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## Title

# CNS macrophages and peripheral myeloid cells in brain tumours

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## Summary

Primary brain tumours (gliomas) initiate a strong host response and can contain large amounts of immune cells (myeloid cells) like microglia and tumour infiltrating macrophages. In gliomas the course of pathology is not only controlled by the genetic make-up of the tumour cells, but also depends on the interplay with myeloid cells in the tumour microenvironment. Especially malignant gliomas like glioblastoma multiforme (GBM) are notoriously immune-suppressive and it is now evident that GBM cells manipulate myeloid cells to support tumour expansion. The pro-tumourigenic effects of glioma-associated myeloid cells comprise a support for angiogenesis as well as tumour cell-invasion, -proliferation and -survival. Different strategies for inhibiting the pathological functions of myeloid cells in gliomas are explored and blocking the tropism of microglia/macrophages to gliomas or manipulating the signal transduction pathways for immune cell activation have been successful in pre-clinical models. Hence, myeloid cells are now emerging as a promising target for new adjuvans therapies for gliomas. However, it is also becoming evident that some myeloid-directed glioma therapies may only be beneficial for distinct subclasses of gliomas and that a more cell-type specific manipulation of either microglia or macrophages may improve therapeutic outcome.

**Neuropathological features of gliomas**

Glial tumours constitute approximately 50% of all newly diagnosed primary brain tumours, with low-grade gliomas accounting for roughly 15% of all brain tumours in adults [106]. The morphology and the cellular markers for gliomas share some similarities with the main macroglial cell-types (i.e. astrocytes, oligodendrocytes) [75]. Histopathologically, gliomas are divided into four different grades (according to the classification scheme by the world health organization, WHO), in which low-grade tumours are defined as grade-I/-II and high-grade gliomas as grade-III and-IV [87]. The presence of mitotic activity is a key feature for distinguishing low-grade from high-grade gliomas [75]. Statistically, low-grade astrocytomas arise roughly in proportion to the relative mass of the different lobes with most common location within the frontal lobes, followed by temporal and parietal lobe lesions [107]. Overall, 5- and 10-year survival rates of ~70% and 50% for grade-I and grade-II gliomas, respectively, have been reported in the literature [25]. The tumour tissue has no obvious signs of neoangiogenesis, infiltrative invasion or inflammation [75]. On the contrary, the outlook for patients with high-grade gliomas is grim. The majority of individuals diagnosed with a grade-IV glioma, which are very heterogeneous tumours and therefore also named glioblastoma multiforme (GBM), have a median progression-free survival of just over half a year and median overall survival of 15-18 months [17], only some subpopulations of patients show median survivals of almost 2 years [54]. GBM is a fast growing, highly angiogenic and invasive tumour and the diffuse growth is a major obstacle for GBM therapy. Other hallmarks of malignant gliomas are break-down of the blood-brain barrier (BBB), many hypoxic areas and necrotic centres [75]. Gliomas are usually diagnosed by neurorimaging (i.e. magnetic resonance imaging) and visualisation of the uptake of a contrast-enhancing agent within the tissue indicates BBB-leakage/breakdown [159]. GBM typically presents as a ragged contrast-enhanced and complex multi-cystic structure. GBM may be diagnosed *de novo*, i.e. in patients without a clinical history for brain tumours (then named primary GBM) or may evolve from lower grade gliomas (secondary GBM) [103].

## Multi-modal glioma therapy

Glioma therapy comprises very different strategies. For patients with deep-seated lesions or lesions located in eloquent regions which are clinically and radiographically indicated as low-grade glioma a conservative management including regular neuroimaging (after obtaining a histological diagnosis) [165], see also [130,150]. Individuals with high-grade gliomas undergo multi-modal treatment combining cytoreductive surgery, radiation- and chemotherapy (using a DNA alkylating agent, Temozolomide; TMZ) [106]. All in all, the approaches to treat high-grade gliomas are largely palliative and substantial efforts are made to improve prognosis and to define markers allowing a stratification of patients into therapeutically relevant phenotypes. Therefore, it was suggested to use gene expression profiling of the tumour mass, which can classify high-grade glioma according to different genetic subtypes (e.g. the proneural, classical or mesenchymal genotype) [19,152,113]. This GBM classification scheme may in the future help to dedicate more individualized and efficient therapies to patients.

## Accumulation of myeloid cells in gliomas

Another striking feature especially of high-grade gliomas is the large number of immune cells, i.e. microglia and macrophages that accumulate in the tumour mass (tumour associated myeloid cells; TAM) [111,7,31]. Peripheral blood-derived macrophages are largely restricted to perivascular areas, the meninx and the choroid plexus in the tumour-free brain, but accumulate in GBM after break-down of the BBB [123]. Microglia is abundant in the tumour-free CNS and comprises between 5% and 10% (depending on region) of all brain cells [71,83]; for comparison - the density of neurons is approximately 20% of cells in the human brain [57]. In GBM the number of TAM can be very high and constitutes up to 30% of the tumour mass [5,161,138,125], in medulloblastomas even 80% of all intra-tumoural cells can carry myeloid cell markers [138]. We observed that the distribution of TAM in GBM is very heterogeneous and the average number of these cells is between 20% and 30% (M. Synowitz and R. Glass, unpublished data). In most studies the quantification of TAM was performed in GBM samples (which is the most frequent intra-axial brain tumour) and TAM were reported to be less abundant in lower grade gliomas.

**Microglia development**

Microglia and bone-marrow-derived monocytes/macrophages have largely overlapping marker profiles and to date there is no unequivocal method to distinguish both cell types in samples resected from human GBM [162]. This matter is not trivial since monocytes of the peripheral blood and microglia have different developmental origin and may have distinct function in physiology and pathology [129]. In contrast to monocyte-dendritic cell progenitors, microglial progenitor cells are generated in the yolk sac after gestational day 8.5, which is before the onset of definitive hematopoiesis (at E10.5, when monocytes arise) [44]. Both, microglia- and monocyte/macrophage-development depends on the myeloid master transcription factor PU.1 (also named spleen focus forming virus proviral integration oncogene, *Spi-1*) [73] and on the activity of the receptor for macrophage colony stimulating factor (CSF1R) [44], while the maintenance of microglial cells during adulthood depends on the activity of the tumour growth factor-β (TGF-β) receptor-1 [20]. Stimulation of CSF1R in microglia is mediated via macrophage colony stimulating factor (M-CSF) and interleukin 34 (IL34) [44]. This signal transduction pathway is not unique for the generation of microglia alone but is also essential for the generation of peripheral tissue-macrophages in the lung, liver (Kupffer cells) or skin (Langerhans cells) [129]. Generation of monocytes and macrophages during definitive hematopoiesis, in contrast to microglia, requires the transcription factor MYB1 and activation of the fms-related tyrosine kinase (FLT3, a cytokine receptor) [133]. After microglia precursors have entered the developing CNS (between E8.5 and E9.5) they differentiate into microglia and appear to self-renew in the brain throughout live [46]. Microglial cells do not need to be replenished from peripheral sources like the bone marrow [3], but previous experiments indicated that a transient ablation of all myeloid cells can lead to the colonisation of the brain with peripheral macrophages, which could then not be distinguished from *bona fide* microglia (in terms of morphology and with older sets of markers) [9]. Such experiments were interpreted to support the notion that monocyte-derived macrophages and microglia largely share the same physiological role. However, today we observe that microglia also have unique roles in the CNS, which are different from classical functions of immune cells [109]. It was e.g. observed that microglia makes

