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Review

The role of microglia/macrophages in glioma maintenance and progression

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Abstract

There is a growing recognition that gliomas are complex tumors composed of neoplastic and non-neoplastic cells, which each individually contribute to cancer formation, progression, and response to treatment. The majority of the non-neoplastic cells are tumor-associated macrophages (TAMs), either from the peripheral origin or intrinsic microglial cells, which create a supportive stroma for neoplastic cell expansion and invasion. TAMs are recruited to the glioma environment, have immune functions and can release a wide array of growth factors and cytokines in response to those produced by the cancer cells. TAMs facilitate tumor proliferation, survival, and migration. Through such iterative interactions, a unique tumor ecosystem is established, which offers new opportunities for therapeutic targeting.

Solid cancers develop within complex tissue environments that dramatically influence tumor growth, transformation, and metastasis. Within the microenvironment of most solid tumors are various non-neoplastic cell types, including fibroblasts, immune system cells, and endothelial cells. Each of these stromal cell types produce growth/survival factors, chemokines, extracellular matrix constituents, and angiogenic molecules with the capacity to change the local milieu in which neoplastic cells grow and infiltrate. In the case of the most common brain tumor (glioma or astrocytoma), monocytes (macrophages and microglia) represent rich potential sources of these stromal factors. Moreover, the fact that as many as 30-50% of the cells in gliomas are microglia or macrophages[1-4] raises the intriguing possibility that targeting microglia and macrophages might emerge as an adjuvant therapy for these difficult to manage cancers. In this review, we discuss the current understanding of these critical stromal elements in glioma.

Origins of glioma associated microglia and macrophages

Microglia are the unique resident macrophages of the central nervous system (CNS). These mononuclear cells are distributed throughout the brain, where they are proposed to function as key immune effector cells of the CNS. Originally discovered and characterized almost a century ago by Pio Del Rio Hortega[5], the tissue origins of microglia and the mechanisms regulating their homeostasis in health and disease have been debated for many decades[6]. Contributing to the confusion was the use of particular experimental systems, including chimera mice generated by bone-marrow (BM) transplantation of lethally-irradiated recipients, and monocyte classification schemes reliant upon the expression of specific cell surface antigens. Using bone-marrow transplantation, investigators concluded that, under homeostatic conditions, a considerable percentage of microglia are replaced by donor-derived monocytes[7]. Similar studies have also suggested that increases in microglial

density in response to CNS damage involve both the expansion of endogenous resident microglia and the active recruitment of BM-derived microglial progenitors from the bloodstream[8-12]. Leveraging analogous methods, other reports demonstrated little or no contribution to the brain microglia pool from circulating progenitors. These studies argued that the expansion of microglia during microgliosis (microglial activation) mainly results from the local expansion of existing resident microglia[13]. These seemingly contradictory findings were finally resolved when chimeric animals generated by parabiosis were employed, which does not require either irradiation or transplantation. Using two models of acute and chronic microglia activation (axotomy and neurodegeneration), no microglial recruitment from the blood circulation was found[13]. In addition, acute peripheral recruitment of monocytes was observed in an experimental mouse model of autoimmune encephalitis (EAE); however, these infiltrating cells vanished following remission and did not contribute to the resident microglia pool[14].

Importantly, recent fate-mapping studies have identified immature yolk sac progenitors as the predominant source of brain microglia. Using sublethally irradiated B6 CD45.2+ newborns with hematopoietic cells isolated from CD45.1^{+} congenic mice, they showed that 3 months after transplantation, 95% of adult microglia remained of host origin. Second, they employed Cx3Cr1$^{GFP/+}$ knock-in mice to show that myeloid cells expressing CD45 and the adult macrophage marker CD11b, F4/80 and CX3CR1 were detectible in the developing brain starting at E9.5. Third, using *Csf1r*-deficient mice, CSF-1R deficiency dramatically reduced the development of microglia, whereas the circulating monocytes were unimpaired. Fourth, leveraging the Rosa26$^{R26R\text{-}eYFP/R26R\text{-}eYFP}$ reporter strain intercrossed with mice in which the tamoxifen-inducible MER-Cre-MER recombinase gene was under the control of one of the endogenous runt-related transcription factor 1 (Runx1) locus promoters, they found that Runx1^{+} progenitors migrate from yolk sac into the brain between E8.5 and

E9.5, where they serve as cells of origin for microglia[15]. Lastly, microglia derived from primitive c-kit^{+} erythromyeloid precursors that subsequently develop into CD45^{+}c-kit^{+}CX3CR1^{neg} immature cells (A1), which then mature into CD45^{+}c-kit^{neg}CX3CR1^{+} (A2) cells following CD31 downregulation and upregulation of F4/80 and colony stimulating factor receptor (CSF1R)[16]. Together, these studies collectively revealed that mouse myeloid progenitors from the blood do not significantly contribute to the pool of adult microglia after birth, establishing that the majority of adult microglia are yolk sac-derived and maintain themselves by virtue of longevity and limited self-renewal[13, 15, 17]. In this regard, resident microglia represent a distinct myeloid cell population (**Fig. 1**).

Whereas the naïve CNS is occupied by resident microglia only, the diseased CNS presents a different picture. In many neuropathological conditions, the blood brain barrier is impaired, resulting in an infiltration of monocytes from the periphery. Understanding the differences between macrophages and microglia is critically important, as it has been well-documented that they can react differently to various types of CNS insults. Recent studies using a complex parabiosis model with highly efficient permanent labeling of blood monocytes showed that peripheral mononuclear cells invade the inflamed CNS during EAE pathogenesis and play a primary role in disease progression to paralysis[14]. In the future, a clear distinction should be made between contributions of microglia and blood monocyte to disease pathogenesis, further underscoring the need to better understand the fate and origin of blood monocytes.

Approaches to distinguishing between microglia and invaded monocytes have traditionally relied on the use of CD45 antibodies to separate resident microglia (CD45^{low}) from macrophages of hematopoietic origin (CD45^{high})[18]. Analysis of human glioma samples by fluorescence-activated cell sorting has revealed that the CD45^{high} population is larger than the CD45^{low} population, suggesting that gliomas contain more recruited monocytes than

microglia[19]. This concept was recently challenged by a study using irradiation chimeras, which demonstrated that the majority of TAMs are intrinsic microglia, and that these microglial cells upregulate their CD45 expression to constitute a significant proportion of the $CD45^{high}$ monocyte population in gliomas[20]. In this study, the investigators protected the head from radiation to avoid a massive infiltration of monocytes due to a disrupted blood-brain barrier. Head-protected irradiation, however, might less efficiently remove all bone marrow cells, such as those residing in the skull bones and they could be a source of unlabeled monocytes mistaken for microglia. Another study using only single staining with either antibody for CX3CR1 or CCR2 concluded that the majority of TAMs are mainly monocyte-derived macrophages ($CCR2^{+}CX3C1^{-}$) and, to a small extent, resident microglia ($CCR2^{-}$ $CX3C1^{+}$) [21]. Although interesting, the study has several limitations, which include the lack of lineage tracing experiments to provide evidence that macrophages were clearly derived from monocytes. Moreover, others have demonstrated that CX3CR1 is expressed by blood monocytes and that this expression is upregulated during monocyte differentiation into macrophages, implying that CX3CR1 does not represent a microglia-specific marker either in the naïve brain[22, 23] or in the context of glioma[24]. The discrepancies in the literature obtained using bone-marrow chimeras and cell surface antibodies highlight the urgent need to re-evaluate these published conclusions and to perform lineage-tracing experiments using reporter mice that accurately distinguish microglia from monocytes/macrophages relative to their distinct roles in glioma formation, maintenance and progression (**Fig. 2**).

