDGFB gliomas and similar proportion of GAMs in GL261 tumors (16% microglia and 6.5% macrophages/monocytes).⁴⁰

The intriguing question is a phenotype of GAMs in different glioma models. Microglia stimulated *in vitro* to glioma-conditioned medium or lipopolysaccharide exhibit responses similar to M1 and M2 activation.⁷⁵ First studies showed that GAMs infiltrating GL261 glioma express M2 phenotype genes: *Arg1*, *Mmp14* (*Mt1-mmp*), *Cxcl14*, *Chi3l3* (*Ym1*).^{76,77} Similar pattern of gene expression was detected in CD11b⁺ cells isolated from rat C6 gliomas and human GBM (unpublished). *Mmp-12* and *Mmp-13* mRNA levels were upregulated in GAMs isolated from GL216 gliomas indicating their pro-invasive function.⁷⁸ Transcriptomic profiles of GAMs from GL261 tumors showed gene overlaps with transcriptomes of M1- and M2b-polarized macrophages.⁴⁰ The analysis of genes characteristic for M1 (*Isg20*, *Il1rn*) or M2 (*Tgfb1*, *Clec7a*, and *Cxcr4*) activation showed a significantly higher expression of selected genes in glioma-associated macrophages, and increased *Il1rn*, *Clec7a*, and *Cxcr4* mRNA levels in glioma-associated microglia. The expression of the selected genes (in particular *Isg20*, *Il1rn*, and *Clec7a*) was lower in GAMs from the RCAS-PDGFB gliomas when compared with the GL261 gliomas.⁴⁰ *Gpnmb* and *Spp1* genes were upregulated in GAMs in both GL261 and RCAS-PDGFB gliomas, with invading macrophages as the major source for *Gpnmb* in the RCAS-PDGFB model, whereas resident microglia were the main source for *Spp1* in both models.⁴⁰

It is apparent that GAMs from rodent models of gliomas (C6, GL261, and less extent RCAS-PDGFB gliomas) show a pro-invasive, immunosuppressive phenotype reminiscent of the M2 phenotype observed in non-CNS tumors. However, intersection of the marker genes of discrete M-type responses points to a distinct phenotype of GAMs, only partially

overlapping with markers of M1/M2 phenotype.⁴⁰ Data on molecular profiles of GAMs from different animal models are still inconclusive and more studies are required to reach a consensus. Interpretation of human GAMs data, which should take into account genomic heterogeneity of tumors and subtypes, is even more complicated.

Lymphocytes in Glioma Models

Comparative analysis of infiltrating lymphocytes into the glioma in patients and RCAS-PDGFB gliomas, have shown that infiltrating CD3⁺CD4⁺ cells comprise only around 3%, and CD3⁺CD8⁺ only 2% of the tumor cell suspension.⁷⁹ Infiltrating lymphocytes when compared with GAMs were of very low frequency. In addition, there is a correlation between infiltrating microglia/macrophages and functional impairment of T cells.⁸⁰ The extent of lymphocyte infiltration differs in experimental gliomas. CD8⁺ cells represented around 22% of infiltrating immune cells in C6 and 9 L gliomas, but only 5% in RG-2 glioma.²⁵ This suggests that two first glioma cell lines possess more immunogenic nature than RG-2 cells, thus the C6 and 9 L models are not very representative for GBM lymphocyte accumulation.⁸¹ An increased frequency of CD11b⁺Gr-1⁺ immature myeloid cells (e-MDSCs) was associated with diminished CD8⁺ T-cell infiltration. In mice with a knockout of the histidine decarboxylase, the enzyme responsible for histamine production, more profound suppression of CD8⁺ T-cell proliferation and functions, associated with increased prostaglandin PGE2 expression levels, was demonstrated.⁸² Maes *et al*⁸³ have shown that depletion of Tregs within the tumor allows infiltration of non-immunosuppressive myeloid cells in mice with GL261 gliomas. This shows that, even though the infiltration of immune cells is very low, it has a big impact on immunosuppressive environment and glioma growth. Owing to a scarce number of studies, there is no sufficient data to compare immune infiltrates between experimental glioma models, in particular transgenic ones. Tumor immunology should be considered when choosing the animal model for preclinical or drug screening studies, as it can influence the therapeutic outcome.