close contact to pruning synapses and can participate in information processing of neurons [72].

**CNS Immunity**

The abundance of TAM in high grade gliomas has been initially been described more than 90 years ago [31] and over several decades researchers focused on delineating the inefficient immune-response of TAM [126,143,39]. It was concluded that the high levels of tumour growth factor-β (TGF-β) that are secreted from glioma cells have immune-modulatory functions, which prevents myeloid cells from inducing a coordinated immune response against the tumour [68]. Indeed, it is now evident that TGF-β signalling is crucial for microglia in physiology and under neuropathological conditions [20]. However, only a sub-population of microglial cells may participate in controlling the adaptive immune response [49]. The current view is that peripheral macrophages and dendritic cells, in addition to microglia, can perform an important role for immune surveillance in the CNS [123]. Although the brain has no lymphatic vessels, which can transport antigen to deep cervical lymph nodes and to specialized dendritic cells, there are other routes by which peripheral immune cell can scan the immune status of the CNS. Peripheral macrophages can encounter antigen from the CNS, even with an intact BBB, by an exchange of interstitial brain fluids through the cribiform plate and through the choroid plexus into the periphery. Especially during auto-immune neuropathology it is evident that the peripheral immune system can mediate strong immunological action within the CNS. One promising way to exploit the peripheral control of CNS immune functions for brain tumour treatment is pursued by immunotherapy approaches [4]. Here e.g. cytokine application, serotherapy or active immunotherapy (to activate peripheral dendritic cells with antigen of tumour-origin) is used to prime the peripheral immune system to attack gliomas [55]. All in all, it is likely that re-activating antigen-presentation especially in bone-marrow derived immune cells (and additionally a relieve from immune-suppressive signals in tumour infiltrating lymphocytes, see below) would revive a functional anti-tumourigenic response of the adaptive immune system.

**Distinguishing microglia from peripheral macrophages**

It was previously suggested that peripheral macrophages can be distinguished from microglia by means of CD11b and CD45 expression levels in the tumour-free mouse brain [58], in tumour models [7] and in human tumours [110]; while both cell types have high levels of CD11b on the plasma-membrane, only macrophages label intensely for CD45 and microglia have low CD45 levels. However, it is not clear how robust these markers really are in a glioma context; i.e. gliomas may modify the CD-molecule expression in TAM. To our experience, patient derived material often does not allow a distinction between the two cell-types based on CD-markers; most glioma biopsies harbour $CD11b^{high}/CD45^{high}$ cells but biopsies containing $CD11b^{high}/CD45^{low}$ populations are infrequent (M. Synowitz and R.Glass, unpublished observation). Peripheral and CNS immune cells were previously also distinguished by generating chimeric animals after sub-lethal irradiation and bone-marrow transplantation (e.g. from donors constitutively expressing green fluorescent protein; GFP) [117]. Microglia is radio-resistant and only the hematopoietic cells in the bone marrow are exchanged by this paradigm. This method requires that the brain is shielded from the radiation beam [94] since otherwise the BBB is opened and peripheral immune cells enter the CNS, populate some brain areas and generate tissue macrophages that share many microglial markers [32,169]. Anyway, the radiation procedure causes a massive release of cytokines into the circulation [134] that may also alter brain physiology. Recently, superior methods have been described to unequivocally separate microglia from peripheral immune cells. Genetic mouse models, which indicate developmental markers specifically for microglia or monocytes and monocyte-derived macrophages were generated and now distinct myeloid-cell populations can be observed without generating pathological side effects. It was reported that *Flt3-cre* induced recombination of an inducible reporter (*flox-STOP-flox-YFP*) very efficiently marks bone marrow-derived myeloid cells but spares microglia [133,46]. Also, a model was established to specifically indicate microglia using recombination (and activation) of an inducible reporter under control of the gene promoter for the fraktalkine receptor (*Cx3cr1*). Timed activation of *Cx3cr1-CreErt2* by Tamoxifen application in transgenic animals crossed to a *flox-STOP-flox* reporter initially marks all myeloid cells, but the physiological turnover of peripheral monocytes and monocyte-derived

macrophage depletes reporter-positive immune cells from the peripheral blood while labelled microglia persists [45]. In another approach to distinguish microglia and blood-borne myeloid cells, microglia was purified from physiological, blood-free brain samples and monocyte-derived macrophages were purified from peripheral blood, then direct RNA sequencing was used to identify differentially expressed genes in both cell-types [58]. Interestingly, it was found that certain purinergic receptors (like $P2Y_{12}$) or surface molecules (like Siglec-H) are exclusive markers for microglia. Future work (e.g. using genetic recombination in transgenic models) will need to show if these differences persist in gliomas; if this is the case the it will be possible to therapeutically treat glioma-associated microglia specifically (e.g. with purinergic compounds) or to identify microglia in glioma immunohistochemically.