TAMs and low-grade glioma

Similar to their high-grade counterparts, the majority of World Health Organization (WHO) grade I and II astrocytomas contain microglia and macrophages[25]. Using CD68 and Iba1 antibodies, the percentage of monocytes in these low-grade tumors has been estimated at

15-30% compared to 10-15% in normal non-neoplastic brain specimens[2]. Depending on the region where the tumor arises, the microglia fraction can be as high as 35-50%, as observed in WHO grade I pilocytic astrocytomas[1]. Interestingly, the percent of proliferating $CD68^+$ cells may be higher in WHO grade I pilocytic astrocytomas (32%) relative to malignant WHO grade III-IV astrocytomas (8.6-13.4%)[26]. The importance of these immune system-like cells to glioma behavior is further underscored by two clinical observations: The number of $CD68^+$ cells increases with increasing malignancy grade[27] and the recurrence-free survival of patients with pilocytic astrocytoma is inversely related to the percentage of $CD68^+$ cells in the tumor[28].

To gain insights into the contributions of microglia to low-grade glioma biology, we have previously leveraged a murine model of neurofibromatosis type 1 (NF1) optic glioma. 15-20% of children with the NF1 inherited cancer predisposition syndrome develop pilocytic astrocytomas involving the optic pathway[29]. These children are born with a germline *NF1* gene mutation (*NF1*+/-) and develop brain tumors following somatic *NF1* gene inactivation in cells of the astroglial lineage [1]. Similarly, *Nf1*+/- mice with somatic *Nf1* gene inactivation in neuroglial progenitors develop low-grade glial neoplasms involving the optic nerve and chiasm[30]. As observed in their human counterparts, these murine low-grade tumors are infiltrated by TAMs[31]. The majority of the TAMs in *Nf1* mouse optic gliomas are $CD11b^{high}$; $CD45^{low}$, thus most likely microglia[32], which are evident early during tumorigenesis[33]. The role of these stromal cells in mediating glioma growth has been revealed by preclinical studies in which pharmacologic (minocycline, c-Jun-NH(2)-kinase inhibition) or genetic (gangciclovir treatment of CD11b- thymidine kinase expressing mouse line) silencing of microglial function results in reduced tumor proliferation[2, 34, 35]. Moreover, *Nf1* optic glioma mice with reduced expression of a chemokine receptor responsible for directional macrophage migration (CX3CR1) demonstrate delayed tumor formation[32]. Collectively, these

data establish critical functions for microglia in murine low-grade glioma formation and maintenance. Importantly, similar requirements for monocytes in another low-grade glial (Schwann) cell tumor have been reported. In these studies, mast cells and macrophages are stromal cell types essential for neurofibroma development and continued growth[36, 37].

While the mechanisms underlying microglia stimulation of low grade glioma growth have not been fully elucidated, *Nf1*+/- TAMs produce paracrine factors and chemokines capable of increasing *Nf1*-deficient astroglial cell proliferation[34]. One such chemokine, stroma-derived factor-1 (SDF-1 or CXCL12), is increased in *Nf1*+/- TAM relative to wild-type (normal) microglia[38, 39]. SDF-1 operating through the CXCR4 receptor promotes optic glioma cell survival, such that CXCR4 inhibition reduces tumor growth *in vivo*. A more complete characterization of TAMs support of tumor maintenance is currently being performed using optimized RNA-sequencing methods[40].

TAMs and high-grade (malignant) glioma

TAM activation

Macrophages and microglia are mononuclear cell types characterized by considerable diversity and plasticity. As such, different types of macrophage activation have been defined following *in vitro* stimulation. The pro-inflammatory M1 phenotype is acquired after stimulation with TLR4 ligands and IFN-γ, while the alternative M2 phenotype occurs after IL-4, IL-10/IL-13 exposure[41]. Alternative macrophage activation can be further subdivided into M2a (Th2 responses, type II inflammation, killing of pathogens, allergy), M2b (Th2 activation, immunoregulation), and M2c (immunoregulation, matrix deposition, tissue remodeling) activation states[41, 42]. These polarized subpopulations of macrophages differ with respect to receptor expression, effector function, and cytokine/chemokine production[43]. Since

the definitions of these mutually-exclusive activation states are based on *in vitro* conditions, they do not translate well to the *in vivo* setting.

Several studies have analyzed the expression of polarization marker genes in TAMs either *in vitro* or *in vivo*[44-46]. Similar to solid tumors arising in other organs, TAMs exhibit alternative macrophage activation, including increased production of anti-inflammatory molecules (e.g. TGF-β1, ARG1, and IL-10) as well as those that support tissue remodeling and angiogenesis (e.g. VEGF, MMP2, MMP9, and MT1-MMP). However, TAMs also produce pro-inflammatory molecules (e.g. TNF-α, IL1-β, and CXCL10)[44, 46-50].

Using RNA microarray analyses, the expression profiles of glioma-associated microglia/macrophages and microglia were compared to those from control animals obtained by CD11b antibody-mediated magnetic activated cell sorting. Approximately 1000 transcripts were differentially expressed by 2-fold or more in glioma-associated microglia/macrophages relative to control microglial cells. This expression pattern had only partial overlap with reported gene signatures for M1, M2a/b/c-polarized macrophages (Figure 3)[51].

In addition, other investigators have performed correlative analyses to determine whether the survival of patients with high-grade gliomas is associated with the expression of either M1 or M2 polarization-specific markers. One such M1 polarization marker, CD74, was found to be expressed by human TAMs, and positively correlated with increased patient survival[52]. In another study, F11R was established as a novel monocyte prognostic marker for glioblastoma that negatively correlates with patient survival and may be critical for defining a subpopulation of stromal cells for future potential therapeutic intervention[53].