Plasticity and Identity of Microglia and Infiltrating Macrophages within the Brain

Microglia originate from hematopoietic progenitor cells during the early embryonic development and share some of the common markers such as CD11b and CD14 with the other peripheral immune cells. However, microglia populate the CNS early in development and have the exclusive functions, such as synaptic pruning, interactions with neurons and other glial cells, maintenance of proper functioning of the neural circuitry.^{84–86} Microglia secrete neurotropic factors that recruits neuronal progenitor cells to the site of injury contributing to regeneration and tissue repair. Even though microglia are capable of self-renewing and maintain their steady state population, peripheral macrophages do not

contribute to the turn-over of microglia under physiological conditions. Experiments with the use of BM transplantation after irradiation or parabiosis (in which two mice are surgically joined) did not support the notion that BM-macrophages enter CNS under normal conditions.^{87,88}

In most of neurologic diseases and CNS cancers, the infiltration of peripheral immune cells into the brain has a very important role in the etiopathology of the disease (for reviews see refs 89,90). In mouse disease models, labeling of cells and the use of chimeras facilitated neuroscientists to distinguish resident cells (CD45^{low}) from the infiltrating ones (CD45^{high}). Various studies on animal models of cerebral ischemia and multiple sclerosis using mouse chimeras showed that microglia maintain their CD45^{low} status even under inflammatory conditions and do not contribute significantly to the CD45^{high} pool of BM-macrophages.^{91,92}

Recent studies comparing gene expression and chromatin landscape of myeloid cells from seven tissues (including CNS) revealed distinct enhancer/promoter landscapes, which reflects the ontogeny and function of an individual type of macrophages. Experiments with transplantation of BM cells from an adult donor into the lethally irradiated recipients have demonstrated that donor cells replace the endogenous embryo-derived macrophages and at the same time acquire a unique epigenetic landscape in accordance with their tissue of migration. Moreover, transplantation of differentiated macrophages from a place of residence into a different microenvironment induced the genes that are specific to the transplanted microenvironment, whereas downregulate genes constituting the identity of its original source.^{93,94} These compelling evidence shows how plastic these cells are and uncovers their capability of reshaping their epigenetic landscape according to the microenvironment. Although all myeloid cells, including microglia, depend on a master transcription factor Pu.1, co-binding of other tissue specific transcription factors mediates the expression of specific gene pattern. For example, the function of macrophages largely depends on the activity of transcription factors Myb and FLT3, whereas microglial functions depends on CSF-1 and FLT3.^{87,88}

Recent transcriptomics studies using microarray and direct RNA sequencing have revealed unique patterns of gene expression in microglia and BM-macrophages. Among all transcripts 40% were shared by both microglia and peripheral macrophages, which indicates their lineage relationship and functional similarities. More than 30% of the transcripts were expressed only in microglia or macrophages. Genes such as *Hexb*, *Trem2*, *Gpr34*, *Tmem119*, *Cx3cr1*, *Siglech*, and *P2ry12*, *P2ry13* were shown to be expressed exclusively in microglia, whereas genes such as *Cxcr7*, *Ifitm3*, *Pilra*, *Itgb2* were enriched on peritoneal macrophages.^{95,96} Functions of those putative discriminating markers are summarized in the Table 2.

The question whether the circulating monocytes entering the CNS acquire microglial signature was addressed by Butovsky *et al*⁹⁷ in an EAE model using Cx3cr1^{GFP/+} chimeric mice. Transcriptomic analysis demonstrated that microglia

Table 2 List of putative microglia and macrophage specific markers and their functions