**Microglial activation and innate immunity**

Classically, the immune cell function of microglia was observed after challenging the brain with bacterial pathogens (e.g. after sterile infection with lipopolysaccharides; LPS), these studies showed that microglia can undergo remarkable morphological changes [76] and become motile [1]. It was speculated that microglia revert from a “resting” state (associated with a stellate cell-shape) towards a motile and activated phenotype (associated with an amoeboid cell-shape) [121]. However, real-time observations by intravital microscopy revealed that microglia under physiological conditions are not immotile (as the term "resting" infers), but constantly scan the environment [121,30,100]. If lesions to the brain parenchyma are encountered, then microglial cells initiate a damage response, which can (depending on the extent of the lesion) also include other brain cells like astrocytes [50]. While the protection of the CNS from infection or injury is certainly a task for microglial cells, current studies also acknowledge the tissue protective role of microglia and show that these cells may contribute to neuronal survival in different neuropathologies [109]. Overall, it is established that microglia has a prominent role during acute inflammation. Here, pathogens activate microglia via stimulation of toll-like receptors (TLR), then microglia phagocytose [124] or kill microorganisms, by releasing reactive oxygen species (ROS)[69] or nitric oxide (NO) [35] and e.g. secrete inflammatory cytokines like tumour necrosis factor- α (TNF-α), interleukin-6 (IL-6),

IL10 and IL12 [62]. The physiological response of microglia to inflammatory pathogens is summarised as the M1-type of microglial activation [16]. Microglial cells also control the resolution of inflammatory events and participate in tissue repair functions including the induction of angiogenesis in lesioned areas [137,16]. During homeostasis the parenchymal cells, in particular neurons, present several plasma membrane molecules and release CX3CL1, which are recognised by microglia and prevent their activation [51,129]. The pro-inflammatory and tissue protective functions of microglia may also become apparent in gliomas but in a tumour environment they were, in most studies, not separated from the inflammatory reactions of monocyte-derived macrophages. Here, gliomas secrete IL4, IL6 and IL10 [139], as well as TGF-β [144] that induce an alternatively activated phenotype in TAM, which is referred to as M2-type of activation [78]. Increased production of prostaglandin E2 ($PGE_2$) by glioma is associated with suppression of T-cell and TAM activation and plays an important role in the generation of an immunosuppressive environment [98]. The cell death pathway molecule FAS-ligand (CD95-L or FASL) was found to be expressed by human GBM and both TAM and T-cells express the FASL receptor (FAS). It was recently reported that FASL contributes to local immunosuppression whereas there is no evidence that GBM-derived FASL induces TAM apoptosis [65]. TGF-β, macrophage-CSF (M-CSF), IL-4 and IL-10 mediate an M2 phenotype of TAM. M2-activated TAM have e.g. reduced MHC-II levels [132], release IL10 [171,157], upregulate GM-CSF, IL-10, CXCL14 [42] and vascular endothelial growth factor (VEGF) [101] and have increased expression of Arginase-1 [37]. M-CSF expression in glioblastomas correlates with the expression of the M2 marker CD163 in TAM [78]. Both microglia and monocyte-derived macrophages share these M1 and M2 features in neuropathology as well as in high-grade gliomas and the NF-κB and the signal transducer and activator of transcription-3 (STAT3) pathways have central roles in shifting TAM between M1 and M2 phenotypes [170,120,77]. Manipulation of the related signalling cascades may become a possibility to promote the anti-tumourigenic M1 type in TAM [59,2]. It should also be noted that a prototypical M1 phenotype (as initiated by LPS) or M2 polarisation (as observed during the resolution of inflammation) is usually not encountered in TAM [37,42]. It was observed that TAM in gliomas have an aberrant immune-type and share both M1 and M2 features (Fig. 1). Currently it is

unknown if myeloid cells homogenously have a mixed M1/M2 type or if there are distinct TAM subsets (subtypes of microglia as well as monocyte-derived macrophages) which are either more in the M1 or the M2 spectrum. Altogether, earlier work suggested that microglial cells were brain macrophages which can switch between "on" and "off" modes of activation – this image is now reverted and we see that microglia and monocyte-derived macrophages are assigned with an array of different brain-specific roles during physiology and pathology, which are not necessarily reflected by morphological changes.

**Adaptive immune functions in gliomas**

Systemic immunosuppression in patients with primary intracranial tumours has been well documented [163]. Deficits in the adaptive immune response of glioma patients are induced by tumour-released immune-modulatory cytokines [39,68] and partly by clinically applied corticosteroids (Dexamethasone) [8], which are given to reduce glioma-associated edema [148]. Glioma patients have low peripheral lymphocyte counts, reduced delayed-type hypersensitivity reactions to recall antigen, impaired mitogen-induced blastogenic responses by peripheral mononuclear cells, and increased levels of $CD8^+$ suppressor T-cells [81]. The lymphocyte deficit involves the T-helper ($CD4^+$) subsets with decreased T-cell activity *in vitro*. Furthermore, there is diminished induction of immunoglobulin synthesis by B-cells *in vitro* from the peripheral blood of patients with intracranial tumour, probably related to diminished T-helper activity [14].

Only activated T-cells can traverse the BBB and gain entry to the brain [123]. Then, brain infiltrating $CD4^+$ T-cells may lose the activated status through the action of TGF-β released from glioma cells or TAM [151], or from TAM secreting IL-10 and C-C chemokine ligand-17 (CCL17), CCL18 or CCL22 [162]. Cytokines like TGF-β have been shown to suppress the production of both IL-1 and human leukocyte antigen (HLA) class-II molecules by antigen-presenting cells, and also suppresses the activation and proliferation of cytotoxic T lymphocyte (CTL) [64]. In GBM patients, systemic immune responses are unable to overcome the immunosuppressive tumour microenvironment and patients have reduced T-cell responses due to a number of factors including impaired T-cell receptor (TCR) signalling, immunosuppressive cytokines, T-cell anergy (mediated by

regulatory T-cells, $T_{reg}$, induced immune suppression), and dysfunctional antigen-presenting cells [97,142]. Another study on GBM has shown that TAM had surface HLA class-II expression but lacked expression of the co-stimulatory molecules CD80, CD86, and CD40 critical for T-cell activation and thus were unable to activate T-cells [60]. Also, in GBM, there is a lack of effector and/or activated CTL and a relative abundance of $T_{regs}$ [158]. One important aspect is a dramatic reduction in the expression of HLA molecules on the surface of tumour cells [67], which weakens their detection by CTL.

Differential activation of STAT3 in TAM can control multiple immunosuppressive pathways in high-grade gliomas [162]. STAT3 activation in TAM is induced by different cytokines of the tumour microenvironment such as IL-10, IL-6, epidermal growth factor (EGF) and fibroblast growth factor. Activated STAT3 is known to reduce the expression of surface molecules necessary for antigen presentation such as MHC-II, CD80, and CD86 [79], as well as to increase the expression of many M2-specific immunomodulatory mediators including IL-10, EGF, VEGF, and various matrix metalloproteinsases (MMPs) [18]. It is currently unclear whether a single dominant molecule or a complex network of molecules is responsible for the immunosuppressive phenotype of glioma TAM, but STAT3 activation appears to play a key role in generating and perpetuating the M2-shifted TAM in gliomas.