Based on the current literature, it is clear that current M1 and M2 classification schemes are not absolute, but constitute relative definitions when studying TAMs *in vivo*. Indeed, TAMs express markers, which are typical for both, the M1 and M2 phenotype. As such, glioma-derived M-CSF induces a shift of microglia/macrophages towards the M2

phenotype, which increases tumor growth[54]. Similarly, mTOR[55] or colony stimulating factor-1 (CSF-1)[54] inhibition shifts to the M1 phenotype. Similar anti-tumor effects were shown by using dopamine or targeting miR-142-3p, which affected the M2-polarisation of TAMs[56, 57]. Based on these studies, the identification of targeting approaches that convert M2 macrophages to M1 macrophages has been suggested as a potential therapeutic strategy to reduce glioma growth. However, other studies have suggested that M1 specific markers or associated pathways positively correlate with glioma growth. For example, IL1β was shown to promote glioma growth[24]. Considering the plasticity and the fact that the M1 and M2 phenotype is a classification defined in cultured macrophages, the phenotype of TAMs *in vivo* is more complex and strategies should rather focus on targeting specific pathways or molecules that TAMs use to interact with gliomas and promote their growth.

TAM recruitment

Microglial cell/macrophages accumulate within and around glioma tissue and acquire an amoeboid morphology. There are many factors which mediate microglia chemoattraction, including chemokines, ligands of complement receptors, neurotransmitters, and ATP. It is presently unclear whether there exist distinct factors that recruit intrinsic (resident) microglia or peripheral macrophages to the tumor. The first chemoattractant factor identified was monocyte chemoattractant protein-1 (MCP-1), also known as CCL2. Ectopic expression of CCL2 in rat glioma cells resulted in a 10-fold higher density of Ox42-positive cells *in vivo* and the tumors generated with CCL2-expressing glioma cells were more than 3 times larger in size, resulting in reduced rat survival[58]. The importance of MCP-1 to human glioma biology has recently been challenged, where a stronger correlation was observed between MCP-3, rather than MCP-1, expression and the density of infiltrating microglia/macrophages[59].

Hepatocyte growth factor/scatter factor (HGF/SF) released by glioma cells similarly functions as a chemoattractant for microglia, but this has only been shown for a microglial cell line[60]. CXCL12 is another potent microglia/macrophage recruiting molecule, especially for attracting TAMs to hypoxic areas[61]. In the normal brain, the receptor for the cytokine CX3CL1 (fractalkine), CX3CR1, is exclusively expressed by microglial cells and has been established as a reliable marker for *in vivo* microglial imaging. The CX3CL1/CX3CR1 signaling cascade plays an important role for neuron-microglia communication, such that deletion of CX3CR1 impairs synapse plasticity during development[62]. Conflicting data exists regarding the importance of CX3CL1 in tumor-directed TAM migration[24, 63, 64].

The growth factor glial cell derived neurotrophic factor (GDNF) was first identified as a released factor from the glial cell line B49 and was found to promote the survival and differentiation of dopaminergic neurons. Mouse and human gliomas also secrete GDNF, which serves as a strong chemoattractant for microglia. When glioma cells were encapsulated in hollow fibers to allow for the passage of substances, but not cells, microglia accumulate around these fibers after brain implantation. GDNF mediated this attraction as shown by GDNF knockdown in the encapsulated glioma or by overexpression of GDNF in an encapsulated fibroblast cell line. Interestingly, the upregulation of GFAP in astrocytes around hollow fibers was not affected by GDNF knock-down, indicating that GDNF specifically acts on microglia[65].

Lastly, CSF-1 is released by glioma cells, where it can also function as a microglia chemoattractant. Treatment of mice with a blood-brain barrier-permeable CSF-1R antagonist reduced the density of TAMs and attenuated glioblastoma invasion *in vivo*[66]. Additionally, granulocyte-macrophage colony-stimulating factor (GM-CSF) can serve as a chemoattractant for microglia, as GM-CSF knockdown reduced microglia-dependent invasion in organotypic

brain slices as well as attenuated the growth of intracranial gliomas *in vivo*[67]. In conclusion, there are many factors that can attract TAMs to the glioma (Figure 4).

TAM regulation of glioma growth and migration

The accumulation of TAMs within and around glioma has raised the question as to whether these mononuclear cells are bystanders or whether they actively influence glioma growth and invasion. Accumulating evidence indicates that TAMs promote glioma growth and invasion. Bettinger et al. (2002) noted that, in the presence of microglial cells, the motility of the murine glioma cells was increased three-fold *in vitro*[68]. In contrast, oligodendrocytes and endothelial cells only weakly promote glioma motility[68]. *In situ*, glioma growth can be monitored using organotypic brain slices. These slices can be depleted of microglia using liposomes filled with the toxin clodronate, resulting in reduced glioma invasion and growth[69]. A complementary *in vivo* approach entails the use of transgenic mice expressing the herpes simplex virus thymidine kinase gene under the control of the CD11b promoter. In the central nervous system, CD11b is specifically expressed by microglia. When ganciclovir was infused into the brain, there was a dramatic reduction in microglia number, which concomitantly attenuated glioma growth *in vivo*[48].

Several factors released from microglia have been reported to promote glioma proliferation and/or migration. Microglia synthesize and release stress inducible protein 1 (STI1), a cellular prion protein ligand, which increases the proliferation and migration of glioblastomas *in vitro* and *in vivo*[70]. In addition, microglia release epidermal growth factor (EGF), which also stimulates glioblastoma cell invasion[66]. This promoting activity by microglia is triggered by CSF-1, which is constitutively released by glioma cells. As described above, CSF-1 is a chemoattractant for microglia and, at the same time, converts microglia into a pro-tumorigenic phenotype[54]. CCL2 is another factor released from human

glioma cell lines and acts on the CCL2 receptor (CCR2) expressed on microglia[71]. CCL2 can trigger the release of IL-6 from microglia, which in turn, promotes the invasiveness of glioma cells[72]. It should be noted that there may be species differences, as it was recently described that mouse microglia do not express CCR2[73].

Transforming growth factor-ß (TGF-ß) also increases the migration of glioma cells through processes that likely involve increased integrin expression and function[74]. TGF-ß is predominantly released from microglia when studied in co-culture systems, such that blocking TGF-ß signaling impairs glioma growth[75]. In addition, TGF-ß2 induces the expression of matrix metalloprotease-2 (MMP-2) and suppresses the expression of tissue inhibitor of metalloproteinases (TIMP)-2, which degrades the extracellular matrix to promote glioma invasion[74]. While antagonizing TGF-ß function was initially considered as a potential anti-tumor therapy, it had severe side effects, since systemic inhibition or lack of TGF-β signaling results in acute inflammation and disruption of immune system homeostasis [75].