Putative markers	Functions	References
<i>Microglia</i>		
Hexb	Hexosaminidase B; expressed in lysosomes, abundant in microglia. Mice lacking both Hexa and Hexb subunits show the pathologic features of the mucopolysaccharidoses and lysosomal storage diseases caused by accumulation of glycosaminoglycans. Defects in the human <i>HEXB</i> gene combined with deficiencies of HexA lead to excessive accumulation of GM2 ganglioside and <i>N</i> -acetylglucosamine oligosaccharides in a recessive lysosomal storage Sandhoff disease.	236
P2ry12	Purinergic receptor, implicated in wound healing and chemotaxis of microglia in response to ATP released from apoptotic neurons.	237,238
Trem2	Triggering receptor expressed on myeloid cells; participates in phagocytosis and inflammatory responses to injury or disease. During infection Trem2 restrains dendritic cells and macrophage phagocytosis, and reduces pro-inflammatory cytokines release through DNAX-activation protein 12 (DAP12) signaling. Trem2 KO mice had attenuated phagocytosis, worst neurological recovery and decreased viable brain tissue. Rare <i>TREM2</i> variants affecting TREM2 function increase risk of Alzheimer disease (AD), Trem2 deficiency prevents microglial clustering around A β deposits in murine AD models. Trem2 is induced upon IL-4 stimulation and expressed in GAMs.	239,240
Gpr34	G $\frac{1}{G}$ protein-coupled receptor of the nucleotide receptor P2Y $\frac{12}{1}$ -like group; involved in microglial chemotaxis; induced upon an inflammatory stimulus. GPR34 deficiency results in morphological changes in retinal and cortical microglia, and reduced phagocytosis. DCs lacking GPR34 have a higher caspase-3/7 activity and increased apoptosis levels.	241
Cx3Cr1	The chemokine CX3CL1/fractalkine receptor, expressed predominantly on microglia; involved in neuron-microglia interaction and immune cell trafficking in the CNS; neuronal CX3CL1 acts as a critical inhibitory signal retaining microglia in quiescent mode and preventing hyper-activation; Cx3cr1 $-/-$ mice had increased tumor incidence and shorter survival times but similar numbers of GAMs, trafficking of inflammatory monocytes into the CNS was increased.	242,243
Siglech	Sialic acid binding Ig-like lectin, expressed on activated microglia, has a role in phagocytosis	244
<i>Macrophages</i>		
CXCR7	Receptor for the chemokine ligand CXCL12/SDF-1; found to be upregulated in TAMs; its targeting inhibits glioma growth and increases survival	245
Ifitm6	Interferon induced transmembrane protein 6; expressed in macrophages from tumor bearing mice; induced upon exposure to TLR agonists	246
Pilra	Paired immunoglobulin-like type 2 receptor Alpha; acts as a cellular signaling inhibitory receptor by recruiting cytoplasmic phosphatases PTPN6/SHP-1 and PTPN11/SHP-2 via their SH2 domains, which blocks signal transduction through dephosphorylation of signaling molecules. PILRA regulates neutrophil recruitment to the inflammatory sites. Pilra $-/-$ mice develop more severe arthritis and have reduced the production of pro-inflammatory cytokines, corroborating the inhibitory role of Pilra during inflammation.	247
Itgb2	Integrin receptor beta2A/macrophage antigen-1 (Mac-1) is a complement receptor CR3 consisting of CD11b (integrin α_M) and CD18 (integrin β_2); Mac-1 represents the predominant β_2 integrin on peripheral mononuclear cells, is expressed on monocytes/macrophages and NK cells, and serves as a receptor for adhesion molecules such as ICAM-1	248

and macrophages maintain their unique molecular signature even under inflammatory conditions. However, some degree of plasticity exists, eg, *Trem2* is expressed only on microglia under normal conditions, however IL-4 stimulation induced *Trem2* in peripheral and infiltrating BM-macrophages. We found that primary microglial cultures exposed to glioma-conditioned medium upregulate *Trem2* mRNA levels,

whereas LPS stimulation sharply downregulates its expression (unpublished). This is in agreement with the previous reports depicting *Trem2* as a M2 phenotype marker induced by IL-4 stimulation, whereas IFN- γ /LPS rapidly down-regulated its level.^{98,99} These results suggest a considerable functional plasticity of microglia and its adaptation to environmental cues.

It has been suggested that in BM transplantation studies immune infiltration to the CNS was exaggerated due to irradiation affecting the blood-brain barrier integrity. In a study using head-protected irradiation chimeras, in which an unspecific influx of donor-derived myeloid cells was reduced, the reduction in the tumor volume was demonstrated. A minor subset of microglia upregulated the CD45 expression, which could contribute to detection of the CD45^{high} population in the tumor.¹⁰⁰ Altogether, recent evidence points out that microglia and macrophages exhibit a unique gene expression pattern under normal conditions, but pathological microenvironment may alter transcriptomic signatures and cell functions. Understanding cellular heterogeneity of microglia and infiltrating macrophages, and dissecting their roles would help in exploiting the peripheral macrophages to manipulate the disease microenvironment.¹⁰¹

FACTORS INVOLVED IN RECRUITMENT AND POLARIZATION OF IMMUNE CELLS IN GLIOMAS

Chemotactic Signals for Myeloid Cells

In malignant gliomas, as in many other cancers, various immune cells accumulate and are polarized within the tumor to acquire new properties, further supporting tumor growth by a variety of growth factors and pro-angiogenic cytokines. Here, we would like to briefly discuss chemoattractants and cytokines that have been shown to stimulate migration and polarization of microglia/macrophages, as well as other types of immune cells, into the tumor site.