**The pro-invasive function of myeloid cells in gliomas**

A specific role for microglial cells in brain tumours was addressed two decades ago [47,143]. Initially, *in vitro* studies showed that microglia, which can be purified from the fetal mouse or from the rat brain and maintained for limited times in cell culture, has profound effects especially on the invasiveness of glioma cells [95,6]. A seminal studies using Boyden chamber migration assays demonstrated that the presence of microglia facilitates the transmigration of glioma cells through a matrix containing barrier [11]. Especially the use of cultivated brain slices, which assures the preservation of original three-dimensional cellular context of the brain in an *in vitro* setting, has advanced our understanding of the specific role of microglia in gliomas [104,91]. Glioma cells, stably expressing a fluorescent reporter protein, can be inoculated into brain slices and the motility of the tumour cells can be monitored in real-time under conditions closely

resembling the *in vivo* situation. These slice cultures are a method of choice when a pharmacological treatments are investigated, since compounds that otherwise do not traverse the blood brain barrier can be applied into the tumour area. Furthermore, microglia can maintain their normal surveillance-function function scanning the brain for potential lesions (see above) and the role of microglial cells in gliomas can be investigated without the intratumoural accumulation of monocyte-derived macrophages. Techniques have been developed to specifically ablate microglia in brain slices without deteriorating the entire cellular architecture of such slice preparations. In tumour-inoculated brain slice preparations the application of Clodronate-filled liposomes was instrumental to uncover important pro-pathological action of tumour-associated microglial cells [91]. The liposomes are rapidly and specifically taken-up by the phagocytic microglia and the payload (Clodronate) induces cytotoxicity. The dying microglia initially promotes astrocyte activation, which ceases after a relatively short interval (of three days). As a control, brain slices can be replenished (after Clodronate application) with exogenously cultivated microglia and the pro-tumourigenic effects are re-installed [91]. The course of tumour progression in microglia-containing versus microglia–depleted slices can be compared in this model. These experiments corroborated that microglia indeed has a pro-invasive effect in gliomas – as postulated from earlier cell culture studies. Conditioned media from the (glioma containing) brain slices had increased metalloprotease-2 (MMP2) activity only when microglia was present and then individual glioma cells invaded deeper into the brain tumour parenchyma. Importantly, this study showed that microglia promotes the activity of MMP2, but does not alter the expression levels of this enzyme. Metalloproteases are synthesised and secreted as inactive pro-forms and only the proteolytic cleavage of a peptide moiety converts the pro-enzyme into its active form [135] Then the active MMP2 can degrade extracellular matrix and facilitate glioma invasion. Subsequent studies with different mouse models supported these *in vitro* findings [93,154,92]. Ablation of myeloid cells by intra-tumoural application (via osmotic mini-pumps) of Ganciclovir in glioma-bearing animals engineered to express the herpes simplex thymidine kinase (HSVTK) gene, which converts of the pro-drug Ganciclovir into an active cell-death inducing agent, under a promoter for CD11b (*Cd11b-Hsvtk*) drastically diminished TAM numbers in

gliomas and largely reduced glioma size [92]. The molecular signalling pathway responsible for the glioma-supporting effect of TAM was uncovered and manipulated in another *in vivo* model. Here, we (and subsequently others) found that glioma cells release a (still unknown) soluble factor that triggers toll-like receptor-2 (TLR2)[154], which promote the activity of mitogen activated kinase (MAPK) p38 and of the TLR signal transducer MYD88 [92] in TAM. The MYD88 molecule induces the expression of another metalloprotease named membrane type-1 metalloprotease (MT1-MMP) on the plasma membrane of TAM. TAM-expressed MT1-MMP converts pro-MMP2 into active-MMP2 thereby facilitating glioma motility (Fig. 2).

The extracellular matrix in the brain has a different biochemical composition and architecture as compared to peripheral tissues like e.g. epithelia [99,33,127]. In epithelial tumours matrix-degradation and disruption of the epithelial layering is a hallmark for tumour progression and malignancy [99]. In the brain extracellular matrix components are less abundant and the matrix is heterogeneous between areas of grey and white matter, basement membranes are predominantly found along the vasculature, the meninges and parenchyma bordering with the ventricles [127]. Metalloproteinase-mediated matrix degradation is therefore associated with different pathological effects as compared to epithelia and can result in increased invasion of glioma cells along the vasculature or also opening of the blood brain barrier [153,10,61]. MMP-activity has also signalling effects and can unmask cryptic amino-acid residues in the matrix[108,155], which stimulate growth factor receptors or bind to different integrins on tumour cells and can thereby support tumour expansion [149].

**The pro-angiogenic role of myeloid cells in gliomas**

Another major role for MMPs on TAM is to initiate and to support the formation of new intra-tumoural blood vessels. The activity of MT1-MMP in concert with MMP2 disrupts basement membranes on blood-vessels and allows endothelial cell sprouting [149]. Additionally, TAM prime endothelial cells to sproute as they synthesize and release TNF-α and also other angiogenic molecules which have direct angiogenic effects or promote the secretion of vascular endothelial growth factor from glioma cells [140,101] (Fig. 3). The sprouting endothelia in gliomas need to be coordinated to form functional

vessels and especially the microglial cells may control this step, too. During development microglia localizes to vessel branch points, guides the endothelial tip-cells towards each other, induces tip- to stalk-cell conversion in the endothelia and thereby assures the building of functional vascular tubes [147]. TAM also express the pro-angiogenic MMP-9 [63] or induce the expression of MMP-9 in glioma (stem) cells [167]. MMP-9 participates in modifying the basement membrane and liberates growth factors like stem cell factor (SCF) [82] that acts as a specific agonist for the tyrosine kinase KIT. The KIT signalling pathway can recruit bone marrow-derived endothelial precursors to tumours and thereby augment the formation of vascular structures by a mechanism termed vasculogenesis [146]. However, another study suggested that vasculogenesis has only a minor role in GBM and that vascular sprouting and vessel cooption are the main drivers for the formation of new blood vessels in gliomas [89]. It is likely that microglial cells have a specific role in the initial steps of intratumoural vascularization (when the BBB is intact and macrophages have not yet invaded the brain), subsequently the sprouting endothelia and later the monocyte-derived macrophages, which invade into the tumour, will also release pro-angiogenic signalling molecules and perpetuate the formation of vascular structures in gliomas. As described above, anti-angiogenesis was regarded as a promising therapeutic strategy [102], but application of the VEGF-A-blocker Bevacizumab has clinically failed [90]. It is currently a matter of intense investigation if TAM can mediate resistance to Bevacizumab by releasing angiogenic factors alternatively to VEGF-A. Myeloid cells accumulating in GBM can promote the acquisition of a mesenchymal tumour-subtype [84,38,34], which is associated with resistance to Bevacizumab [114,115]. However, as with most pathways controlled by TAM, support of angiogenesis is only one side of the intra-tumoural actions of these immune cells in gliomas. Myeloid cells also inhibit intra-tumoural vessel formation e.g. by liberating angiostatins from the extracellular matrix, by activity of the urokinase type plasminogen activator [74]. In brain tumours the net effect of TAM pro- and anti-tumourigenic activity is on the tumour supporting side, but there may be a possibility to shift this balance and promote the anti-tumour effects of glioma associated microglia or of blood-borne macrophages that invaded into primary brain tumours.