MMP-2 enzyme is released in a pro-form, which is larger and needs to be cleaved in order to become active. The prominent enzyme for pro-MMP-2 cleavage is the membrane bound metalloprotease MT1-MMP. Under normal conditions, microglia do not elaborate MT1-MMP, but, when exposed to glioma cells, upregulate MT1-MMP expression. Microglial MT1-MMP expression then increases glioma growth in organotypic slices. In this regard, slices obtained from MT1-MMP- deficient mice harbor significantly smaller tumors. Moreover, when microglia were depleted from MT1-MMP deficient organotypic slices, glioma growth was further reduced, indicating that MT1-MMP is not the only glioma-promoting factor expressed by microglia. In human glioma samples, MT1-MMP expression positively correlates with the increasing glioma malignancy grade[48].

The involvement of the Toll-like receptor (TLR) signaling cascade in glioma-microglia interactions was initially inferred by the observation that deletion of MyD88, an

adapter protein of most Toll-like receptors, inhibits MT1-MMP induction in microglia. Toll-like receptors are prominent detectors of DNA fragments or bacterial cell wall components, important for mediating immunologic responses to pathogens [76]. TLR2 was identified as the major TLR involved in triggering MT1-MMP upregulation in microglia. In this manner, gliomas implanted into *Tlr2*-deficient mice were significantly smaller, and the survival of these mice was prolonged. TLR2 forms heterodimers with TLR1 and TLR6, which is important for modulating MT1-MMP expression: Deletion of both TLR1 and TLR6 results in reduced MT1-MMP expression. In addition, treatment with TLR2-neutralizing antibodies reduced glioma-induced microglial MT1-MMP expression as well as attenuated glioma growth[77].

In a screen for endogenous ligands released from glioma cells, versican was identified as a candidate molecule for triggering TLR2 signaling [78]. Versican exists as different splice variants, V0, V1 and V2. The V0/V1 isoforms are highly expressed in mouse and human gliomas, and reduced glioma versican expression is associated with decreased microglial MT1-MMP expression *in vitro* and *in vivo*. Furthermore, inoculation of versican-silenced gliomas resulted in smaller tumors and longer survival rates relative to controls. Importantly, the impact of versican signaling on glioma growth depends on the presence of microglia. The ability of glioma-produced versican to trigger increased TLR2 expression converts microglia into a pro-tumorigenic phenotype characterized by the up regulation of MT1-MMP and MMP9 expression. This feed-forward loop establishes an interdependent circuit of cellular interactions that increases glioma growth and invasion[79].

TAMs not only target glioma cells, but also affect angiogenesis to indirectly affect tumor growth. Signaling through the receptor for advanced glycation endproduct (RAGE) plays an important role in the process. RAGE ablation abrogated angiogenesis, which could be reconstituted with wild-type microglia or macrophages. This TAM activity correlated with

the expression of VEGF, an important pro-angiogenic factor[80]. Figure 5 summarizes the possible mechanisms by which TAMs influence glioma growth.

The effects of microglia/macrophages on glioma stem cells

Glioblastomas contain a subpopulation of cells with stem cell-like properties (self-renewal, multi-lineage differentiation) capable of reconstituting the native tumor following implantation into naïve hosts. These glioma stem cells (GSCs) reside within the perivascular niche, where they can be highly resistant to radiation and chemotherapy[81-83]. Figure 6 illustrates the complexity and cellular composition of perivascular niche. The importance of GSCs to microglia attraction is underscored by the finding of a positive correlation between the density of GSCs and TAMs, indicating that GSCs may recruit TAMs more efficiently than their more differentiated neoplastic counterparts[84]. A recent study showed that GSCs release periostin, which accumulates in the perivascular niche. Periostin acts as a chemoattractant for TAMs, which is mediated by signaling through the integrin receptor $\alpha_v\beta_3$[21]. TAMs also influence the properties of GSCs, in that TGF-ß released from TAMs induce MMP-9 expression and increase GSC invasiveness[50]. In addition, naïve microglia can reduce the sphere-forming ability of human stem cells to suppress glioma growth, while microglia or monocytes cultured from glioma patients have lost this anti-tumorigenic potential[85]. Supernatants from glioma stem cells likewise inhibit the phagocytosis activity of TAMs and induce the secretion of interleukin-10 and TGF-β1[86].

Microglia and macrophages as targets for glioma therapy

For several decades, our understanding of glioma biology was largely driven by studies focused on the genetic and molecular changes that occur within cancer cells and their contributions to deregulated cell growth. Over the past several years, work from numerous

laboratories, including our own groups, has revealed that glioma growth is dependent upon growth regulatory signals that emanate from the tumor microenvironment. In this regard, it is important to recognize that brain tumors are complex microcosms in which the communication between neoplastic and non-neoplastic cells will influence not only gliomagenesis[87], but also may modify glioma responses to standard therapy The identification of these glioma microenvironment-derived signals represents an initial step towards developing stroma-directed glioma therapies, with the longstanding goal of combining these therapies with neoplastic cell-targeted therapies.

In this regard, the induction of HIF-1 following glioma radiation results in the recruitment of bone-marrow-derived myeloid cells, partially due to the activation of stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4. Activation of SDF-1/CXCR4 promotes vasculogenesis and tumor recurrence. These findings support the notion that better outcomes for glioblastoma might be achieved using combination of radiotherapy and the clinically-approved small molecule inhibitor of CXCR4 signaling (AMD3100)[88]. These observations were further supported using a different glioma model, showing that tumor-secreted SDF-1 is one important factor in radiotherapy-induced tumor invasiveness, where it exerts its primary effect through macrophage mobilization and tumor revascularization[89]. Similar observations were made when human recurrent glioblastomas were treated with anti-angiogenic therapy. The increased TAM number correlated with poor survival, suggesting that TAMs may participate in the escape from anti-angiogenic therapy, and therefore represent a potential biomarker of resistance as well as a logical therapeutic target for recurrent glioblastoma treatment[90]. In support of these human correlative data, murine glioma studies revealed that glioblastoma resistance to anti-VEGF therapy is associated with increased myeloid cell infiltration[91].

Interfering with CSF-1 signaling is another potential approach to targeting TAM regulation of glioma growth. Pyontek et al. used an inhibitor of the CSF-1 receptor in a mouse proneural GBM model to increase survival and shrink established tumors [54]. Periostin has also emerged as an interesting target to attenuate the tumor-supportive phenotype of TAMs by interrupting integrin $\alpha v\beta_3$ signaling [21]. Interfering with this pathway with a blocking peptide impaired TAM recruitment. One could also exploit the interaction of TAMs with glioma initiating cells. In a drug screen, Amphotericin B was identified as a molecule that enhanced the microglial impact on brain tumor initiating cell (TIC) cell cycle growth arrest and differentiation [85], whereas Stat3 inhibition was shown to activate TAMs and inhibit glioma growth in mice [92].