Hepatocyte growth factor (HGF)/scatter factor (SF) is a soluble ligand for transmembrane tyrosine kinase receptor c-Met,^{102,103} required for wound healing and development.¹⁰⁴ In malignant gliomas, expression of HGF/SF and its receptor c-Met was found both in GAMs and glioma cells isolated from GBM tissues,^{102,103} and positively correlated with a grade of malignancy^{103,105,106} and inversely with patient mortality.¹⁰⁷ *In vitro* studies revealed that HGF/SF secreted by glioma cells induces migration of microglial cells and BM-macrophages.¹⁰² Moreover, HGF/SF-c-Met signaling stimulates proliferation, migration, and invasiveness of human glioma cells *in vitro*,^{105,108} as well as glioma growth and angiogenesis *in vivo*.^{109,110} The expression of c-Met and HGF/SF secretion are induced by hypoxia¹¹¹ and radiation,¹¹² pointing to their role in tumor angiogenesis and glioma radio-resistance.

The monocyte chemotactic proteins (MCPs) belong to the MCP subgroup of the C-C chemokines family. CCL2 (MCP-1) by binding to its receptor CCR2 promotes chemotaxis and stimulates migration of immune cells, such as macrophages, monocytes, NK and T cells to the site of inflammation or injury. CCL2 is overexpressed in many cancers, including GBM and astrocytoma.^{113–116} Levels of MCP-1 were correlated to numbers of GAMs in tumor tissues,^{114,115} grade of malignancy and poor clinical prognosis.¹¹⁷ CCL2 supports proliferation and migration of GAMs to the tumor site and activates inflammatory responses

in these cells.¹¹⁸ Moreover, gliomas expressing higher levels of CCL2 have more blood vessels.⁵⁶ The role of distinct MCPs in glioma pathology is not yet clear, as it was shown that several tested glioma lines predominantly express MCP-3, but not MCP-1 mRNAs, and infiltration of GAMs in human gliomas was correlated with MCP-3, but not MCP-1 expression.¹¹⁹ MCP-3 promotes chemotaxis of immune cells by binding to CCR1, CCR2, and CCR3 receptors (while MCP-1 binds exclusively to CCR2).¹²⁰ Consequently, MCP-3 is able to attract not only monocytes, T cells and NK as MCP-1 does, but it attracts also dendritic cells.¹²¹ Human monocytes express both CCR1 and CCR2 receptors on their surface,¹²² thus only MCP-3 can activate both of them. Knockdown of MCP-1 expression in murine GL261 gliomas did not decrease of GAM accumulation.¹²³ Interestingly, CCR2A is frequently overexpressed in GBM cells and reduction of CCL2 level suppressed migration of these cells.¹²⁴

Glial-derived neurotrophic factor (GDNF) contains seven conserved cysteine residues typical for all members of the TGF- β superfamily, but shares no more than 20% homology with family members. GDNF binds to GDNF receptor- $\alpha 1$ (GFR- $\alpha 1$), which leads to activation of tyrosine kinase RET signaling.^{125,126} GDNF acts as a trophic factor promoting survival and differentiation of a various neuronal cell populations in the CNS.^{127,128} In both human and rat, GDNF expression was detected not only in neurons,^{129,130} but also in other types of cells such as astrocytes,^{131,132} glioma cell lines,^{133–135} and activated microglia/macrophages after injury.¹³⁶ GDNF expression was highly upregulated in glioma cell lines and human high-grade gliomas.¹³⁷ GDNF is involved in chemoattraction of different cells,^{138,139} including microglia.¹⁴⁰ Overexpression of GDNF in fibroblasts favored microglia attraction, whereas silencing of GDNF expression in GL261 glioma cells decreased accumulation of GAMs and reduced tumor growth *in vivo*. GDNF promotes glioma cell migration in an autocrine manner.^{141,142}

GAM POLARIZING FACTORS

The macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor 1 (CSF-1), is a secreted cytokine expressed by a variety of cells, such as macrophages, endothelial cells, fibroblasts, osteoblasts, and smooth muscles.¹⁴³ CSF-1 is involved in the differentiation, proliferation and survival of monocytes, macrophages, and BM progenitor cells. In osteopetrotic mice (op/op), carrying a single mutation in the *Csf-1* gene, a lack of CSF-1 prevents growth of macrophages and osteoclasts, thus leads to decrease in a number of macrophages and monocytes, both in blood and the tissues.^{144–146} CSF-1 R, a receptor for CSF-1, is expressed on monocytes, tissue macrophages, and monocyte-derived dendritic cells, and can bind not only CSF-1, but also IL-34.¹⁴⁷ Binding a ligand to CSF-1 R induces phosphorylation of receptor followed by recruitment and activation of Src, PI-3 K, and Cbl kinases along with downstream signaling pathways.^{148,149}