Adenosine-5-triphosphate (ATP) was shown to stimulate the production of chemokines and CCl2 (MCP-1) and interleukin-8 (IL-8) in gliomas [66]. Nucleosides like adenosine modulate in an auto- and paracrine fashion the fine-tuning of the tumour-stroma-interaction [15]. Extracellular adenosine itself is not only a passive product of tumour induced ischemia, hypoxia and necrosis but also actively released due to altered purine metabolism [85]. Most of the signalling actions of extracellular adenosine are mediated by G-protein-coupled cell-surface receptors that are divided into four subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ [40]. Adenosine has several inhibitory effects on M1 macrophage activation which are mediated by $A_{2A}$ receptors [53] and increases M2 macrophage activation [28]. VEGF production by macrophages is stimulated through $A_{2A}$ receptors and therefore it can support angiogenesis [122]. Microglial cells express functional $A_{1A}$ receptors and in gliomas selective stimulation of $A_{1A}$ inhibits the TAM activation [88,145].

**Blocking the tumour supporting phenotype of myeloid cells**

While the abundant expression and release of TGF-β in gliomas was initially attributed only to the tumour cells, it now established that myeloid cells also generate TGF-β and thereby mediate important pathological effects [164,68,166]. In addition to blunting adaptive immune responses TGF-β was also observed to have direct effects on the highly aggressive subset of stem-like glioma cells especially in tumours of the mesenchymal genotype [112]. Application of Amphotericin-B can induce a tumour stem cell suppressive phenotype in TAM and mediates profound therapeutic effects in mouse models [131].

Of note, the ablation of myeloid cells in a mouse glioma model (using *Cd11b-Hsvtk* transgenic animals; see above) has also shown anti-tumour effects of TAM, previously. Here, systemic application of Ganciclovir was used which may have preferentially reduced the number of monocytes in the peripheral blood and monocyte-derived macrophages (which accumulate in gliomas) and under these settings an anti-tumourigenic role for myeloid cells in gliomas was observed [43]. The anti-tumourigenic function of the macrophage population was addressed to their antigen-presenting capacity and to their ability to activate T-lymphocytes. Again, these results suggest that TAM have the capacity to mediate both pro- and anti-tumour effects and that it will be

interesting to dissect the function of intra-tumoural monocyte-derived macrophages or microglia in order to establish cell-type specific targets for adjuvans therapies for gliomas.

The *Flt3-cre* [46] and *Cx3cr1-CreErt2* [45] based models, in addition to newly established cell-surface markers [58], will advance our understanding of the cell-specific traits of microglia- or monocyte-derived macrophages in gliomas. Nevertheless, it is likely an oversimplification to regard either immune-cell population as homogenous [119]. There is solid evidence that microglia are heterogenic with respect to different brain areas and that they also undergo changes during aging [52]. These differences may not cause too much variance in experimental glioma models where tumour localisation, timing of tumourigenesis and genetic background of gliomas can be tightly controlled [23]. However, when analysing patient-biopsies heterogeneity is an issue since gliomas are located to different brain areas, patient-age can differ by several decades and gliomas are caused by a range of genetic mutations with consequences for cell-signalling and immunogenicity [87,103]. One way of patient stratification is to obtain glioma samples and to investigate the gene expression pattern assigning tumours to distinct genetic subclasses [19,152]. Preclinical glioma models indicated that this approach is important to identify individuals that may respond to myeloid-directed brain tumour therapies [118,12]. Blockade of CSFR1 signalling specifically in TAM by a small molecule inhibitor (BLZ945) had very robust anti-tumourigenic effects in a mouse glioma model resembling the proneural GBM-type [118]. Importantly, BLZ945 largely abrogated the tumour supporting function of TAM in a mouse model and the beneficial effects of CSFR1-inhibition were associated with the induction of a gene-expression signature that also served to predict improved survival in human GBM of the proneural subtype but not in other GBM subtypes. Another CSFR1 inhibitor (PLX3397) inhibited glioma cell invasion in an orthotopic implantation model using immune-competent mice [26]. However, in our hands CSF1R-inhibition did not mediate any anti-tumour effects, when using a related glioma mouse model (M. Synowitz and R. Glass, unpublished observations); suggesting that subtle differences in experimental procedures may have profound impact, when investigating TAM. Several studies suggest that inter-individual differences between gliomas, in particular the genetic subtype of tumours, can be of

importance to predict the outcome of immune-cell targeting therapies [84,38,34]. This is not surprising since the genetic subtype is determined from the entire tumour biopsy, which includes TAM. During progression and relapse GBM can convert from the proneural to the mesenchymal type [13,21,27] and it was shown that TNF-α release form intra-tumoural myeloid cells and TNF-α induced NF-kappa-B signalling in stem-like glioma cells can promote the conversion of the GBM genotype [12]. The shift from the proneural towards the mesenchymal glioma phenotype can promote radiation resistance in a subset of the proneural tumours. This has profound therapeutic implications, as NF-κB blockade may be an important strategy to improve the outcome of radiotherapy. Altogether, the studies on the tumour-modulating effects of TAM in gliomas show that the expression signature has some predictive value for the application of e.g. BLZ945, but also indicate that the proneural subtype is not homogenous and that some proneural gliomas are sensitive to a TAM-induced mesenchymal drift. Hence, a better understanding of the gene expression pattern of TAM in glioma is necessary to e.g. stratify patients according to new myeloid cell-targeting adjuvans treatments that comprise CSFR1-inhibition or NF-κB blockade plus irradiation. The molecular analysis of tumour biopsies and different glioma cell subsets has largely advanced our understanding of the pathological processes in malignant brain tumours. Similar techniques have already been used in several pioneering studies to uncover prognostic markers and signalling pathways of myeloid cells in gliomas or neuropathological disease. With more studies on the genetic make-up of microglia and monocytes-derived macrophages in glioma mouse models we will obtain a more detailed picture on the potential therapeutic targets in subsets of TAM. The larger challenge ahead is to obtain similar data on TAM in human brain tumours, where we do not have easy access to relevant control samples from the tumour-free human brain (so different controls form post-mortem material and epilepsy surgery will be necessary) and where it will be difficult to prove if the markers distinguishing microglia and blood-borne macrophages in the mouse brain also apply to human cells.