Minocycline, an antibiotic, interferes with the process of microglia activation. Using a rat model of glioma demonstrated synergistic activity when systemic BCNU (chemotherapy) treatment was combined with the local delivery of minocycline to impair microglia activation[93]. Currently, investigators at the University of Utah are recruiting patients for a phase I clinical trial using minocycline as adjuvant therapy (ClinicalTrials.gov Identifier: NCT02272270). Additionally, immunotherapy using activated natural killer (NK) cells combined with the antibody mAb9.2.27 directed against the proteoglycan Neuroglial-2 (NG2) showed beneficial effects, which were partly due to a reversal of the tumor-promoting effects of TAMs[94]. Collectively, these studies suggest that TAMs modify the glioma response to standard and anti-angiogenic therapy.

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Conclusions

It is now evident that TAMs home to the evolving glioma and interact in a complex fashion with the tumor environment to promote glioma growth in mouse models and in human patients (Figure 6). However, there are still many unanswered questions. It is not clear what

factors are truly responsible for mediating the interaction between glioma cells and microglia/macrophages. In this respect, we do not know how microglia and macrophages interact within the tumor, and whether they acquire distinct properties and execute distinct functions. It remains also an open question as whether TAMs acquire different functional phenotypes depending on individual glioma types (low-grade versus high-grade, glioblastoma molecular subtypes). Similarly, even within a given tumor, TAMs might interact differently with different neoplastic cell types (GSCs, differentiated astrocytoma cells). Nonetheless, after decades of applying treatments directed against the tumor cells directly, TAMs have emerged as novel targets for therapeutic intervention. Further investigation into the mechanisms and interactions between TAM populations and the variety of neoplastic and non-neoplastic cells in these tumors may one day yield novel glioma treatment strategies.

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Figure Legends

Fig. 1. Microglia and monocytes have distinct cellular origins. Under steady-state conditions, these different mononuclear cell populations reside in separate locations. In adult life, monocytes are generated from hematopoietic stem cells (HSCs) that differentiate into granulocyte-macrophage progenitors (GMPs) and then into monocyte-dendritic cell progenitors (MDPs). Mature Ly6C^{hi}, CCR2^{+}, CX3CR1$^{low/int}$ inflammatory monocytes are released into the circulation, where they can migrate to tissues in response to specific pathological conditions. These cells can also give a rise to circulating monocytes. Microglia originate from yolk sac progenitors in the neuroepithelium beginning around embryonic day 8.5 in the mouse. In the adult brain they express high levels of CX3CR1, CD11b, F4/80, low levels of CD45 and no CCR2.

Fig. 2. Microglia and monocytes converge in high-grade glioma (HGG). HGG cells induce local inflammation that compromises the integrity of the blood-brain-barrier (BBB) and results in Ly6C^{hi}, CCR2^{+}, CX3CR1$^{low/int}$ monocytes infiltrating into the tumor. Once within the CNS, they can differentiate into tumor-associated macrophages and become nearly indistinguishable from activated resident microglia.

Fig. 3 Comparison of TAMs with M1 and M2a/b/c-stimulated macrophage datasets (from [51]. Data sets from http://www.ebi.ac.uk/arrayexpress (Data set: E-GEOD-32690; [95]), containing data of macrophages that were stimulated for 24 h *in vitro* into different polarization states (M0 (unstimulated), M1 (IFNγ + LPS), M2a (IL4), M2b (IFNγ + complexed Ig), and M2c (Dexamethasone)) were compared with glioma-regulated genes in a TAMs data set. A: A graphical representation of the overlap of upregulated genes in TAMs and the four

macrophage data sets. The TAMs gene expression profile shows the greatest overlap with M1, and M2b polarized macrophages. The number of overlapping genes is indicated as number. B: Using Gene Set Enrichment Analysis (GSEA) indicate that only a minority of genes upregulated in TAMs were also induced in the M1 to M2c phenotype, 59.5% of the genes that were upregulated in TAMs were not regulated in any of the four macrophage phenotypes.

Fig. 4. Glioma cells release several factors, which attract microglial cells and/or peripheral monocytes to the tumor tissue.

Fig. 5. (A) Microglial cells release several factors which promote glioma cell invasion. (B) Microglia release TGF-ß, which triggers the release of pro-MMP2 from glioma cells. Pro-MMP2 is then cleaved into active MMP2 by microglia-expressed MT1-MMP. Microglial MT1-MMP expression is stimulated by versican, which is released from glioma cells. Versican activates TLR2/p38-MAP-kinase signaling in microglial cells, which leads to the up regulation of MT1-MMP. TLR2 signaling in microglia also triggers MMP9 release.

Fig. 6. Stroma-tumor interactions create a biological system comprised of feed-forward circuits that drive glioma growth and invasion. (A) Microglia and macrophages are recruited to the tumor by tumor bulk, glioma stem cells (GSCs) and endothelial cells. (B) These recruited and reprogrammed microglia and macrophages secrete soluble factors that both expand the tumor bulk and GSCs as well as (C) stimulate angiogenesis to establish a supportive niche for GSCs. (D) In addition, the interaction between non-neoplastic astrocytes and tumor cells can further support tumor growth. Lastly, each cellular component has the

capacity to change the extracellular matrix to further facilitate glioma proliferation, survival, and expansion.