CSF-1 is expressed in GBM cells, normal human astrocytes, and in operative specimens of human gliomas. The CSF-1 receptor, encoded by the *c-Fms* gene, is also expressed in human gliomas, which suggests autocrine/paracrine interactions.¹⁵⁰ Murine GL261 glioma cells secrete CSF-1, which stimulates microglia invasion, and PLX3397, an inhibitor of CSF-1 R signaling, reduces the number of GAMs and GL261 glioma invasion *in vivo*.¹⁵¹ Treatment of mice bearing RCAS-PDGFB gliomas with PLX3397 increased survival and regressed established tumors.¹⁵² CSF-1 R blockade reduced intracranial growth of patient-derived glioma xenografts. However, GAMs were not depleted in treated mice, which suggests a negligible role of CSF-1 in glioma-microglia communication. Stimulation with granulocyte-macrophage CSF (GM-CSF/CSF-2) and IFN- γ facilitated GAM survival in animals with CSF-1 R inhibition. Expression of M2 activation markers (*Adm*, *Mrc1*, *Arg1*, *F13a1*) decreased in surviving GAMs, which is consistent with impairment of tumor-promoting functions.¹⁵² In both studies, Csf-1 levels were not compared with those in non-transformed astrocytes. In a MAFIA-based glioma model, temporal ablation of the CSF-1 R⁺ population affects the myeloid population in gliomas and results in switching to M1 phenotype, which was associated with decreased tumor cell proliferation, angiogenesis, and growth.⁷⁴

We found the low expression and secretion of Csf-1 in murine GL261 glioma cells and human glioma cells (at similar levels as in non-transformed astrocytes).⁷⁶ CSF-1 mRNA levels were not significantly upregulated in GBMs in comparison to normal brains and WHO grade I juvenile pilocytic astrocytomas.⁷⁷ Growth of GL261 gliomas and accumulation of GAMs were not affected Csf-1 deficient mice.⁷⁷ These conflicting results may stem from differences in applied animal models, selection of GBM cell lines and lack of specificity of a pharmacological inhibitor.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)/colony-stimulating factor 2 (CSF-2), is a cytokine secreted by macrophages, T cells, NK cells, mast cells, endothelial cells, and fibroblasts. CSF-2 stimulates survival, proliferation and differentiation of hematopoietic myeloid cells, and in combination with other factors also hematopoietic multipotent precursors and differentiated cells of other lineages.¹⁵³ CSF-2 binds to a dimeric receptor (CSF-2 R), which contains a ligand-specific α -chain subunit binding CSF-2 with a low affinity, and a β -chain subunit, which is able to bind CSF-2, IL-3, and IL-5.^{154,155} The structure of CSF-2 R allows its sequential activation,^{146,156} thus CSF-2 can have distinct effect on survival or proliferation of myeloid cells, depending on concentration. Activation of CSF-2 R stimulates PI-3 K, JAK-STAT, and MAPK signaling pathways.¹⁵⁷

Currently data regarding expression of CSF-2 and its receptor in gliomas are conflicting. CSF-2 mRNA was found to be upregulated in human and rodent glioma tissues, and GBM cell lines^{76,158–161} and high expression of CSF-2 R was

observed in those samples.¹⁶¹ Sawamura and colleague reported that CSF-2 was produced by 10 glioma cell lines *in vitro* but CSF-2 receptor was not expressed. No CSF-2 protein was detected in the cerebrospinal fluid of three malignant glioma patients.¹⁶² Further studies demonstrated that CSF-2 and CSF-2 R expression is highly upregulated in GBMs in comparison to low-grade gliomas and normal brain tissue.¹⁶¹ We demonstrated highly upregulated CSF-2 expression (but not CSF-1) in GBMs. Kaplan–Meier survival curves acquired from the Rembrandt depository showed correlation between CSF-2 expression and poor survival of glioma patients.⁷⁷ Csf-2 expression was high in GL261 glioma cells and its knockdown reduced accumulation of GAMs, impaired GL261 glioma growth in mice, and prolonged their survival.⁷⁷ We corroborated these results by the analysis of TCGA data set and found upregulation of CSF-2 expression in a subset of GBMs (unpublished). Upregulated CSF-2 expression in human GBMs and the autocrine or paracrine CSF-2 effects on glioma proliferation *in vitro* have been reproducibly demonstrated.^{161,163} CSF-2 was upregulated in both human and mouse glioma microenvironment compared with normal brain or peripheral blood samples, and stimulated the IL-4R α expression on MDSCs.¹⁶⁴ In conclusion, most data show the increased expression of CSF-2 in GBMs and several studies point to a critical role of this cytokine in accumulation and polarization of GAMs and MDSCs.