**The tropism of myeloid cells to gliomas**

In the past, additional TAM targeting strategies in preclinical glioma models also had beneficial effects. Application of the clinically approved immune-suppressive drug cyclosporine-A could strongly inhibit the pro-tumourigenic effects of myeloid cells in glioma [96,141,24]. Other investigator used stimulation of TLRs in glioma cells and in TAM as a therapeutic paradigm [48,36,70]. Another way to interfere with the tumour promoting role of myeloid cells in gliomas is to blunt the intra-tumoural accumulation of these immune cells by blocking chemoattractive signalling. Experiments using encapsulated glioma cells showed that glioma-released soluble factors have major role in guiding myeloid cells towards the tumour mass. Here, glial-derived neurotrophic factor (GDNF) predominated chemoattraction of microglia in vitro, GDNF induced a tropism for myleoid cells towards gliomas in vivo and GDNF-knockdown in gliomas had a therapeutic function [80]. Other studies showed that CCL2 (MCP-1), CCL5, CCL7, VEGF-A [116,105,168] or (in hypoxic areas) stromal derived factor-1 [160] have prominent tropic function for myeloid cells in gliomas (Fig. 2). In contrast to the function of CX3CR1 in physiology, which promotes a resting state in microglia [51], the same receptor can promote myeloid cell recruitment to human brain tumours and thereby support tumour expansion [56], but had no pathological role in a mouse model [86]. A recent publication suggests hypoxia-induced Semaphorin 3A (Sema3A) as a potent attractant for TAM by triggering VEGF-receptor-1 phosphorylation through the associated holoreceptor, composed of Neuropilin-1 (NRP1) and PlexinA1/PlexinA4. Importantly, whereas NRP1 levels are down-regulated in the hypoxic environment, Sema3A continues to regulate TAM in an NRP1-independent manner by eliciting PlexinA1/PlexinA4-mediated stop signals, which retain them inside the hypoxic niche [22]. Again, it is presently not known if these many different chemotropic molecules are active either in different glioma subtypes, or glioma models of distinct genetic backgrounds, if all myeloid cells respond equally to these pro-migratory cues or if there are distinct immune cell-subtypes which have a preference for the one or the other chemoattractive signalling pathway.

**Future directions**

To date most studies on myeloid cells in glioma have uncovered specific pro- or anti-tumourigenic function of myeloid cells in the untreated tumour. Especially in pre-clinical models distinct roles of TAM in tumour-antigen presentation, promotion of glioma-invasion or angiogenesis were described. These data were backed up with findings from human biopsies, with the caveat that humans usually are treated with Dexamethasone before surgical tumour resection. Some exciting data are also available on the impact of myeloid cells on glioma treatment using irradiation [12,128]. It was observed that myeloid cells can support glioma cell survival and prevent the accumulation of anti-tumourigenic reactive oxygen after radiochemotherapy. The myeloid cells provide alternative metabolites to glioma cells (quinilonic acid instead of NADH), which prevent the generation of reactive oxygen species that mediate some of the beneficial effects of radiochemotherapy [128]. Also it was shown that the composition of myeloid cell-subtypes is altered throughout radiochemotherapy, with as yet unknown consequences for the therapy of glioma relapse [136]. One important topic that has not received much attention so far is the role of microglia during tumour formation and progression form low-grade to high-grade stages. It is postulated that gliomas arise from neoplastic transformation of stem and precursor cells (NPCs) in the brain or from de-differentiating astrocytes [23,41]. Microglia has been proven to be of importance for the turnover of NPCs [29,156] and it remains to be shown if microglia (e.g. during brain inflammation) contributes to the acquisition of somatic mutations in pre-neoplastic brain cells, which can drive tumourigenesis. Importantly, it was recently observed in a transgenic mouse model that CSFR1 blockade (by BLZ945) in myeloid cells can largely prevent/ slow-down malignant progression of neoplastic brain cells into high-grade gliomas [118]. Given that there are often little treatment options for patients with low-grade gliomas it will be important to explore if tackling the glioma-associating microglia can prevent the malignant transformation of lower grade tumours. For basic science studies investigating the role of microglia in gliomas there is the advantage that lower-grade gliomas have an intact BBB, which implies that nor monocyte-derived macrophages have entered the

tumour area, and hence specifically the pathological impact of glioma-associated microglia can be studied in biopsies from human tumours.

Altogether, we have now firm evidence that TAM are pathologically important and potential targets for adjuvans treatment of gliomas. Currently, we see an array of pro- and anti-tumourigenic effects of microglia or monocyte-derived macrophages, which make it difficult to directly translate immune cell-directed therapeutic approaches into clinical procedures. Uncovering the inter-individual heterogeneity of TAM in brain tumours and defining distinct pro- or anti-tumourigenic functions in microglia or blood-borne macrophages (and in subtypes of these cells) will help to set-up promising therapies and to locate patients that may profit from new TAM-targeting treatments.