Figure 1

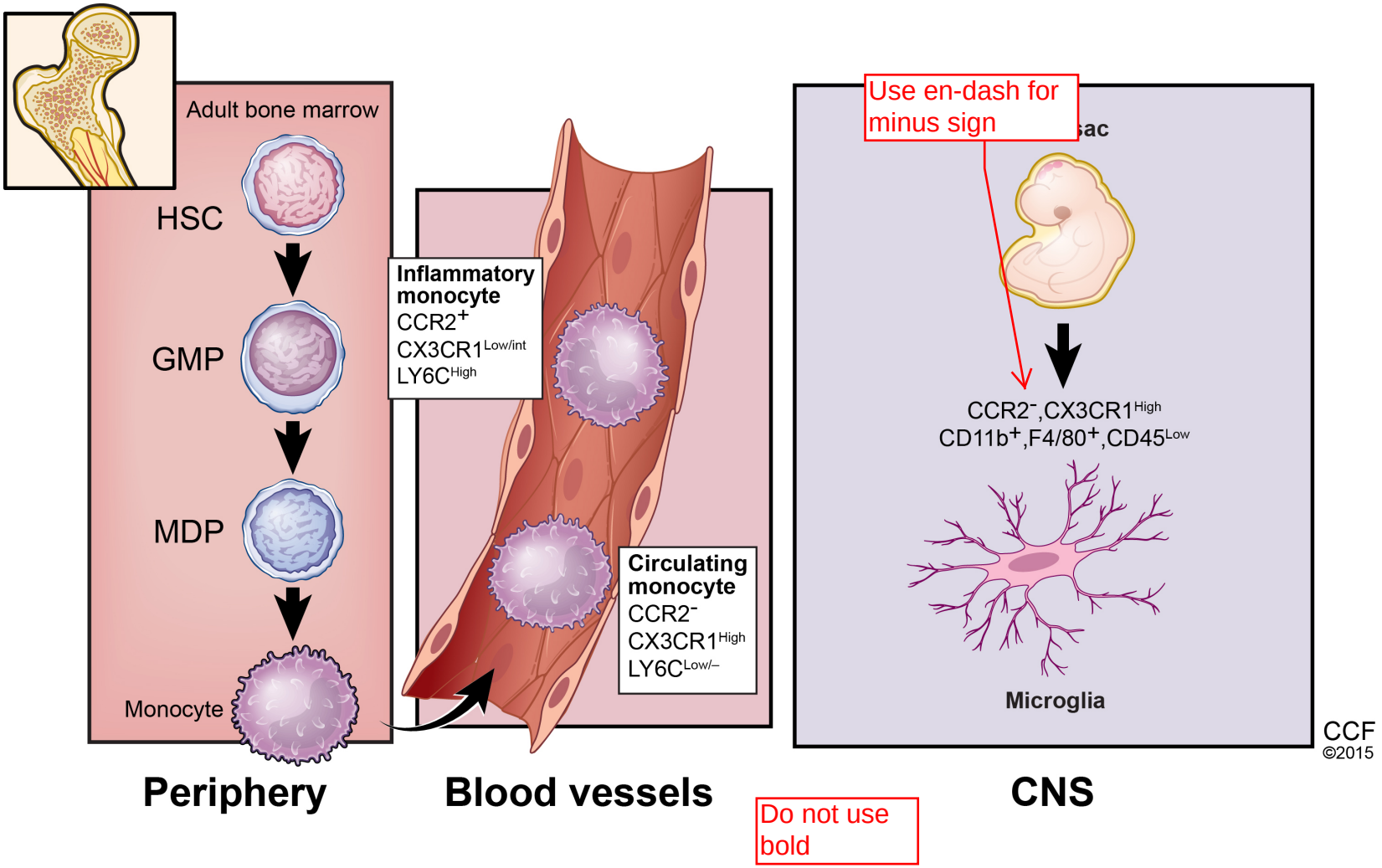
Adult bone marrow
HSC
GMP
MDP
Monocyte
Inflammatory monocyte
CCR2+
CX3CR1Low/int
LY6CHigh
Circulating monocyte
CCR2-
CX3CR1High
LY6CLow/–
Use en-dash for minus sign
CCR2-,CX3CR1High
CD11b+,F4/80+,CD45Low
Microglia
CCF ©2015
Periphery
Blood vessels
CNS
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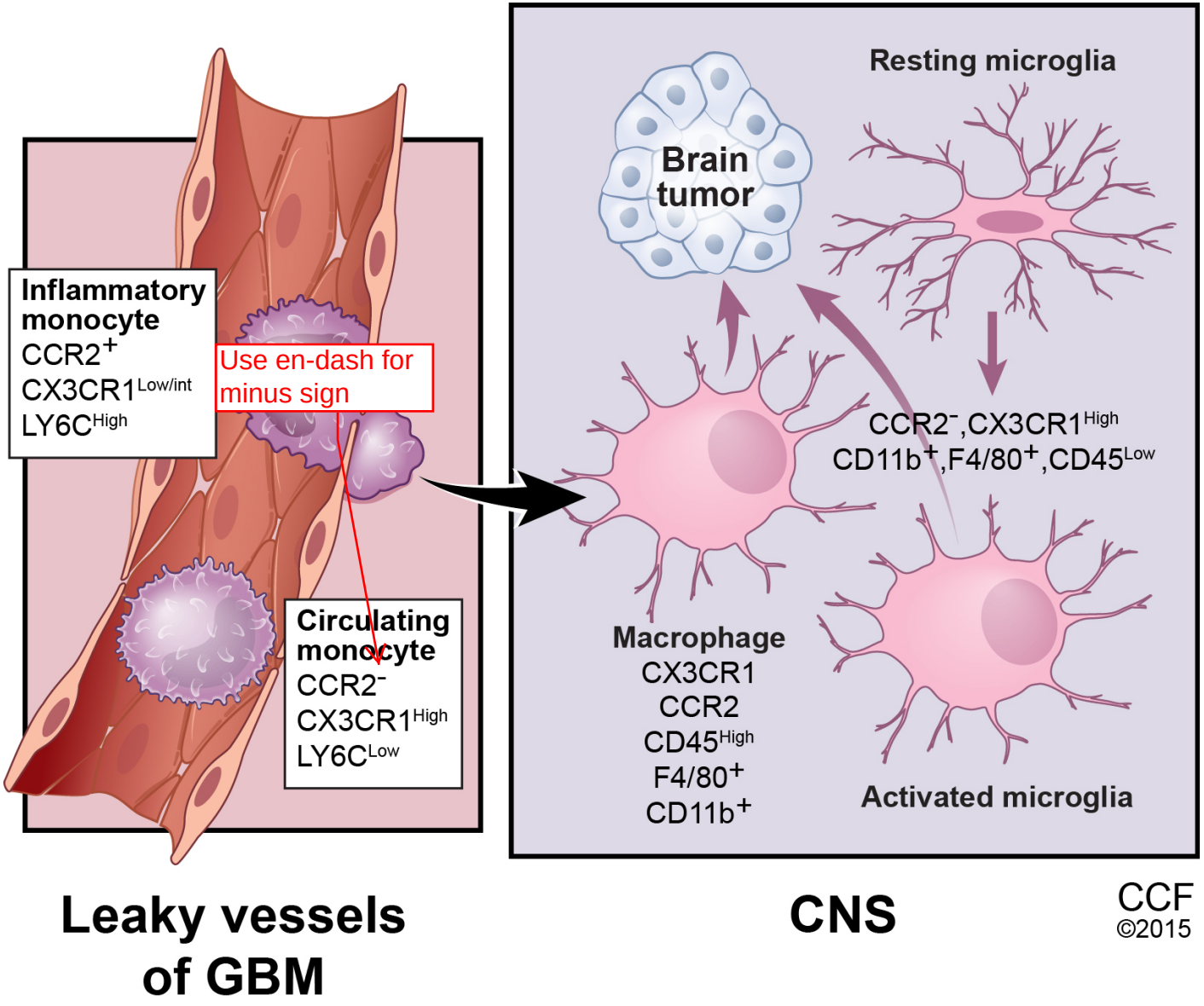
Figure 2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Inflammatory monocyte
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CX3CR1Low/int
LY6CHigh
Circulating monocyte
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CX3CR1High
LY6CLow
Leaky vessels of GBM
Brain tumor
Resting microglia
CCR2-,CX3CR1High
CD11b+,F4/80+,CD45Low
Macrophage
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CCR2
CD45High
F4/80+
CD11b+
Activated microglia
CNS
CCF
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Figure 3