Transforming growth factor-beta (TGF- β) belongs to the TGF- β superfamily consisting three isoforms: TGF- β 1, TGF- β 2, and TGF- β 3 in humans.¹¹⁴ TGF- β is a pleiotropic cytokine able to bind to a TGF- β R complex composed of type I and type II receptors. Upon activation, transcription factors Smad2 and Smad3 are phosphorylated and form homo- and heterotrimeric complexes with a common mediator Smad4. These complexes translocate to the nucleus, where they regulate transcription (for a review see ref. 165). Activity of TGF- β /SMAD pathway in human gliomas was negatively correlated with clinical prognosis.¹⁶⁶

Glioma-derived TGF- β is a soluble cytokine which regulates cell proliferation, differentiation, invasiveness (by inducing MMP expression and endothelial–mesenchymal transition), and angiogenesis.^{167,168} TGF- β 1, TGF- β 2, and TGF- β 3 are differentially expressed in glioma cells *in vitro* and glioma tissues,^{169–171} with TGF- β 2 expression highly upregulated in malignant gliomas.^{170,171} TGF- β 2 has been shown to induce immunosuppressive polarization of immune cells,¹⁷⁰ especially GAMs.¹⁷² TGF- β , together with prostaglandin E2 (PGE2), diminishes activation of GAMs through an inhibition of MHC I and MHC II expression on glioma and microglial cells.¹⁷³ Glioma-conditioned medium induced production of PGE2 in rat microglial cultures.¹⁷⁴ TGF- β treatment reduced a phagocytic activity of GAMs and IFN- γ -induced production of inflammatory cytokines IL-6, IL-1, and TNF- α .¹⁷⁵ TGF- β was also described as an inhibitor of T_C and NK cells activation and differentiation.¹⁷⁶ Downregulation of TGF- β expression in glioma cells led to increased

susceptibility to lysis by both NK cell and T_c cells,¹⁷⁷ which could be explained by the fact that TGF- β downregulates NKG2D receptor on the NK and T_c cells.¹⁷⁸ Microglial cells produce TGF- β 1 when exposed to glioma.^{179,180} Microglia-derived TGF- β 1 strongly increased human and rat glioma cell invasion *in vitro* and in immunocompromised mice.¹⁸⁰ TGF- β secreted by glioma cells is implicated into a functional shift of GAMs to the immunosuppressive, pro-invasive phenotype, which supports tumor growth. A dual function of TGF- β in gliomas has been suggested: TGF- β secreted by glioma exerts immunosuppression by shifting GAMs to the immunosuppressive phenotype, whereas GAMs-derived TGF- β promotes tumor progression by upregulation of its own receptors: TBRI and TBRII on glioma cells.¹⁸¹

Osteopontin (OPN/SPP1) is a chemokine-like, secreted phosphoglycoprotein, which belongs to a small integrin-binding ligand N-linked glycoproteins (SIBLINGs) family. OPN has distinct roles intra- and extracellularly, and is expressed by many cells: macrophages, neutrophils, dendritic cells, natural killer cells, epithelial cells, smooth muscle cells, osteoblasts, Purkinje cells, and neoplastic cells. Originally, OPN was described as a protein involved in osteoclast adherence and bone remodeling, however its role in pathophysiologic processes, including cancer progression, and immune responses was also shown.^{182–184} Overexpression of *SPP1* (a gene encoding osteopontin)¹⁸⁵ and elevated protein levels were found in GBMs,¹⁸⁶ and correlated with poor patient prognosis.¹⁸⁷

Multi-functionality of osteopontin is related to numerous post-translational modifications, including phosphorylation, glycosylation, proteolytic cleavage by thrombin and matrix metalloproteinases 3 and 7, as well as alternative splicing of *Spp1* transcripts. Three different isoforms of human osteopontin were detected: OPN-a (a full-length protein), OPN-b (without exon 5), and OPN-c (without exon 4).¹⁸⁸ In glioma cells OPN-c stronger induced MMP-2, MMP-9, and uPA (urokinase plasminogen activator) expression, than OPN-a and OPN-b.¹⁸⁹ Expression pattern of different isoforms and a type of post-translational modification is cell-specific and may affect the potential role of osteopontin in cancer.¹⁹⁰