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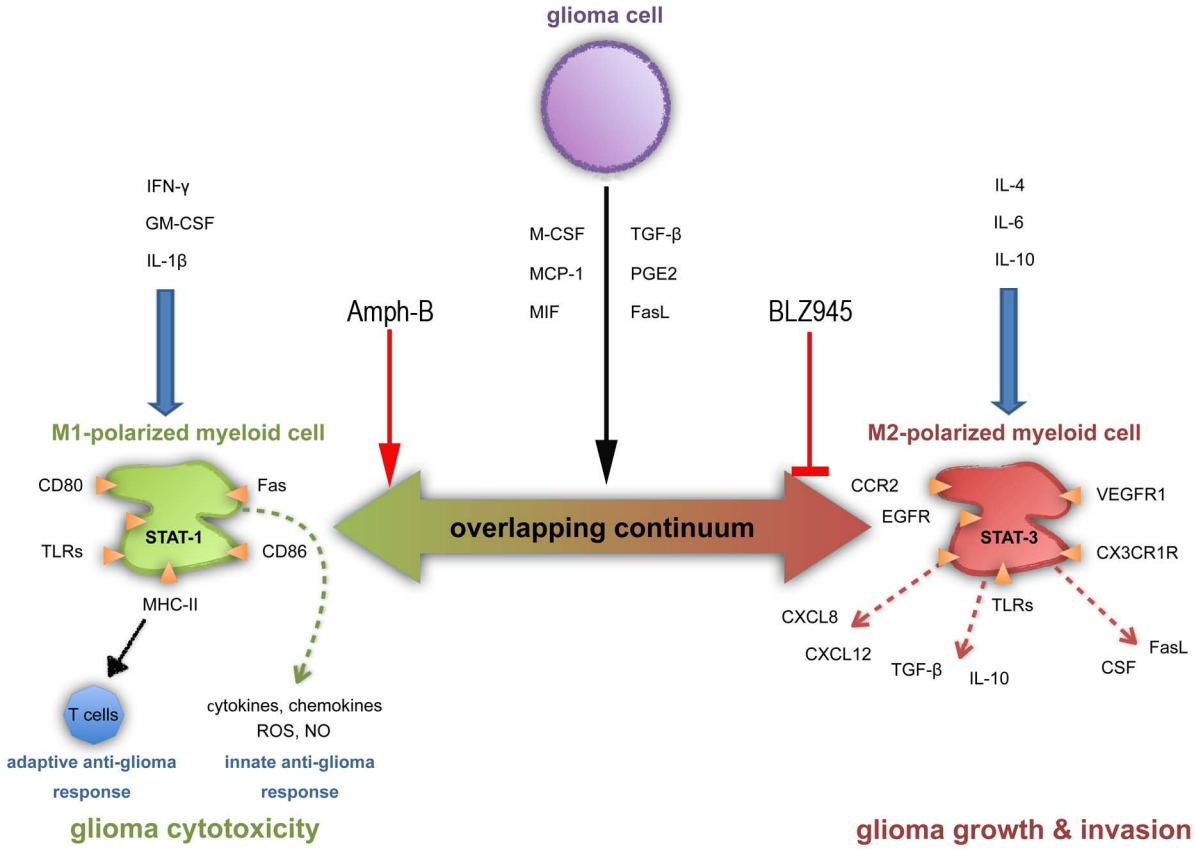
**Legends**

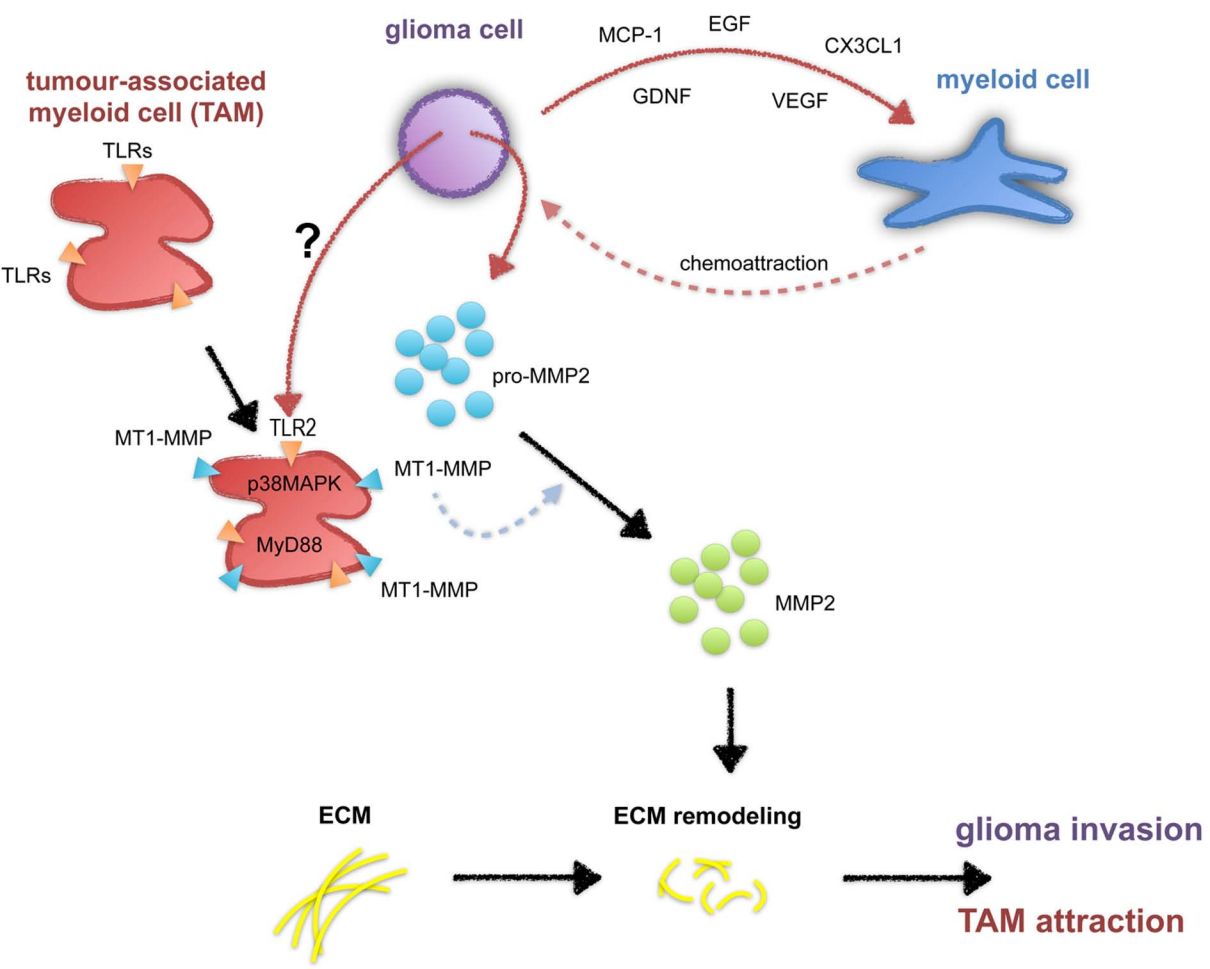
**Figure 1. Myeloid cell polarization in glioma.** Glioma-derived factors (black arrow) induce glioma-infiltrating myeloid cells (TAM) to adopt an immune-phenotype that is predominated by markers for the M2-type (alternatively activated), but M1-markers (classically activated) are also present. The M1-shifted TAM mediate anti-tumour effects (e.g. by initiating an adoptive immune response or by releasing cytotoxic oxygen- or nitrogen-radicals) whereas M2-shifted TAM drive tumour-growth, -invasion and angiogenesis (by releasing tumour cell-supporting cytokine, chemokines and angiogenic growth factors). M1- and M2-types are largely controlled by the signal transducer and activator of transcription (STAT) signalling pathways (STAT-1 and STAT-3) and can be induced in microglia and macrophages by different cytokines and growth-factors (blue arrows). Drugs like Amphotericin-B (Amph-B; a clinically approved anti-fungal agent), BLZ945 or PLX3397 (CSFR-1 inhibitors) are able to efficiently modulate glioma-polarized myeloid cells; Amph-B can induce TAM activation (and TAM-mediated tumour stem cell suppression), while CSFR-1 inhibitors block acquisition of the M2 phenotype in TAM.