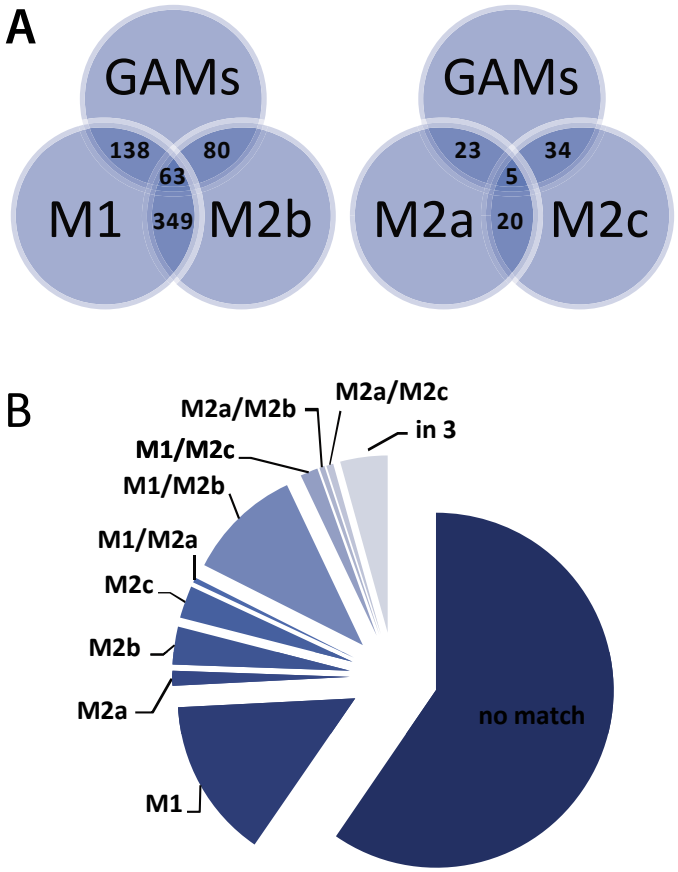

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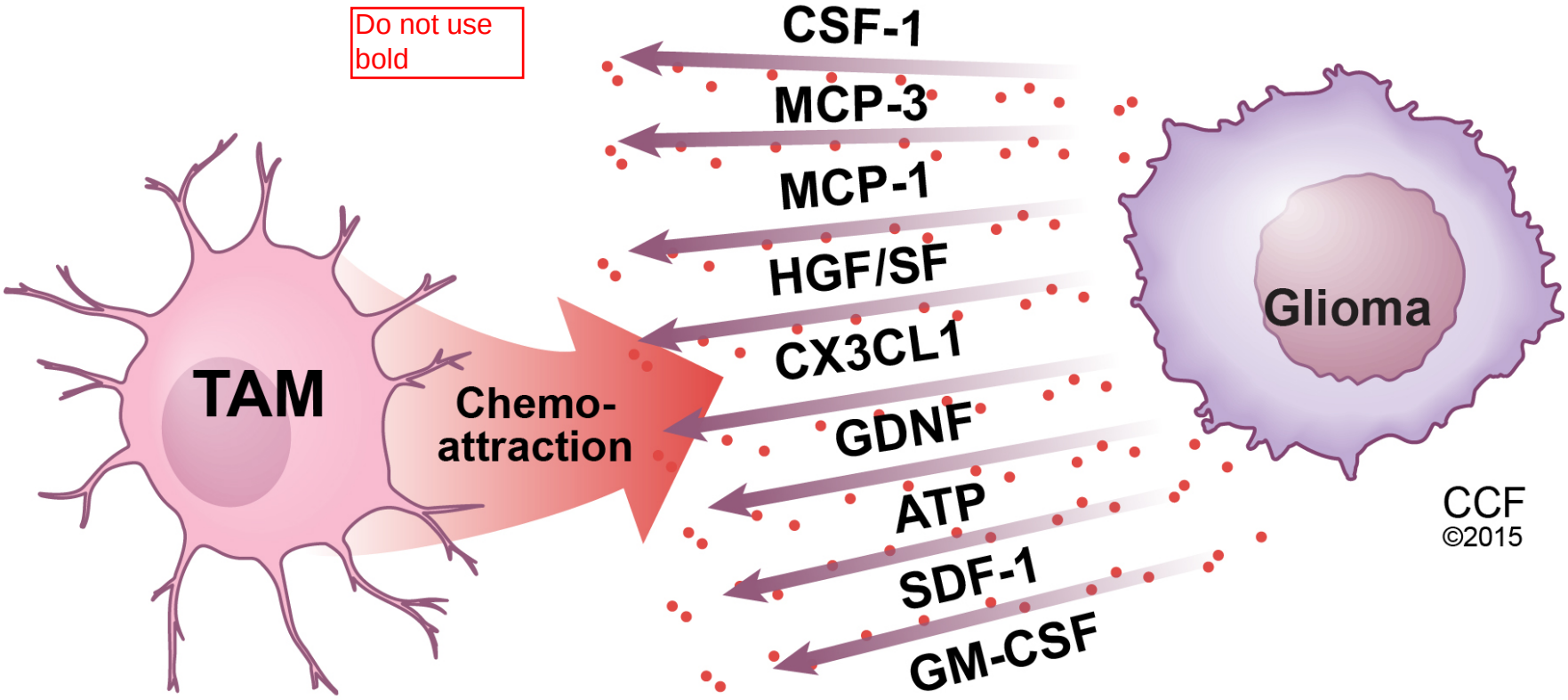
Figure 4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CSF-1
MCP-3
MCP-1
HGF/SF
CX3CL1
GDNF
ATP
SDF-1
GM-CSF
TAM
Chemo-attraction
Glioma
CCF
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Figure 5