Osteopontin contains an arginine-glycine-aspartate (RGD) motif, which binds to integrin receptors $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu \beta 1$, $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, and $\alpha \nu \beta 6$,^{191,192} whereas OPN cleaved by thrombin exposes C-terminal SVVYGLR motif, which binds to $\alpha 4\beta 1$, $\alpha 9\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 4$ integrins.^{193–195} Such integrins are present on variety of cells, including endothelial cells, macrophages, neutrophils, and lymphocytes.^{196–199} In human gliomas, osteopontin enhanced attraction of leukocytes and GAMs to the tumor site in the RGD-dependent manner and protein itself was co-localized with neutrophils and macrophages.²⁰⁰ Thrombin-cleaved fragments of osteopontin were abundant in malignant glial tumors.²⁰¹ Giachelli *et al*²⁰² reported that both a full-length and thrombin-cleaved osteopontin induce chemotactic migration of monocytes,

macrophages, neutrophils and lymphocytes. OPN can also act in an autocrine manner and interacts with CD44v6, a specific variant of CD44 receptor expressed on neoplastic cells. Recent data show that osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem-cell phenotype and promotes aggressive tumor growth.^{203,204} High levels of *Spp1* mRNA were detected in dendritic cells and GAMs infiltrating murine GL261 gliomas,⁴⁰ but there is no information regarding protein form and processing.

We demonstrated that C6 rat glioma cells overexpress *Spp1* and secrete the processed protein, which participates in the pro-invasive, immunosuppressive activation of microglia. Pre-treatment with the RGD peptide, blocking interaction between a ligand and a integrin receptor, reduced the expression of genes characteristic for the protumorigenic microglia phenotype and abolished activation of focal adhesion kinase (FAK) as well as downstream kinases, such as Akt and ERK in cultured microglial cells. We found that osteopontin, produced by non-neoplastic cells, is a primarily pro-inflammatory factor, but sequential processing by thrombin and MMP-3 and -7 in glioma cells, generates a microglia-activating form devoid of the pro-inflammatory activity. In rat experimental gliomas, knockdown of osteopontin in glioma cells did not affect accumulation of GAMs, but blocked their polarization into the protumorigenic phenotype and tumor growth.²⁰⁵ Our findings provide the evidence for the role of glioma-derived osteopontin in polarization of infiltrating myeloid cells to the immunosuppressive, tumor supportive phenotype.

Periostin (POSTN), also known as osteoblast-specific factor 2 (OSF-2), is a multifunctional, evolutionarily conserved extracellular matrix (ECM) protein.²⁰⁶ POSTN also contains cysteine-rich domain, four internal repeats, fascilin domain, and hydrophilic C-terminal domain.²⁰⁷ Owing to the splice alterations at the C terminus, at least eight isoforms of periostin can be found in humans.²⁰⁸ POSTN interacts with cells through various integrin receptors and is an essential downstream effector of TGF- β superfamily signaling. The protein is involved in many processes in cancer development and progression, such as growth, invasiveness, and metastasis.^{209–211} Tumorigenic properties of periostin are associated with acting through Akt/PI3K and Wnt signaling pathways.^{210,212} A recent study demonstrated that POSTN is one of the key cytokines involved in attraction and activation of GAMs in malignant gliomas.²¹³ POSTN was abundantly expressed in glioma stem-like cells (GSCs) and its silencing led to significant reduction of GAMs infiltration, which indicated that POSTN is involved in attraction of GAMs by less differentiated glioma cells. Knockdown of POSTN inhibited tumor growth *in vivo* and increased survival of mouse xenografts. Moreover, knockdown of POSTN significantly reduced a number of M2-like GAMs (reduced expression of *Fizz-1*, *Cd163*, and *Arg1*) favoring GAMs expressing M1-like markers (*MHC II* and *CD11c*). Overexpression of

POSTN in GSCs promoted recruitment of M2-like GAMs *in vivo*. The exact mechanism is yet to be understood, however, the proposed mode of action is through integrin-dependent activation of phosphatidylinositol 3-kinase signaling, which supports activation of macrophages. POSTN binds to $\alpha v\beta 3$ integrin on GAMs and the use of neutralizing $\alpha v\beta 3$ integrin antibody or a synthetic RGD peptide blocked this activation. The authors concluded that a majority of GAMs were derived from circulating monocytes, rather than from microglia as they were abundant around blood vessels, both in case of murine xenografts and human malignant brain tumors.²¹³ POSTN secreted by glioma may also act in an autocrine manner by binding to the αv integrins on their surface and increasing tumor invasiveness.²¹⁴ In human GBM tissues, GAMs density was positively correlated with POSTN protein expression.²¹³ TCGA database analysis revealed that the *POSTN* level is higher in GBM than in low-grade gliomas, and high *POSTN* expression is associated with poor patients survival.²¹³