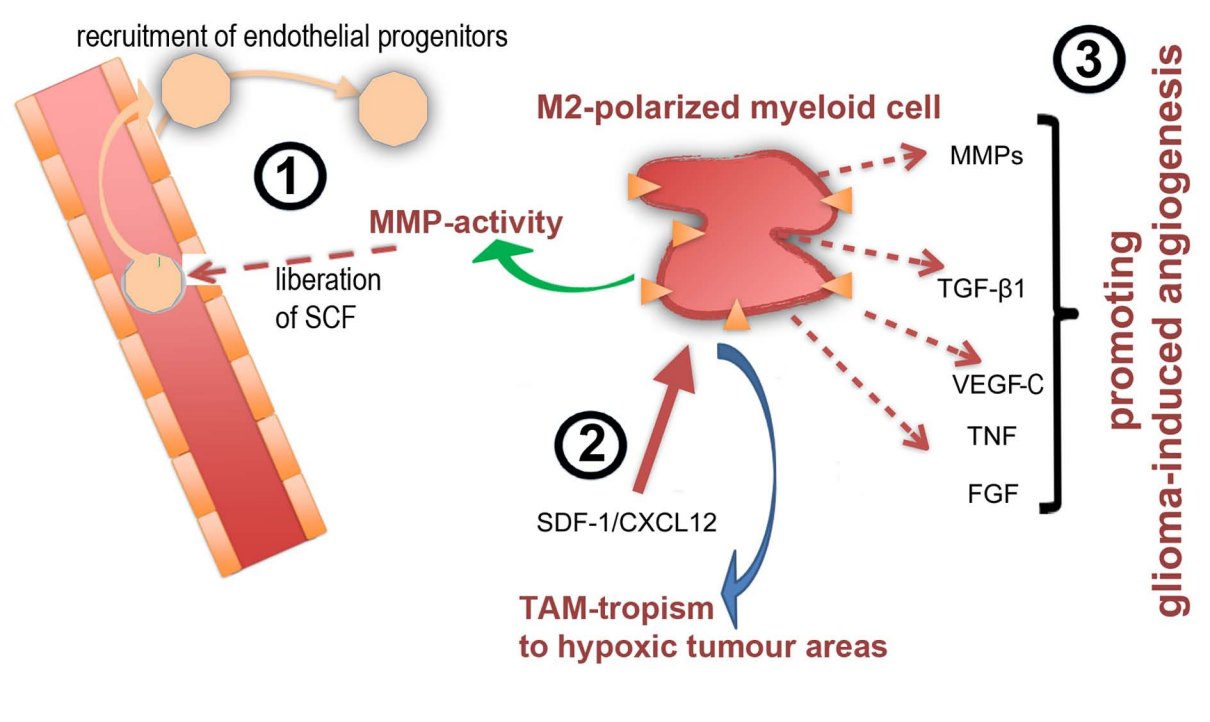
**Figure 2. Glioma chemoattract myeloid cells and induce the a pro-invasive mechanism**. Chemoattractants that have been shown to stimulate myeloid cell migration into the gliomas include CSF-1, GDNF, M-CSF and GM-CSF. An unidentified glioma-released factor (?) stimulates toll-like receptor-2 (TLR2) in TAM and subsequently the p38-MAPK- and MYD88-pathway, then MT1-MMP is expressed and on the plasma-membrane of TAM. MT1-MMP on TAM proteolytically cleaves glioma-derived pro-MMP-2 (an inactive pro-enzyme), which is thereby converted into the active form (MMP2) that degrades the extracellular matrix (ECM) and promotes glioma invasion; matrix degradation can activate other TLRs in TAM and promote the accumulation of additional TAM.

**Figure 3. Mechanisms by which glioma-associated myeloid cells promote tumour angiogenesis.** TAM have increased activity of MMPs (like e.g. MMP9) that promotes the release of stem cell factor, which is a chemoattractive signal for bone marrow-derived

endothelial progenitors; thereby TAM potentially participate in vasculogenesis **(1).** The chemokine CXCL12 induces the tropism of TAM to hypoxic areas that are not well vascularised or have aberrant vasculature **(2)**, which may foster angiogenesis in these regions by the mechanisms describe in point-3. TAM activate ECM-modifying enzymes (MMPs) and release a variety of soluble factors (growth factors and cytokines) which have direct angiogenic effects **(3)**.


glioma cell
IFN-γ
GM-CSF
IL-1β
M-CSF
MCP-1
MIF
TGF-β
PGE2
FasL
IL-4
IL-6
IL-10
Amph-B
BLZ945
M1-polarized myeloid cell
M2-polarized myeloid cell
CD80
Fas
TLRs
STAT-1
CD86
MHC-II
overlapping continuum
CCR2
EGFR
VEGFR1
STAT-3
CX3CR1R
TLRs
CXCL8
CXCL12
TGF-β
IL-10
CSF
FasL
T cells
cytokines, chemokines
ROS, NO
adaptive anti-glioma response
innate anti-glioma response
glioma cytotoxicity
glioma growth & invasion


glioma cell
MCP-1
EGF
CX3CL1
GDNF
VEGF
myeloid cell
tumour-associated
myeloid cell (TAM)
TLRs
TLRs
?
chemoattraction
pro-MMP2
MT1-MMP
TLR2
p38MAPK
MyD88
MT1-MMP
MT1-MMP
MMP2
ECM
ECM remodeling
glioma invasion
TAM attraction


recruitment of endothelial progenitors
1
MMP-activity
liberation
of SCF
M2-polarized myeloid cell
3
MMPs
TGF-β1
VEGF-C
TNF
FGF
promoting
glioma-induced angiogenesis
2
SDF-1/CXCL12
TAM-tropism
to hypoxic tumour areas