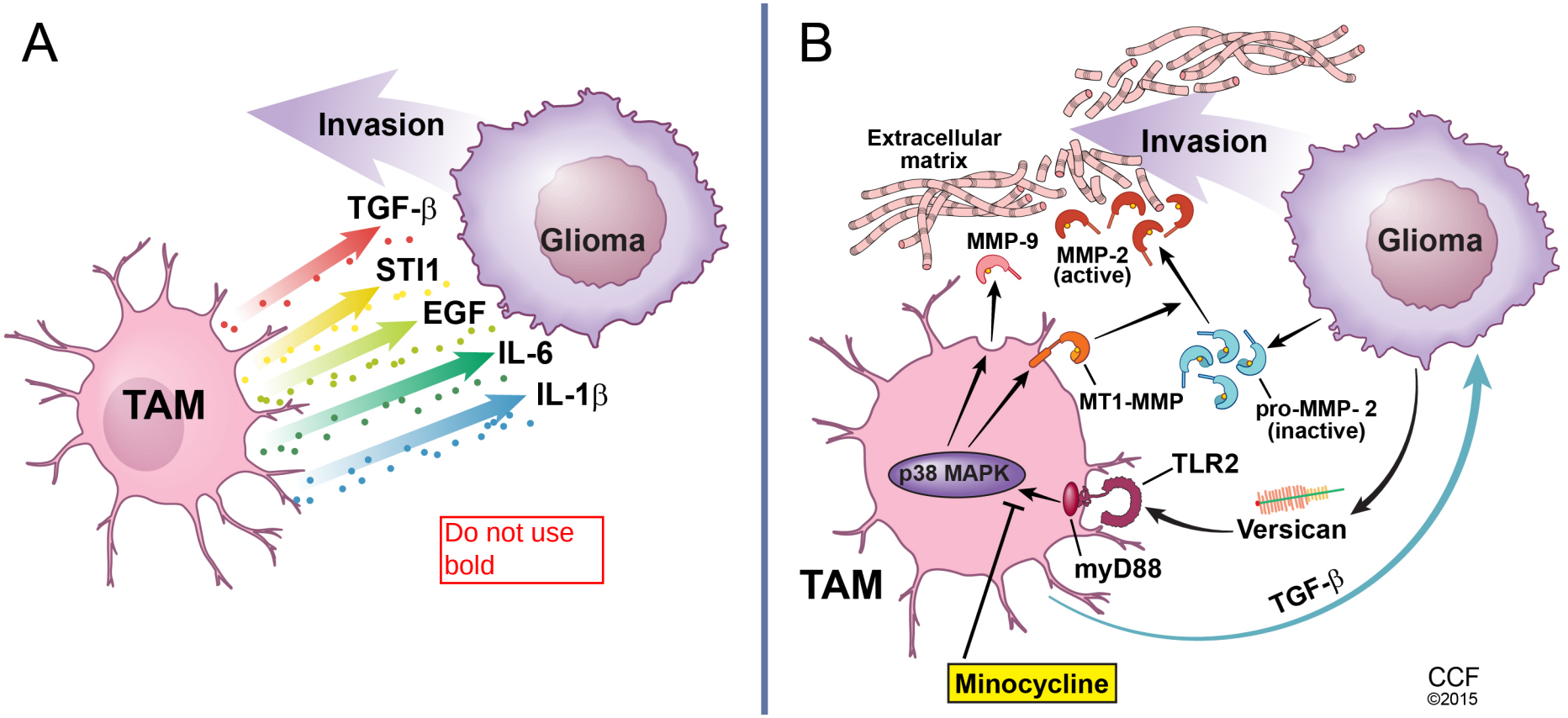
A
Invasion
Glioma
TGF-β
STI1
EGF
IL-6
IL-1β
TAM
Do not use bold
B
Extracellular matrix
Invasion
Glioma
MMP-9
MMP-2 (active)
MT1-MMP
pro-MMP- 2 (inactive)
p38 MAPK
TLR2
Versican
myD88
TAM
TGF-β
Minocycline
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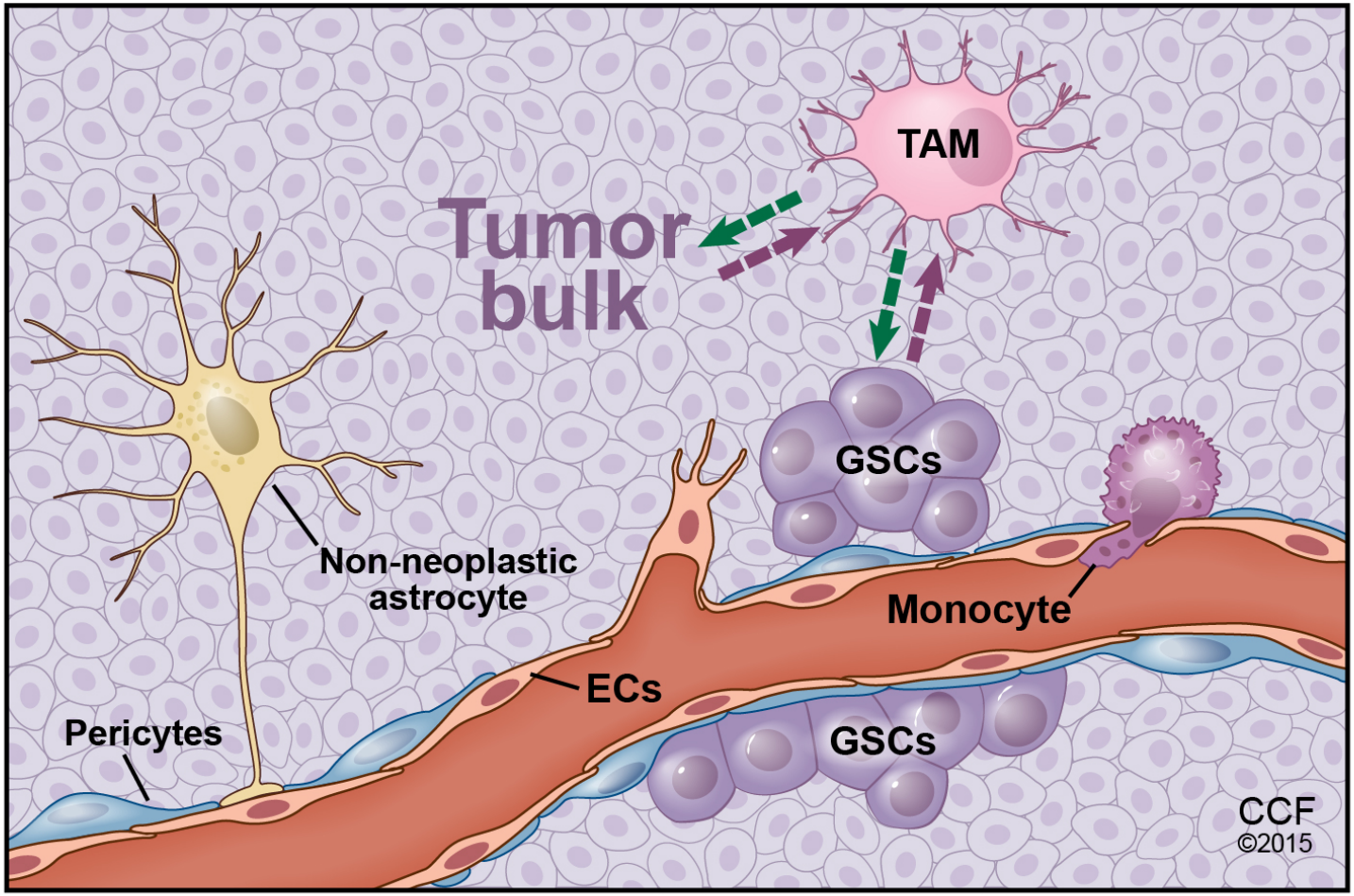
Figure 6



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