Findings of two glioma-derived ligands that polarize GAMs in the glioma microenvironment via integrins, open a new perspective in glioma therapy. Factors secreted by glioma such as OPN and POSTN that bind to the integrins on immune cells are potential candidates for therapeutic interventions. Preclinical and clinical research on integrins in cancer, particularly on $\alpha v\beta 3$ and $\alpha v\beta 5$, revealed their substantial

potential as therapeutic targets for the treatment of different cancer types.²¹⁵ Cilengitide is a cyclic peptide (cyclo(-RGDfV-)), that selectively binds to the target integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ present on the surface of endothelial cells, macrophages, and neoplastic cells. Cilengitide has been shown to act by inhibiting the FAK/Src/AKT pathway,²¹⁶ and preclinical studies in mice revealed efficacious tumor regression. Initial clinical trials have shown promising results of cilengitide treatment in GBM therapy, but when combined with radiation and temozolomide in the phase III CENTRIC clinical trial, the drug failed to provide additive benefits.²¹⁷ Integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 8$ are differentially expressed in GBM. In the CORE phase II trial, cilengitide was more effective in GBM patients with higher $\alpha v\beta 3$ levels in tumor cells.²¹⁸

Versican is a chondroitin-sulfate proteoglycan, which forms complexes with hyaluronan and is cleaved at specific peptide bonds by ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type 1 motifs) proteases. ADAMTS proteases are expressed in coordinate manner and generate a bioactive versican fragment containing the N-terminal G1 domain—versikine.²¹⁹ It has been shown that microglia promote glioma expansion through upregulation of membrane type 1 matrix metalloproteinase (MT1-MMP, MMP14), which activates glioma-derived MMP-2.²²⁰ This upregulation is mediated by the Toll-like receptor (TLR)2

Figure 2 Summary of glioblastoma-immune system interactions. The expression of all presented factors/cytokines is upregulated in GBMs and inversely correlates with patient survival.

signaling.²²¹ TLRs belong to the superfamily of pattern recognition receptors that classically activate and mediate pro-inflammatory responses in innate immune cells by recognizing invading pathogens. *TLR2* is highly expressed in all gliomas compared with non-tumor tissue, and patients with high *TLR2* expression had reduced survival compared with patients having lower *TLR2* expression.²²²

In glioma cells, synthesis of versican is primarily stimulated by TGF- β 1.²²⁰ TGF- β 2 has been shown to interact with versican V1 and support glioma proliferation and invasion.²²¹ In the ECM, versican interacts with various partners, such as tenascin-R, fibulin-1, fibrillin-1, fibronectin, P- and L-selectin, and various chemokines via the globular domains or GAG chains.²²³ Versican can also bind to the epidermal growth factor receptor, the cell-surface proteins CD44 and integrin β 1. Versican has been shown to act on macrophages through toll-like receptors (TLR2 and TLR6), and promote inflammatory cytokine production and Lewis lung carcinoma cell metastasis.²²⁴ The splice variants V0/V1 of versican were expressed in mouse and human glioma cell lines and tissues, and versican released from glioma promoted tumor invasion through TLR2 signaling in GAMs.²²⁵

CONCLUSIONS

Combined work from a number of laboratories worldwide supports the thrilling concept that gliomas rely on tumor infiltrating microglia/macrophages during development and progression. GAMs produce a large number of cytokines, interleukins, and growth factors that directly stimulate glioma cell growth and invasion, or create a more permissive tumor microenvironment (Figure 2). These changes establish a supportive environment, rich in extracellular substrates, and facilitating glioma growth or invasion. Moreover, glioblastomas produce a large number of cytokines (IL-6, TGF β) and small molecules (osteopontin, periostin, versican) that act on the glioma cells to increase glioma stem-cell self-renewal, and support cell proliferation, survival, and/or invasion of more differentiated glioma cells. Identifying the cellular and extracellular matrix-dependent relationships unique to the glioma microenvironment may provide exceptional opportunities to develop effective treatments to target these symbiotic associations that support glioma progression.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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